



Age-related changes in medial septal cholinergic and GABAergic projection neurons and hippocampal neurotransmitter receptors: relationship with memory impairment

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Abstract

The hippocampus, which provides cognitive functions, has been shown to become highly vulnerable during aging. One important modulator of the hippocampal neural network is the medial septum (MS). The present study attempts to determine how age-related mnemonic dysfunction is associated with neurochemical changes in the septohippocampal (SH) system, using behavioral and immunochemical experiments performed on young-adult, middle-aged and aged rats. According to these behavioral results, the aged and around 52.8% of middle-aged rats (within the “middle-aged-impaired” sub-group) showed both impaired spatial reference memory in the Morris water maze and habituation in the open field. Immunohistochemical studies revealed a significant decrease in the number of MS choline acetyltransferase immunoreactive cells in the aged and all middle-aged rats, in comparison to the young; however the number of gamma-aminobutyric acid-ergic (GABAergic) parvalbumin immunoreactive cells was higher in middle-aged-impaired and older rats compared to young and middle-aged-unimpaired rats. Western Blot analysis moreover showed a decrease in the level of expression of cholinergic, GABAergic and glutamatergic receptors in the hippocampus of middle-aged-impaired and aged rats in contrast to middle-aged-unimpaired and young rats. The present results demonstrate for the first time that a decrease in the expression level of hippocampal receptors in naturally aged rats with impaired cognitive abilities occurs in parallel with an increase in the number of GABAergic neurons in the MS, and it highlights the particular importance of inhibitory signaling in the SH network for memory function.

Keywords Aging · Cognition · Medial septum · Hippocampal receptors · Rats

Introduction

Biological aging is one of the most complex organic processes which can lead to cognitive decline (reviewed by da Costa et al. 2016). Cognitive impairment has also been

shown to be associated with changes in specific areas of the brain. Morphological studies indicate that structural changes, such as a decreased density of synapses and spines and reduced gray matter and volume, occur in regions of the brain involved in cognition (Bonifazi et al. 2018; Guerra-Gomes et al. 2018). The hippocampus, a structure fundamental for cognitive functions, is very vulnerable to aging (Foster et al. 2012; Morrison and Baxter 2012) and is subjected to many age-related molecular and cellular changes (Serrano-Pozo et al. 2011; Moodley and Chan 2014), as well as to changes in the neuronal connectivity (Kelly et al. 2006; Wilson et al. 2005).

One key modulator of the hippocampal neural network is the medial septum (MS), a part of the basal forebrain (Hangya et al. 2009; Roland and Savage 2009; Kanju et al. 2012). Septohippocampal (SH) projections predominantly include cholinergic and gamma-aminobutyric acid-ergic

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(GABA-ergic) fibers (Rye et al. 1984), although there are some glutamatergic (Sotty et al. 2003) and neuropeptide projection neurons as well (Peterson and Shurlow 1992). Lesions or inactivation of MS neurons impair hippocampal-dependent forms of learning and memory (Lecourtier et al. 2011; Dashniani et al. 2015, 2020). Studies investigating the effects of selective lesion of MS cholinergic neurons have resulted in both impairments (Paban et al. 2005; Cai et al. 2012) and intact performance on a variety of spatial tasks (Winters and Dunnett 2004; Dashniani et al. 2020). Previous studies, including ours, have revealed the significance of GABAergic SH projections in spatial memory (Pang et al. 2011; Lecourtier et al. 2011; Dashniani et al. 2020). It has been shown that rats with GABAergic lesions in MS exhibited an impaired spatial reference memory (Lecourtier et al. 2011; Dashniani et al. 2020). So far, there is no evidence that MS glutamatergic neurons can modulate hippocampal-dependent memory function.

In the absence of disease, the relationship between cognitive aging and alterations in the SH system remains relatively understudied. Furthermore, the few studies that have investigated the role of SH projections in cognitive aging have produced contradictory results. Some studies report that cholinergic basal forebrain neurons decline with age, which mediates age-related spatial learning impairments (Fragkouli et al. 2005; Fadda et al. 2000), whereas others have found no relationship between decline in cholinergic cell numbers and the loss of cognitive abilities. Moreover, certain studies have reported that cholinergic neurons remain relatively stable in number at advanced ages (Ypsilanti et al. 2008; McQuail et al. 2011). There is little information on the possible alterations of the GABAergic systems in relation to aging. It has though been demonstrated that the densities of GABAergic SH projection neurons in the MS remain largely constant during aging (Rubio et al. 2012), while an increase in the number of GABAergic SH projection neurons have also been shown in old rats with impaired spatial abilities (Bañuelos et al. 2013).

It should be noted that studies have shown that most basic cellular characteristics of hippocampal principal cells do not change with advanced age (for review see Rosenzweig and Barnes 2003). Therefore during aging, changes in hippocampal cognitive function do not appear to be attributable to changes in neuron number or basic cellular physiology. It is believed that structural changes in synaptic connections affect functional connectivity in the hippocampus during aging (reviewed by Rosenzweig and Barnes 2003; Chawla and Barnes 2007). The importance of different types of neuroreceptor for the function of the hippocampal network and their role in hippocampal dependent memory processes has been discussed widely (Gu and Yakel 2011; Nathan et al. 2013; Sadigh-Eteghad et al. 2015; Ghafari et al. 2017). These studies indicate

that SH projection plays an important role in the functional properties of hippocampal circuits, particularly in the intrinsic neural mechanisms involved in memory function.

In spite of the crucial known role of the SH system in learning and memory, especially its sensitivity to the effects of aging, alterations in SH projections and the subsequent effects on the hippocampal receptor expression associated with memory decline in natural aging are far less defined. Predominantly some animal models of chemically induced aging (Garceza et al. 2018) or neurodegenerative diseases (Martín-Belmonte et al. 2019, 2020, 2021) are used as tools for this purpose. Considering the role of cholinergic and GABAergic MS neurons for hippocampal-dependent memory by modulating the hippocampal neural network, in the present study, first time we examined the relationship between cognitive aging and age-related changes in (i) the MS cholinergic and GABAergic projection neurons and (ii) the level of expression of glutamatergic, cholinergic and GABAergic receptors in the hippocampus during natural aging.

The present study was designed to ascertain whether age-related alterations in the expression level of hippocampal neuroreceptors are associated with changes in the number of cholinergic and GABAergic MS projection neurons and mnemonic dysfunction during natural aging. Immunohistochemical methods were employed to determine the number of cholinergic and GABAergic projection neurons in the MS, the classical Nissl histological method was used to reveal if above mentioned changes was a consequence of age-dependent general neuronal loss, whilst Western immunoblotting was used for the study of the following neurotransmitter receptors in the hippocampus—two types of cholinergic receptors (AChR)—Alpha7 nicotinic acetylcholine receptor ($\alpha 7$ nACh) and muscarinic (mAChR-M1), GABAergic receptors (the $\alpha 1$ subunit of GABAA) and two types of glutamatergic receptors (GluR)—the NR2B subunit of *N*-methyl-D-aspartate (NMDA) and the GluR1 subunit of α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)—in behaviorally characterized young, middle-aged and old rats. All these receptors play an essential role in long-term potentiation (LTP) and/or long-term depression (LTP) in the hippocampus, processes thought to underlie learning and memory (Ge et al. 2010; Kakegawa et al. 2004; Kroker et al. 2011; Cheng and Yakel 2015).

The data obtained reveal for the first time a decrease in the expression level of hippocampal neurotransmitter receptors in naturally aged rats with impaired cognitive abilities, which is associated with an increase in the number of MS GABAergic neurons and indicates the particular importance of inhibitory signaling in the SH network for memory function during aging.

Materials and methods

The detailed description of methods is provided in Supplementary Materials (see Supplementary Materials-1—Materials and Methods), whereas below they are provided shortly.

Subjects

Male outbred albino rats served as subjects—aged 5 months (group—young-adult; $n = 15$), 15 months (group—middle-aged; $n = 36$) and 22 months (group—aged; $n = 23$) at the start of experimentation. The number of animals of different ages is based on the two facts: (a) the high mortality of older rats and (b) that two sub-groups (unimpaired/impaired) clearly emerged from the results of the middle-aged rat group spatial memory task. Based on these facts the number of animals in different age groups, sufficient for adequate statistical processing of results, was determined. At the end of behavioral testing, there was 81% percentage survival in the 22-month-old cohort and 100% in the 5 and 15 months old. The final number of rats in the groups in which the statistical analysis was carried out was: young-adult ($n = 15$), middle-aged ($n = 36$) and aged ($n = 18$).

The animals were procured from the Laboratory Animal Division of the I. Beritashvili Center of Experimental Biomedicine. All experimental procedures were conducted in accordance with the European Communities Council Directive Guidelines for the care and use of laboratory animals (2010/63/EU-European Commission) and approved by the animal care and use committee at the I. Beritashvili Center of Experimental Biomedicine.

Experimental design

Within the present study, habituation to both the environment and objects was assessed using an open field, and long-term spatial memory via a Morris water maze (MWM) task. A schematic representation of the experimental design is shown in Supplementary Materials-1 (Fig. S1). A week before the start of the behavioral experiments, each rat was numbered with earrings to be later identified. Accordingly, the data obtained in the present study (in each experiment) were known for each rat. The experiment was conducted first in the open field (days 1–3) and, given that testing in the MWM is quite stressful for the animal, thereafter in the maze (days 4–8). At the end of the behavioral experiments, six rats from each group were used by random sampling in the histological studies and six in immunoblotting.

Behavioral study: apparatus and procedures

Open field

The open field square arena ($65 \times 65 \times 75$ cm) was enclosed by wooden walls and illuminated by a 60 W light bulb mounted 1 m above the area used for the behavioral test. The floor of the arena was divided into 16 equal squares using white lines. During the sessions, four different (by color, shape and size) objects were simultaneously present in the open field. Habituation to the environment and objects was assessed using an open field test, as previously detailed (Dashniani et al. 2015). In brief, the rats were individually given three habituation (3 min) sessions (days 1–3), each of which was separated with a 24-h delay. Locomotor activity was assessed by counting the number of grids crossed by each animal when moving in the open field. The decrease in the number of crossings between session 1 and session 3 was taken to be a measure of habituation to the environment. The habituation score (difference score) was defined by subtracting the number of crossings in session 3 from the number of crossings during session 1 (Bolivar and Flaherty 2003; Lee et al. 2005). The amount of time spent by each animal during object exploration was recorded. The habituation score (difference score) was defined by subtracting the sum of the time spent exploring the four different objects in session 3 from the sum of time spent on the same objects during session 1—the larger the habituation score data, the greater the habituation.

Morris water maze

Long-term spatial memory was assessed in a Morris water maze (Morris et al. 1982). The test consisted of a training phase (days 4–7; four trials per day) and a retrieval phase. The task was adapted from Liu et al. (2015). A probe trial was used to evaluate memory retention, which was carried out 24 h after task acquisition. In the probe trial, the platform was removed from the pool and the rats were placed in the pool from a novel starting point. The time spent in each of the four quadrants was recorded and used for further analysis of memory retention performance. Spatial memory of the hidden platform location is indicated by preference for the test (Q_{test}) over opposite (Q_{opp}) quadrant (Ge et al. 2010). Following spatial reference memory testing, the rats were given a single session with four trials of cue training to assess sensorimotor abilities and their motivation to escape (day 8). The task was adapted from Bañuelos et al. (2013).

Histological evaluation

At the end of the behavioral experiments, a random sample ($n = 6$) for each group of animals was used for the

histological studies. To determine the effect of aging on the population of MS neurons, choline acetyltransferase-immunoreactive (ChAT-ir) and parvalbumin-immunoreactive (PV-ir) neurons as well as the total number of neurons were counted in the MS. Quantification of MS neurons was performed by microscopic examination of serial coronal sections stained with cresyl violet. Immunohistochemical methods were employed to determine the ChAT-ir, and PV-ir projection neurons in the MS. All reagents and buffers were purchased from Abcam and immunostaining was conducted under the appropriate protocols (<https://www.abcam.com/protocols/immunostaining-paraffin-frozen-free-floating-protocol>).

Electrophoresis and immunoblotting

Electrophoresis and immunoblotting were run in a random sample ($n=6$) for each group of animals. Each animal was deeply anaesthetized, via the respiratory route, by exposure to ~ 15 ml diethyl-ether for approximately 2 min, with continuous heart rate monitoring. The rats were decapitated when their heart rate reduced to approximately one beat per second. After decapitation, the hippocampus was removed and immediately frozen on dry ice. Tissue from the hippocampus of each rat formed one individual sample. The details of subcellular fractionation, Images of Western immunoblotting and calculation of relative amounts of proteins are provided in Supplementary Materials-1 (Fig. S3).

Statistical analysis

Levene's test for the equality of variance and K-means clustering (MacQueen 1967) was carried out by "R" (R Development Core Team 2016), whereas all other statistical analysis was performed using SigmaStat statistical software. All behavioral data were analyzed using a two-way repeated measures (one factor repetition), a one- or two-way analysis of variance (ANOVA). Further post hoc comparisons or two-tailed t tests were used, where appropriate. All the data are presented as a mean \pm standard error of the mean (SEM). Differences were considered significant when $P < 0.05$.

Results

Behavioral study; cognitive performance in young, middle-aged and aged rats

Middle-aged rats consists from two significantly different subgroups: with impaired and unimpaired memory

The methods of classification of middle-aged rats are described in detail in Supplementary Materials-1.

As mentioned above, spatial reference memory was assessed via a MWM task in a probe trial 24 h after task acquisition (MWM task—day 8; Supplementary Materials-1—Fig. S1). Spatial memory of the hidden platform location is indicated by preference for the Q_{test} over Q_{opp} . The results of the Levene's test for the equality of variance showed that the data obtained in the spatial reference memory test in young-adult, middle-aged and aged groups are not homogeneous; the middle-aged group has a larger variance than the other two groups (Supplementary Materials-1—Fig. S4). We applied the well-known k-means clustering algorithm (MacQueen 1967) on both time spent in the Q_{opp} and Q_{test} for identifying two homogeneous sub-clusters within the middle-aged group (Supplementary Materials-1—Fig. S5). A first cluster, named "middle-aged-unimpaired" (MA/U) contains 17 rats with high time spent in the Q_{test} and low time spent in the Q_{opp} , indicating preserved long-term spatial memory. The second cluster, named "middle-aged-impaired" (MA/I) contains 19 rats that do not show the preference for Q_{test} , indicating impaired spatial memory capability. Following the separation of the middle-aged group in MA/I and MA/U, the Levene's test is not significant anymore ($P=0.067$), that is, the four groups are now homogeneous. Accordingly, the analysis of the data obtained in the study was conducted over four groups of animals: young-adult (Y; $n=15$), middle-aged-unimpaired (MA/U; $n=17$), middle-aged-impaired (MA/I; $n=19$) and aged (A; $n=18$).

Although the classification of the middle-aged sub-groups is based on the data obtained in the spatial reference memory test in the MWM at the end of the behavioral experiments, the results of the experiments in the open field and MWM are presented in chronological order and the presented statistical analysis of behavioural, immunohistochemical and biochemical data were carried out on four groups.

Open field

Habituation to the environment is impaired in MA/I and aged rats

The two-way repeated measures ANOVA for locomotor activity (the number of grid crossings) showed a significant effect of group ($F_{3,206}=7.152$, $P < 0.001$), a significant effect of session ($F_{2,206}=352.989$, $P < 0.001$) and interaction between group and session ($F_{6,206}=25.673$, $P < 0.001$). A significant difference between sessions was found for the locomotor activity of the young-adult ($F_{2,44}=283.999$, $P < 0.001$), MA/U ($F_{2,50}=144.673$, $P < 0.001$), MA/I ($F_{2,56}=103.268$, $P < 0.001$) and aged ($F_{2,53}=4.554$, $P < 0.015$) groups. Although all groups showed a decrease in locomotor activity, when analyzing the number of grid crossings on day 3 (Tukey Test), the rats of MA/I and aged groups demonstrated significantly more grid crossings on day 3 compared to rats of young-adult and MA/U groups (Y vs. MA/I— $P < 0.001$; Y vs. A— $P < 0.001$; MA/U vs.

MA/I— $P < 0.001$; MA/U vs. A— $P < 0.001$). It was also revealed that the aged rats showed significantly fewer grid crossings on day 1 than the rats of young-adult, MA/U and MA/I groups ($P < 0.001$, for all comparisons). Figure 1A shows the effect of aging on the number of grid crossings [a post hoc (Tukey Test) analysis of the differences between sessions 1, 2 and 3 for the four separate groups].

The decrease in the number of grid crossings between session 1 and 3 was taken to be a measure of habituation to the environment. Figure 1B reveals the effect of aging on habituation to the environment. The one-way ANOVA for the habituation score data showed a significant effect of group ($F_{3,68} = 47.729$, $P < 0.001$). While the post hoc analysis (Holm-Sidak method) showed no significant difference between young-adult and middle-aged-unimpaired rats ($t = 0.675$, $P = 0.502$), and a significant difference between young-adult and aged rats ($t = 10.427$, $P < 0.01$); between young-adult and MA/I ($t = 4.323$, $P < 0.01$), between MA/I and MA/U ($t = 3.757$, $P = 0.025$), and between MA/I and aged rats ($t = 6.543$, $P = 0.013$). These results indicate that habituation to the environment deteriorated in MA/I and aged rats.

Habituation to the objects is impaired in middle-aged and aged rats The amount of time spent by each animal during object exploration was recorded. The two-way repeated measures ANOVA for object exploration showed a significant effect of session ($F_{2,206} = 49.724$, $P < 0.001$) and group ($F_{3,206} = 34.615$, $P < 0.001$), and a significant interaction between group and session ($F_{6,206} = 10.667$,

$P < 0.001$). The one-way ANOVA showed significant differences between sessions for the object exploration of the young-adult group ($F_{2,44} = 51.811$, $P < 0.001$) and the MA/U group ($F_{2,50} = 28.769$, $P < 0.001$). While no significant differences were found in the object exploration of the MA/I ($F_{2,56} = 1.325$, $P = 0.274$) and aged groups ($F_{2,53} = 0.213$, $P = 0.809$). Analyzing the time spent on object exploration on day 3 (Tukey Test), the rats in the MA/I and aged groups exhibited significantly longer on object exploration than the young-adult and MA/U groups (Y vs. MA/I— $P < 0.001$; Y vs. A— $P < 0.001$; MA/U vs. MA/I— $P < 0.001$; MA/U vs. A— $P < 0.001$). Figure 2A shows the effect of aging on the time spent on object exploration [a post hoc (Tukey Test) analysis of the differences between sessions 1, 2 and 3 for the four separate groups].

The decrease in time spent exploring the four objects between session 1 and session 3 was taken to be a measure of habituation towards the objects. The one-way ANOVA for the habituation score data showed a significant effect of group ($F_{3,68} = 18.022$, $P < 0.001$). The post hoc analysis showed a significant difference between young-adult and all three groups (Y vs. MA/U— $t = 3.719$, $P = 0.02$; Y vs. MA/I— $t = 5.799$, $P = 0.001$; Y vs. A— $t = 6.562$; $P = 0.001$), a significant difference between MA/U and aged groups ($t = 3.070$, $P = 0.017$); and no significant difference between the MA/I and aged groups ($t = 0.710$, $P = 0.481$). These results indicate that middle-aged and aged rats were impaired in the habituation to the objects. Figure 2B shows the effect of aging on habituation to the objects.

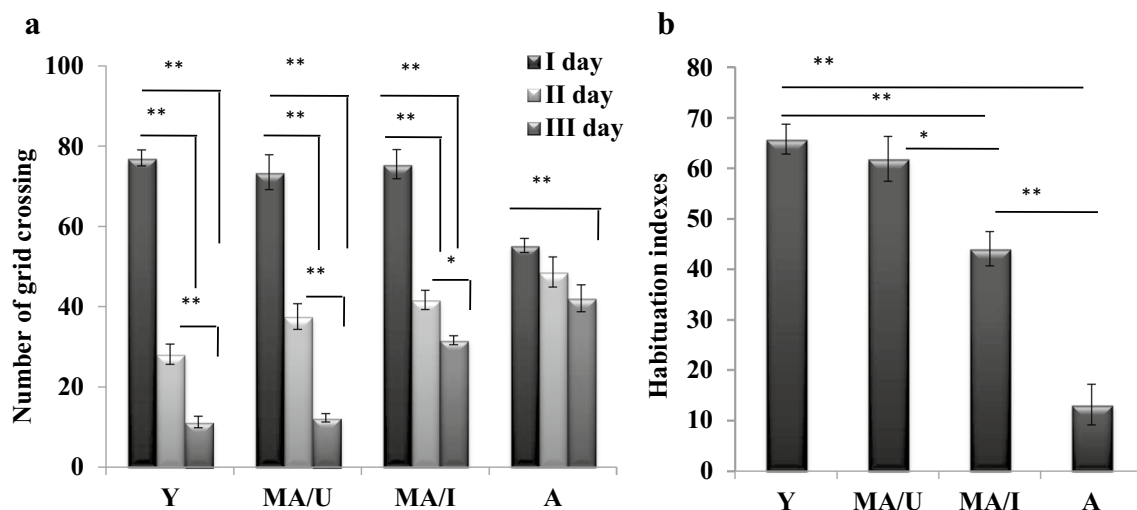


Fig. 1 The effect of aging on habituation to the environment in the open field (Y—young-adult, MA/U—middle-aged-unimpaired, MA/I—middle-aged-impaired and A—aged). **A** The number of grid crossings in sessions 1–3 in the open field. The rats of all groups showed a significant difference between sessions 1 and 3 in the num-

ber of grid crossings. $**P < 0.000$, $*P < 0.02$. **B** The habituation score (difference score—DS) for the number of grid crossings. $**P < 0.01$, $*P < 0.03$. Note: habituation to the environment deteriorated in middle-aged-impaired and aged rats. The data is given as a mean \pm SEM

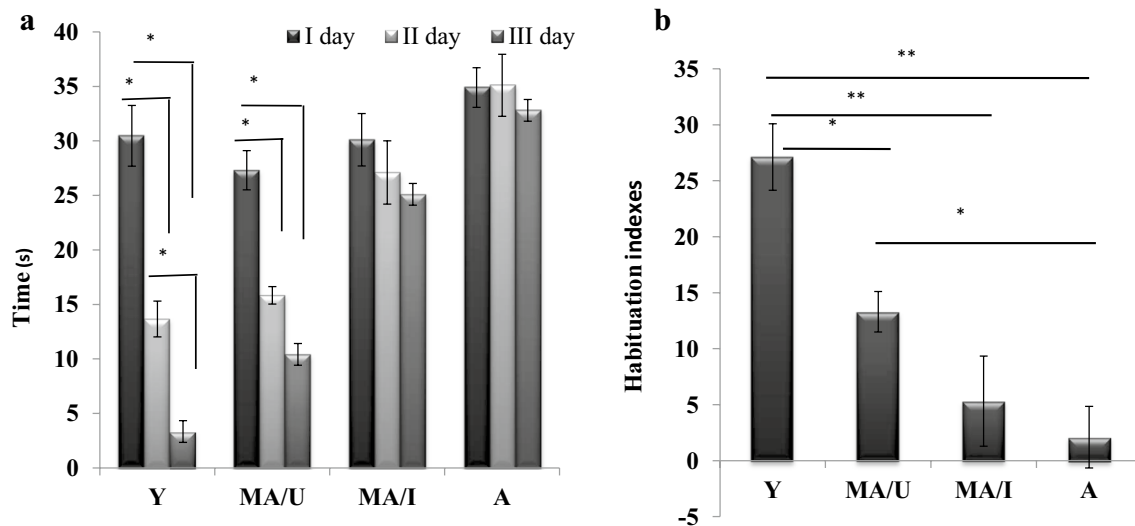


Fig. 2 The effect of aging on habituation to the objects in the open field (Y—young-adult, MA/U—middle-aged-unimpaired, MA/I—middle-aged-impaired and A—aged). **A** The time spent for object exploration in sessions 1–3 in the open field. Rats in the young-adult and the middle-aged-unimpaired groups showed significant differences between sessions for object exploration; there were no sig-

nificant differences in the rats of the middle-aged-impaired and aged groups. $*P < 0.000$. **B** The habituation score (difference score—DS) for object exploration. $**P < 0.001$, $*P < 0.02$. Note: the middle-aged and aged rats were impaired in habituating to the objects. The data is given as a mean \pm SEM

Morris water maze

Spatial reference memory is impaired in MA/I and aged rats The two-way repeated measures ANOVA for the learning process revealed significant main effects for group ($F_{3,275} = 13.777$, $P < 0.001$), day ($F_{3,275} = 641.709$, $P < 0.001$) and the interaction group \times day ($F_{9,275} = 2.984$, $P = 0.002$), thus indicating variations in animal's ability to locate the submerged platform. The one-way ANOVA showed significant differences between days for the latency in reaching the hidden platform of the young-adult ($F_{3,59} = 102.829$, $P < 0.001$), MA/U ($F_{3,67} = 30.239$, $P < 0.001$), MA/I ($F_{3,75} = 180.323$, $P < 0.001$) and aged ($F_{3,71} = 213.165$, $P < 0.001$) groups. When analyzing training phase days 1–4 animals of all groups showed significantly decreased latency in reaching the hidden platform. There are significant differences in escape latency between four training days in all groups (post-hoc Tukey test $P < 0.001$, for all training days in each group; Supplementary Materials-1—Table S1), although rats in the MA/I and aged groups exhibited a longer latency reaching the hidden platform on day 4 in comparison to the young-adult and MA/U groups (Y vs. MA/I— $P = 0.004$; Y vs. A— $P < 0.001$; MA/U vs. MA/I— $P < 0.001$; MA/U vs. A— $P < 0.001$). There was no significant difference found between the young-adult and MA/U, or between the MA/I and aged groups in reaching the hidden platform on day 4 (Fig. 3A).

A probe test was performed 24 h after task acquisition to assess spatial reference memory. Spatial memory

of the hidden platform location is indicated by preference for a Q_{test} over the Q_{opp} . An ANOVA was used to examine between-group differences on probe-test performance indices. In the 24 h after training, the two-way ANOVA showed a significant effect of group ($F_{3,137} = 7.488$, $P < 0.001$), a significant effect of quadrant ($F_{1,137} = 145.377$, $P < 0.001$) and significant interaction between group and quadrant ($F_{3,137} = 58.697$, $P < 0.001$). The results of the post hoc (Tukey Test) analysis of the differences for time spent in Q_{test} and Q_{opp} identified a significant difference between quadrants in the young-adult and MA/U groups (Y— $P < 0.001$; MA/U— $P < 0.001$). While there was no significant difference between time spent in the quadrants for the aged ($P = 0.567$) and MA/I ($P = 0.598$) groups (Fig. 3B).

To determine whether rats in each groups learned the location of the hidden platform, the time spent in the target quadrant was compared to 15 s (chance level), using a one-sample t test. During the probe test performed 24 h after task acquisition, the trained rats in the young-adult and MA/U groups spent significantly longer than chance (15 s, the dotted lines on Fig. 3B) in the Q_{test} where the hidden platform was located during the training trials (Y: 21.061 ± 1.181 , $t = 5.130$, $P < 0.001$; MA/U: 25.656 ± 1.1219 , $t = -8.741$, $P < 0.001$). In contrast, the rats of the aged groups spent significantly less time than chance within the Q_{test} (13.758 ± 0.448 , $t = 2.770$, $P = 0.009$), and no significant difference between the time spent in the target quadrant and chance level in MA/I group (13.789 ± 0.844 , $t = 1.423$, $P = 0.163$). It should be

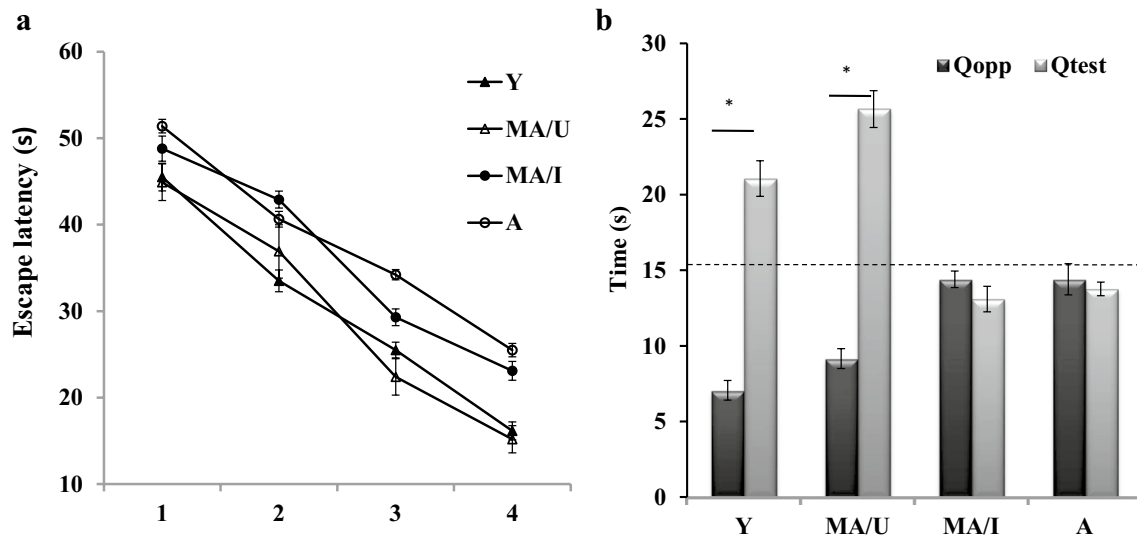


Fig. 3 The effect of aging on spatial learning and memory function assessed in the Morris water maze test (Y—young-adult, MA/U—middle-aged-unimpaired, MA/I—middle-aged-impaired and A—aged). **A** The average escape latencies of rats in days 1–4 of the training phase. The rats of all groups showed significantly decreased latency reaching the hidden platform. **B** Probe-test performance of rats from different groups 24 h after training. The dotted line indicates the chance level – 15 s (the quadrant time expected from a random search of the water maze). Long-term spatial memory for the location of the hidden platform is indicated by preference for the Q_{test} over Q_{opp} . Note: The rats in the middle-aged-impaired and aged groups exhibited a retention deficit 24 h after training. The data is given as a mean \pm SEM. $*p < 0.001$

noted that without exception, all young rats spent much longer than 15 s in the Q_{test} , while for aged rats an almost equal time was spent in every quadrant. The results suggest that 24 h later the rats in the MA/I and aged groups could not remember the information acquired during training.

In the visible platform task, a one-way ANOVA analysis showed no significant latency differences between groups in reaching the platform ($F_{3,68} = 0.309, P = 0.819$). These results show that there were no differences in the sensorimotor abilities or escape motivation between groups (data not presented).

Age and cognitive comparisons of ChAT, PV immunoreactive and the Nissl stained cells numbers in the MS

To determine the effect of aging on the population of MS neurons, ChAT-ir and PV-ir neurons, as well as the total number of neurons were counted in the MS. The mean number of ChAT-ir and PV-ir neurons in the MS in different groups of rats and the representative microphotographs of the ChAT and PV positive staining of MS sections are presented in Figs. 4, 5. The representative microphotographs of MS Nissl staining sections and the data of Nissl

counts the chance level – 15 s (the quadrant time expected from a random search of the water maze). Long-term spatial memory for the location of the hidden platform is indicated by preference for the Q_{test} over Q_{opp} . Note: The rats in the middle-aged-impaired and aged groups exhibited a retention deficit 24 h after training. The data is given as a mean \pm SEM. $*p < 0.001$

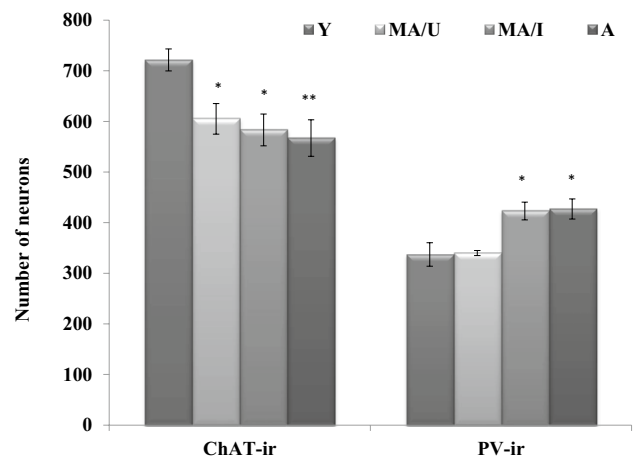


Fig. 4 The morphological changes in the MS of young-adult (Y), middle-aged-unimpaired (MA/U), middle-aged-impaired—(MA/I) and aged (A) rats. The choline acetyltransferase immunoreactive (ChAT-ir) cell number significantly reduced in MA/U, MA/I and aged rats relative to young ($**P < 0.001, *P < 0.02$); the parvalbumin immunoreactive (PV-ir) cell number significantly increased in MA/I and aged rats relative to young ($*P < 0.001$). Note: A reduction with age in cholinergic neuron numbers in the MS was not related to spatial learning ability, rather the increase in the number of PV-ir neurons in middle-aged-impaired and aged rats confirms the importance of these neurons in cognitive function. The data is given as a mean \pm SEM

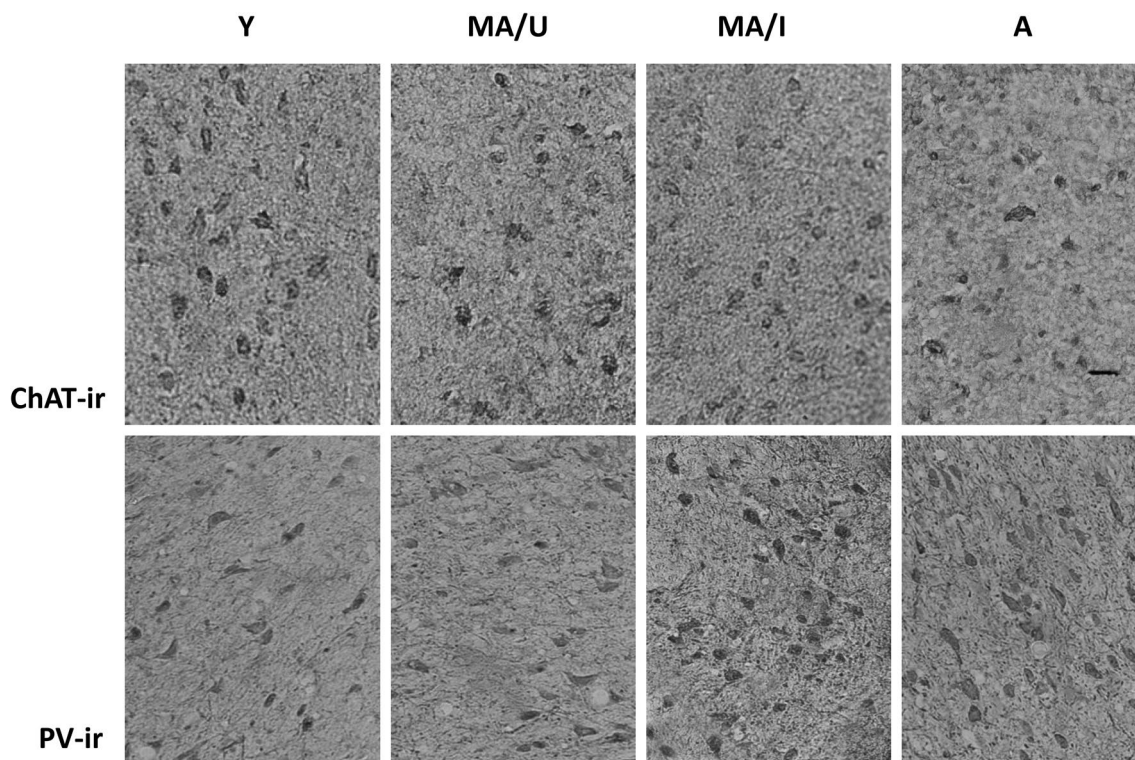


Fig. 5 Representative microphotographs of choline acetyltransferase (ChAT) and parvalbumin (PV) positive staining of MS sections for different groups of rats. Immunoreactivity for PV and ChAT was used to visualize the GABAergic and cholinergic MS neurons, respectively. The results showed a significant decline in the number of MS

ChAT-ir cells in aged (A), middle-aged-unimpaired (MA/U) and middle-aged-impaired (MA/I) rats relative to young-adults (Y). The PV-ir cell number was greater in middle-aged-impaired and aged rats relative to the young. Scale bar 40 μ m

stained cell numbers are presented in Supplementary Materials-1 (Fig.S2).

Number of ChAT immunoreactive neurons is decreased in middle-aged and aged rats

The one-way ANOVA revealed significant effect of the group ($F_{3,23} = 5.261$, $P = 0.008$). Post hoc (Holm-Sidak) analysis of differences showed a significant reduction in the number of ChAT-ir cells in aged rats relative to young (-21% , $t = 3.206$, $P = 0.009$). A comparison of the ChAT-ir cells among the young and the middle-aged rat sub-groups (unimpaired and impaired) indicated a similar trend toward decreased ChAT-ir cell numbers with age (-16% , $t = 2.702$, $P = 0.013$; -19% , $t = 3.206$, $P = 0.010$, respectively). There was no significant decrease in the number of ChAT-ir neurons in aged animals compared to the MA/U or MA/I groups ($t = 0.879$, $P = 0.390$; $t = 0.375$, $P = 0.712$, respectively).

Number of PV immunoreactive neurons is increased in MA/I and aged rats

One-way ANOVA revealed significant effect of the group ($F_{3,23} = 9.998$, $P < 0.001$). The results of the post hoc (Holm-Sidak method) analysis of the differences, relative to young, showed a significant increase in the number of PV-ir cells in aged and in MA/I rats ($+27\%$, $t = 4.16$, $P = 0.006$; $+26\%$, $t = 3.859$, $P = 0.008$, respectively). There was no significant difference in the number of PV-ir cells in MA/I rats relative to aged rats ($t = 0.157$, $P > 0.05$).

The Nissl stained cell number is stable with age and across cognitive groups

To investigate whether age- and cognition-related alterations in ChAT-ir and PV-ir neurons estimates contributed to an overall difference in neuron number in MS, Nissl stained cells were quantified. A one-way ANOVA analysis showed no significant differences between groups ($F_{3,23} = 0.510$, $P = 0.680$). Although the mean number of Nissl stained cells was numerically lower in MA/U, MA/I

and aged rats relative to young. However this difference was not significant (Supplementary Materials—Fig. S2).

The ratio of changes in the number of ChAT and PV immunoreactive neurons to the total number of cells

The ratio of ChAT-ir and PV-ir neurons to the total amount of neurons (TN) in MS was estimated by dividing the number of these neurons in each animal by the number of Nissl stained neurons in the same animals. A one-way ANOVA analysis for ChAT-ir/TN and PV-ir/TN neurons showed significant differences between groups ($F_{3,23} = 3.612, P = 0.032$; $F_{3,23} = 12.657, P < 0.001$, respectively). The differences between the groups follow the same trend as for the separate ChAT-ir analysis; the young group has significantly higher ratio as compared to all other groups (Post hoc Holm-Sidak method: Young vs aged— $t = 2.922, P = 0.009$, Young vs MA/U $t = 2.688, P = 0.010$ and Young vs MA/I $t = 2.308, P = 0.013$). There was no significant difference in aged rats relative to MA/I rats ($t = 0.234, P > 0.05$). Post hoc analysis of PV-ir/TN showed a significant differences in aged rats relative to young ($t = 4.783, P = 0.009$) and MA/U ($t = 4.401, P = 0.010$) rats; there was no significant difference between MA/U and young rats ($t = 0.382, P > 0.05$).

Based on these analyses, we conclude that the differences observed for ChAT-ir or PV-ir neurons between the groups does not reflect the changes in total number of neurons, but are specific to these types of cells.

Changes in neuroreceptor amounts; MA/I and aged animals express the similar patterns of differences

We investigated expression level of cholinergic, GABAergic and glutamatergic receptors in the hippocampus of behaviorally characterized rats. Sample films of internal standards and calibration plots for NR2B, GluR1, $\alpha 1$ GABAA, muscarinic M1 and $\alpha 7$ nACh are shown in Supplementary Materials-1 (Fig. S3).

NR2B subunits of the NMDA receptors are decreased in MA/I and aged groups

The one-way ANOVA showed a significant effect of group on the amount of NR2B ($F_{3,23} = 7.19, P = 0.002$). The planned comparisons revealed that the mean amount of the NR2B subunit of the NMDA receptor in the hippocampus decreased significantly in the MA/I and aged groups compared with the young-adult (MA/I vs. Y— $t = 2.45, P = 0.034, DF = 10$; A vs. Y— $t = 2.88, P = 0.016, DF = 10$) and the MA/U groups (MA/I vs. MA/U— $t = 3.21, P = 0.009, DF = 10$; A vs. MA/U— $t = 4.04, P = 0.002, DF = 10$). The difference between the young-adult and MA/U groups and between the MA/I and aged groups was not significant (Fig. 6).

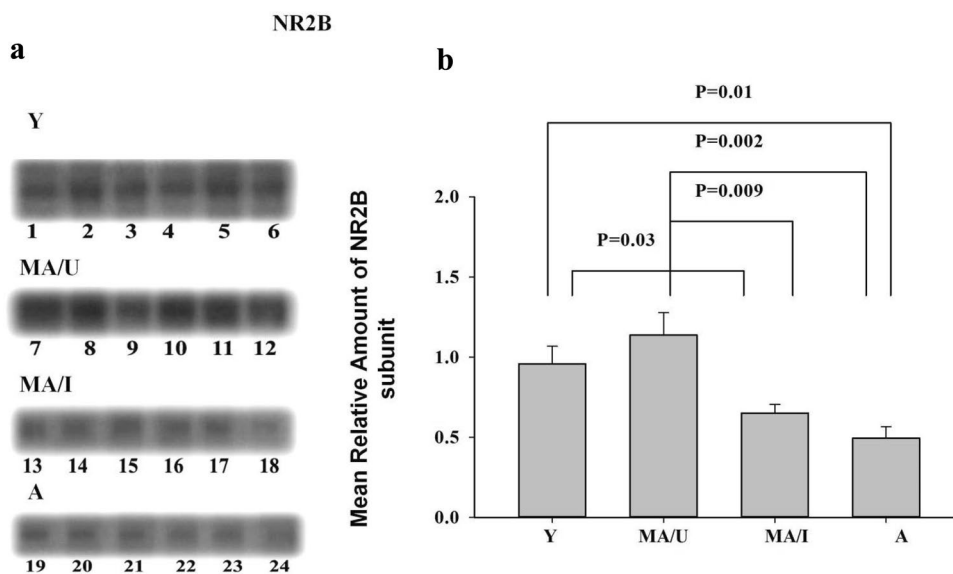


Fig. 6 Sample film (a) and mean levels (mean \pm standard error of the mean) (b) of hippocampal NR2B in different groups of rats. Each lane corresponds to one sample: lanes 1–6 are from the young-adult (Y) group; lanes 7–12 from the middle-aged-unimpaired (MA/U) group; lanes 13–18 from the middle-aged-impaired (MA/I) group; and lanes 19–24 from the aged (A) group. The gels also contained

irrelevant intercalated lanes, which have been excluded from the representative image. The full blot images for all neuroreceptors are provided as supplementary figures S6–S15. The same applies to the Figs. 7, 8, 9 and 10. Note: Young and middle-aged-unimpaired rats express a higher level of NR2B

GluR1 subunits of the AMPA receptors are decreased in MA/I and aged groups

GluR1 levels were significantly influenced by the group factor (one-way ANOVA; $F_{3,23} = 18.96$, $P = 0.000$). The mean amount of the GluR1 subunit of the AMPA receptor in the hippocampus of the MA/I and aged rats is significantly less than in the young-adult (MA/I vs. Y— $t = 4.47$, $P = 0.002$, $DF = 10$; A vs. Y— $t = 2.70$, $P = 0.022$, $DF = 10$) and the MA/U groups (MA/I vs. MA/U— $t = 6.94$, $P = 0.000$, $DF = 10$; A vs. MA/U— $t = 5.34$, $P = 0.000$, $DF = 10$). These results indicate a significant difference between the MA/I and aged groups ($t = -2.73$, $P = 0.021$, $DF = 10$). The difference between the young-adult and MA/U groups was not significant (Fig. 7).

Fig. 7 Sample film (a) and mean levels (mean \pm standard error of the mean) (b) of hippocampal GluR1 in different groups of rats. Each lane corresponds to one sample: lanes 1–6 are from the young-adult (Y) group; lanes 7–12 from the middle-aged-unimpaired (MA/U) group; lanes 13–18 from the middle-aged-impaired (MA/I) group; and lanes 19–24 from the aged (A) group. Note: Young and middle-aged-unimpaired rats express a higher level of GluR1

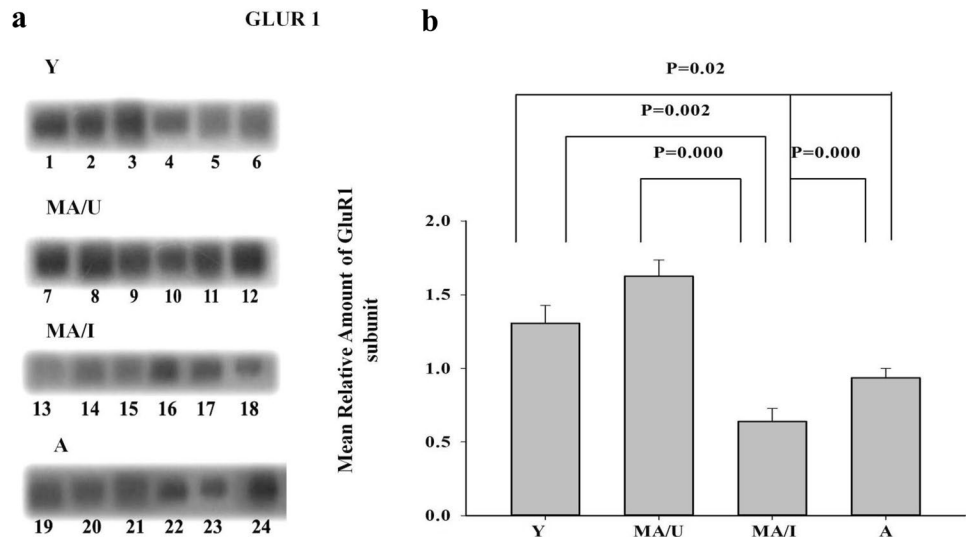
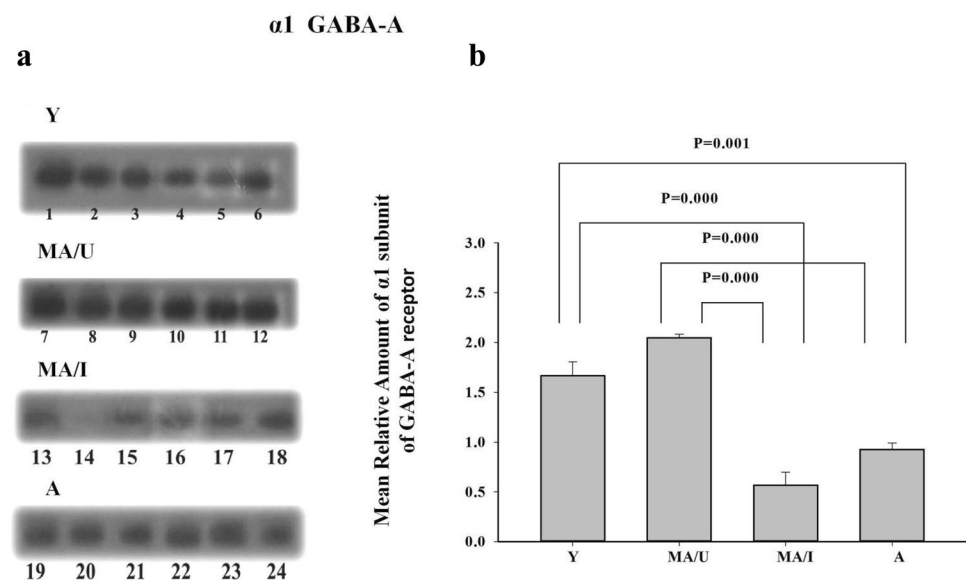


Fig. 8 Sample film (a) and mean levels (mean \pm standard error of the mean) (b) of the hippocampal $\alpha 1$ subunit of GABAA in different groups of rats. Each lane corresponds to one sample: lanes 1–6 are from the young-adult (Y) group; lanes 7–12 from the middle-aged-unimpaired (MA/U) group; lanes 13–18 from the middle-aged-impaired (MA/I) group; and lanes 19–24 from the aged (A) group. Note: Young and middle-aged-unimpaired rats express a higher level of $\alpha 1$



The $\alpha 7$ nACh receptors are decreased in MA/I and aged groups

The influence of the group factor was significant on changes of $\alpha 7$ nACh levels ($F_{3,23} = 4.80, P = 0.011$). The mean amount of the $\alpha 7$ nACh receptor in the hippocampus decreased significantly in the MA/I and aged groups compared to the young (MA/I vs. Y— $t = 2.61, P = 0.02, DF = 10$; A vs. Y— $t = 3.17, P = 0.010, DF = 10$) and the MA/U groups (MA/I vs. MA/U— $t = 2.30, P = 0.03, DF = 10$; A vs. MA/U— $t = 2.72, P = 0.021, DF = 10$). The difference between the young-adult and MA/U, and between the MA/I and aged groups was not significant (Fig. 9).

The M1 receptors are decreased in MA/I and aged groups

The one-way ANOVA showed a significant effect of the group factor on the amount of M1 ($F_{3,23} = 4.46, P = 0.015$). The MA/I and A groups expressed significantly less M1 cholinoreceptor compared to the young-adult (MA/I vs. Y— $t = 2.24, P = 0.04, DF = 10$; A vs. Y— $t = 2.98, P = 0.014, DF = 10$) and the MA/U groups (MA/I vs. MA/U— $t = 2.44, P = 0.035, DF = 10$; A vs. MA/U— $t = 3.01, P = 0.01, DF = 10$). The difference between young-adult and MA/U and between the MA/I and aged groups was not significant (Fig. 10).

A comparison of expression levels of NR2B, GluR1, $\alpha 1$ GABAA, muscarinic M1 and $\alpha 7$ nACh hippocampal

Fig. 9 Sample film (a) and mean levels (mean \pm standard error of the mean) (b) of hippocampal $\alpha 7$ nACh in different groups of rats. Each lane corresponds to one sample: lanes 1–6 are from the young-adult (Y) group; lanes 7–12 from the middle-aged-unimpaired (MA/U) group; lanes 13–18 from the middle-aged-impaired (MA/I) group; and lanes 19–24 from the aged (A) group. Note: Young and middle-aged-unimpaired rats express a higher level of $\alpha 7$

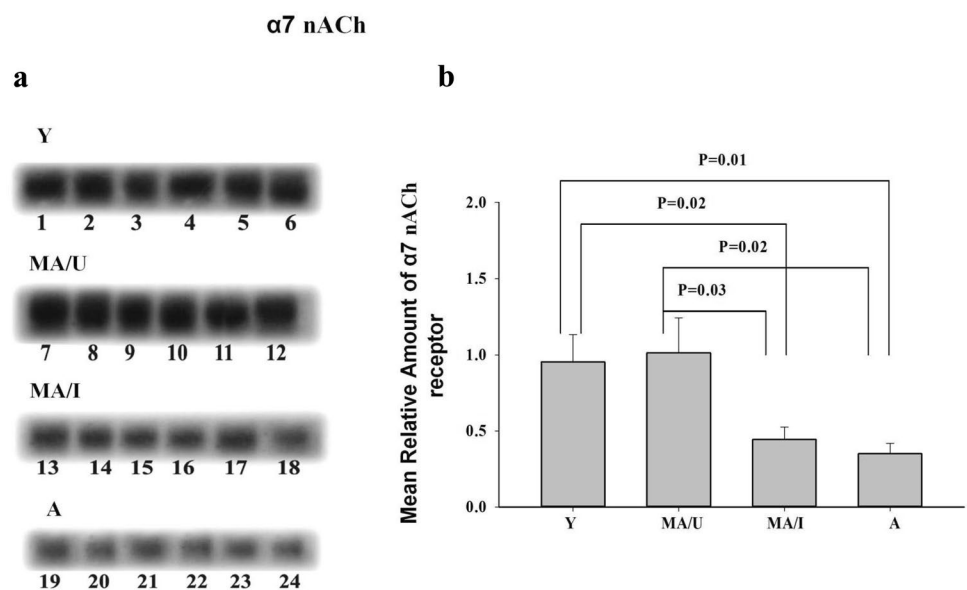
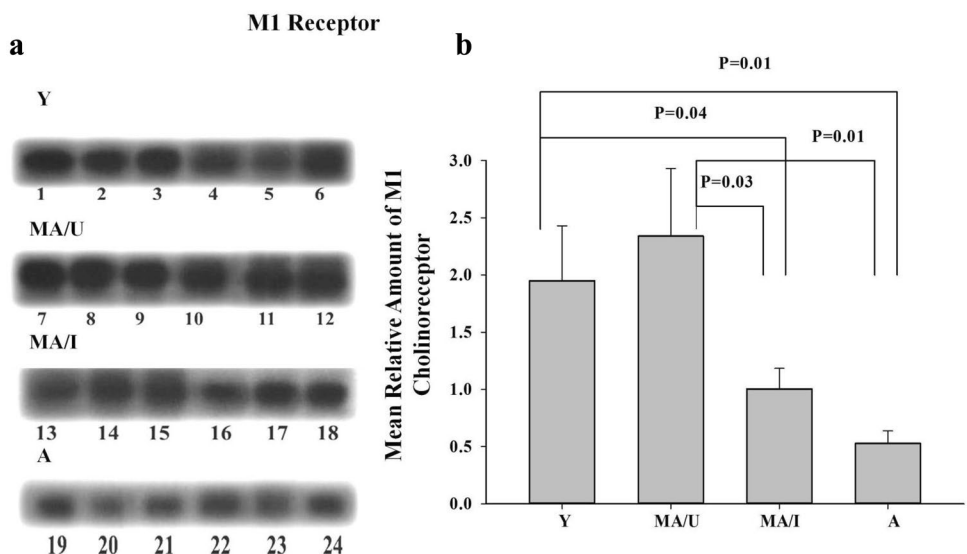


Fig. 10 Sample film (a) and mean levels (mean \pm standard error of the mean) (b) of hippocampal M1 in different groups of rats. Each lane corresponds to one sample: lanes 1–6 are from the young-adult (Y) group; lanes 7–12 from the middle-aged-unimpaired (MA/U) group; lanes 13–18 from the middle-aged-impaired (MA/I) group; and lanes 19–24 from the aged (A) group. Note: Young and middle-aged-unimpaired rats express a higher level of M1



receptors among young, aged and middle-aged rats subgroups indicate a significant decline in the expression level of all these receptors in the hippocampus of middle-aged-impaired and aged rats in comparison to young-adult and middle-aged-unimpaired rats.

Discussion

The present study aims to reveal whether age related mnemonic dysfunction is paralleled with changes in the amounts of hippocampal neuroreceptors and the immunoreactivity of cholinergic and GABAergic cells in the MS. The data obtained indicate significant age associated changes in the SH system. The present results demonstrate for the first time that a decrease in the expression level of cholinergic, GABAergic and glutamatergic receptors in the hippocampus of naturally aged rats with impaired cognitive abilities, runs parallel with an increase in the number of GABAergic neurons in the MS, and it further identifies the particular importance of inhibitory signaling for memory function in the SH network.

Cognitive performance in young, middle-aged and aged rats

The results of experiments in a water maze showed that all rats exhibited decreased latency in finding the hidden platform across the training trials. During the probe test, which was performed 24 h after task acquisition, the young-adult and around 47.2% of middle-aged trained rats showed normal spatial learning and memory abilities in the MWM task, however approximately 52.8% of middle-aged rats showed impaired spatial reference memory in the maze; these rats constituted the “middle-aged-impaired” group. Thus, rats within the middle-aged-impaired and aged groups exhibited a retention deficit 24 h after training. This result is consistent with studies that have reported the poorer performance of aged rats in MWM tasks (Taridi et al. 2014; Hamezah et al. 2017). It should also be noted that preliminary studies have shown that short-term spatial memory is not impaired in middle-aged and aged rats (unpublished data). Although rats in the middle-aged-impaired and aged groups exhibited a longer latency in reaching the hidden platform on day 4 compared to rats in the young-adult and middle-aged-unimpaired groups, learning does in fact take place.

To investigate the effect of natural aging on the habituation process, we chose a non-associative task in which there was no clear reward. In this open field task, aged rats displayed low level exploratory activity and impaired habituation towards a repeated spatial environment. In the first session, middle-aged rats with impaired spatial memory exhibited similar locomotor activity to that of young

and middle-aged rats with intact spatial memory, yet they showed impaired habituation like aged rats. Accordingly, we can conclude that impaired habituation to the environment in aged rats does not depend on changes in locomotor activity. Thus, the experiments in the open field indicate that middle-aged-impaired and aged rats showed impaired habituation to the environment. A similar conclusion was made in the study by Garceza et al. (2018), in which habituation to a new environment was evaluated in an experimental model of aging induced by the long-term administration of D-galactose and in naturally-aged rats. Our results reveal that rats in the middle-aged-impaired and aged groups were impaired in the habituation to objects. It should be noted that there was also a significant difference between middle-aged rats with intact spatial memory and young rats in habituating to objects. The fact that objects habituation was impaired in middle-aged rats with intact spatial memory suggests that age-related changes also occur in regions outside the hippocampus, including the structures involved in processing information on object familiarity. It should be noted that reduced habituation to objects also means impairment in processing information on object familiarity and consequently in object recognition memory. It is widely accepted that the perirhinal cortex plays an important role in object recognition memory (Aggleton et al. 2010).

It should be highlighted that the various learning procedures and tasks include different sensory, motor and motivational demands that may decline during aging and reduce learning and memory performance in aged rats, which could have several interpretations regarding the involvement of specific brain structures (e.g., the hippocampus) on different forms of learning and memory (discussed by Manrique et al. 2007). The results of the present study highlight that impaired habituation in the open field is independent of changes in motor activity and that there are no differences in sensorimotor abilities or escape motivation between the different ages (the escape latencies are similar in all groups) within a MWM visible platform task.

MS cholinergic (ChAT immunoreactive) and GABAergic (PV immunoreactive) neurons

The present study provides important information about age-related changes in the population of MS projection neurons, and how these changes are associated with mnemonic dysfunction and alterations in hippocampal neurochemistry. It has been shown that MS manipulation can affect various forms of memory (Givens and Olton 1990; Fitz et al. 2008; Roland et al. 2014; Okada et al. 2015; Gangadharan et al. 2016). The comparative analysis of our behavioral and immunohistochemical results revealed that, age-related impairment of habituation or spatial reference memory is not associated with a decrease in the number of ChAT-ir

neurons, since middle-aged rats, with changes only in the number of ChAT-ir MS neurons, had an intact memory. However, the increase in the number of PV-ir neurons in middle-aged-impaired and aged rats confirms the importance of these neurons in cognitive function. The changes in ChAT-ir and PV-ir neurons are not the consequence of the changes in total amount of neurons and thus are specific to the aging and cognition processes. These results are consistent with the study from Bañuelos et al. (2013), which found that a reduction with age in cholinergic neuron numbers in the rostral basal forebrain was not related to spatial learning ability, rather the increase in GABAergic projections (Glutamic acid decarboxylase 67 immunopositive—GAD67 immunopositive) was observed only in older rats with impaired spatial abilities. In this study an overall difference in neuron number in rostral basal forebrain NeuN (neuronal nuclear antigen—a biomarker for neurons) immunopositive cells were also quantified. Authors found that NeuN immunopositive cell number was stable with age and across cognitive groups.

Hippocampal receptors

As previously mentioned, alterations in SH projections and the subsequent effects on the hippocampal receptor expression associated with memory decline in natural aging are far less defined. In the present study, in parallel with our immunohistochemical assessment of age-related changes in the MS, we investigated the expression level of cholinergic, GABAergic, and glutamatergic receptors in the hippocampus for all age groups of rats. The Western Blot analyzes showed a significant decline in the cholinergic ($\alpha 7$ nACh and M1), GABAergic (the $\alpha 1$ subunit of GABAA) and glutamatergic (the NR2B subunit of NMDA and the GluR1 subunit of AMPA) receptors in the hippocampus of middle-aged-impaired and aged rats in comparison to young-adult and middle-aged-unimpaired rats. Thus, middle-aged-unimpaired rats with alterations to only MS cholinergic neurons do not show a significant decline the mean level of the hippocampal receptors evaluated in this study, whereas middle-aged-impaired and aged rats with alterations in the MS cholinergic and GABAergic neurons show a significant effect on the mean level of hippocampal receptors.

The importance of inhibitory signaling in the septohippocampal network for memory function

The results of the present study indicate that rats with age-related mnemonic disfunction have an increased number of GABAergic neurons in the MS, with a significant decrease in the expression of all, including GABAA, receptors. Therefore, it can be assumed that a decrease in the content

of GABAA receptors may be a response to increased inhibitory input from the MS. The results of our previous study (Dashniani et al. 2020) demonstrated that unlike selective lesions of MS cholinergic neurons, lesions of GABAergic neurons cause memory impairment with significant concomitant changes in the expression level of receptors in the hippocampus—a significant increase in the expression of the $\alpha 1$ subunit containing GABAA receptors, and a decrease in others. Since alterations in GABAergic MS neurons during natural aging, as well as in experiments with GABAergic MS lesions, regardless of the direction of the change, cause memory impairment, it is clear that modulation of inhibitory transmission is important for the normal functioning of the septohippocampal network and, accordingly, hippocampal function.

As mentioned above, MS GABAergic neurons are specifically connected with hippocampal inhibitory interneurons (Freund and Antal 1988). As such, the importance of this connection for memory processes has been widely discussed (Dwyer et al. 2007; Pang et al. 2011; Gonzalez-Sulser et al. 2014; Albert-Gascó et al. 2018). The significance of MS GABAergic neurons in hippocampal dependent memory function was supported by studies demonstrating the key role of MS GABAergic neurons in generating hippocampal theta, an oscillatory activity implicated in learning and memory (Buzsáki 2002). PV-ir neurons have moreover been shown to exhibit a bursting activity closely associated with hippocampal theta waves (Borhegyi et al. 2004; Bassant et al. 2005). It is noteworthy that descending projections from the GABA projection neurons of the hippocampus also provide descending feedback regulation of the MS (Toth et al. 1993; Gulyas et al. 2003; Yuan et al. 2017). Manseau et al. (2008) showed that hippocamposeptal inhibition facilitates theta rhythmic discharges in MS GABAergic neurons. The authors concluded that the hippocampal theta is the result of complex interactions between the septum and the hippocampus, and that GABAergic neurons have particular importance in generating hippocampal theta. Alongside this brief literature review on the particular role of GABAergic neurons in the generation of hippocampal theta and our conclusions that age-related changes in the number of GABAergic neurons in the MS plays a key role in memory deficits during natural aging, it is possible to conclude that inhibitory networks are particularly vulnerable to age-related dysfunction, and that such dysfunction can seriously affect cognition.

Conclusion

In summary, our results demonstrate: (a) an age-related decrease in the number of cholinergic and an increase in number of GABAergic neurons in the MS; (b) an age-related decreased expression of cholinergic, GABAergic

and glutamatergic receptors in the rat hippocampus; (c) the important contribution of alterations in the SH network to age-related mnemonic dysfunction; and (d) the particular importance of inhibitory signaling in the septohippocampal network for memory function. Further research into the causal mechanisms responsible for age-related SH network dysfunction may thus reveal new targets for the treatment of age-related memory decline.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00221-022-06354-2>.

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Author contributions MB and MD contributed to conception and design; MB, MD, VL and RS contributed to analysis and interpretation of data in the manuscript; GB, LK, NC, MC, and LT managed the data collection. Statistical analysis was done by MB, RS and VL. RS and TN revised the manuscript; all authors agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Availability of data and material All primary data are provided in Supplementary Materials-2.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethics approval All experimental procedures were conducted in accordance with the European Communities Council Directive Guidelines for the care and use of Laboratory animals (2010/63/EU—European Commission) and approved by the animal care and use committee at the I. Beritashvili Center of Experimental Biomedicine.

Consent for publication All co-authors have agreed to the submission of final manuscript.

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