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Preservation of Hippocampal Neuron Numbers and Hippocampal Subfield Volumes in Behaviorally Characterized Aged Tree Shrews

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ABSTRACT

Aging is associated with a decreased ability to store and retrieve information. The hippocampal formation plays a critical role in such memory processes, and its integrity is affected during normal aging. We used tree shrews (*Tupaia belangeri*) as an animal model of aging, because in many characteristics, tree shrews are closer to primates than they are to rodents. Young and aged male tree shrews performed a holeboard spatial memory task, which permits assessment of reference and working memory. Upon completion of the behavioral measurements, we carried out modified stereological analyses of neuronal numbers in various subdivisions of the hippocampus and used the Cavalieri method to calculate the volumes of these subfields. Results showed that the working memory of aged tree shrews was significantly impaired compared with that of young animals, whereas the hippocampus-dependent reference memory remained unchanged by aging. Estimation of the number of neurons revealed preserved neuron numbers in the subiculum, in the subregions CA1, CA2, CA3, and in the hilus of the dentate gyrus. Volume measurements showed no aging-related changes in the volume of any of these hippocampal subregions, or in the molecular and granule cell layers of the dentate gyrus of tree shrews. We conclude that the observed changes in memory performance in aging tree shrews are not accompanied by observable reductions of hippocampal neuron numbers or hippocampal volume, rather, the changes in memory performance are more likely the result of modified subcellular mechanisms that are affected by the aging process. J. Comp. Neurol. 468:509–517, 2004. © 2003 Wiley-Liss, Inc.

The hippocampal formation is an essential structure for the acquisition and consolidation of various forms of memories, including episodic-declarative and associative memories, and classical conditioning (Squire, 1992; Eichenbaum, 1999), which are subject to functional impairments over the course of aging (Rapp and Heindel, 1994; McEchron et al., 2001; Small et al., 2002; Wu et al., 2002). Many experimental paradigms are described that test for hippocampus-dependent memories both in humans and animals (Squire, 1992; Eichenbaum, 1999). In search of structural changes that underlie behavioral and cognitive impairments, various techniques are used to study aging-associated anatomical changes of the hippocampal formation (Harding et al., 1994; Keuker et al., 2001).

The hypothesis of a substantial age-related hippocampal neuron loss has been challenged since the introduction of modern stereological counting methods. In normally

aging humans, only a selective neuron loss was found in the subiculum and hilus (West, 1993) and in the subiculum and CA1 region (Simic et al., 1997). However, stereological analyses in young and aged rhesus monkeys revealed a preservation of hippocampal neurons (West et al., 1993; Berman et al., 1997; Keuker et al., 2003a). Moreover, several stereological studies in rodents allow the conclusion that hippocampal neuron numbers do not decline with advanced age (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Calhoun et al., 1998).

Whereas hippocampal neurons do not disappear in significant numbers with advanced age, age-related memory impairment may be reflected in a reduced hippocampal volume. Apparently, the hippocampal volume in humans correlates with deficits in hippocampus-dependent recognition memory (Golomb et al., 1994, 1996; Lupien et al., 1998). Magnetic resonance imaging (MRI) volumetric analyses in humans demonstrated that aging is associated with a reduction of the hippocampal volume (Jack et al., 1997; Schuff et al., 1999; Tisserand et al., 2000; Bigler et al., 2002). To date, postmortem volumetric studies of the human hippocampal formation have not shown such consistency. One study in cognitively normal humans aged between 16 and 99 years revealed a negative correlation between hippocampal volume and age (Simic et al., 1997). However, another postmortem study in humans aged 45 to 86 years showed no age-related change in total hippocampal volume (Harding et al., 1998).

The number of animal studies that have examined age-related changes in hippocampal volume is surprisingly small, and the data reported are conflicting. A preliminary study in rhesus monkeys reported a negative correlation between hippocampal volume and age (Berman et al., 1997). In contrast to this primate study, the hippocampal volume of aged Sprague-Dawley rats was increased compared with that of adults (Amenta et al., 1998). Old Fischer 334 rats had increased volume of the outer two-thirds of the molecular layer of the dentate gyrus, the total molecular layer, the hilus, and the regio inferior (corresponding to CA3; Coleman et al., 1987). More subtle changes were observed in the dentate gyrus of aged Long-Evans rats, in that the middle portion of the dentate gyrus molecular layer was selectively decreased, whereas the inner molecular layer was correspondingly expanded (Rapp et al., 1999). However, only very old, but not aged deer mice had a reduced hippocampal volume compared with young individuals (Perrot-Sinal et al., 1998). Even so, the volume of the CA1 region and dentate gyrus of young, adult, and aged C57BL/6J (B6) mice was the same (Calhoun et al., 1998).

There is extensive evidence that the hippocampal formation is associated with spatial memory performance. Consequently, many aging studies have aimed at establishing a link between spatial memory impairment and changed neuron numbers in, and volume of the hippocampal formation. However, hippocampal neuron numbers were preserved in aged rats, whereas hippocampus-dependent spatial memory was impaired only in a subgroup of aged rats (Rapp and Gallagher, 1996; Rasmussen et al., 1996). In non-human primates, preliminary studies have combined the analysis of hippocampal neuron numbers and hippocampal volumes in the assessment of non-spatial hippocampus-dependent memory only (West et al., 1993; Berman et al., 1997). In humans, MRI analysis showed a reduction of the hippocampal volume in those persons who performed more poorly in a spatial maze (Lupien et al., 1998).

In the current study, we aimed to further investigate age-related changes in hippocampus-dependent memory and its structural correlates in the hippocampal formation. We used a spatial memory task prior to the evaluation of hippocampal neuron numbers and hippocampal volumes in tree shrews (*Tupaia belangeri*), a non-rodent mammalian species. Originally regarded as primitive primates (Le Gross-Clark, 1956), tree shrews are nowadays considered an intermediate between insectivores and primates and are placed in the separate order Scandentia (Starck, 1978; Martin, 1990). The life span of the day-active tree shrews in the wild is still unknown, but under laboratory conditions the longevity is approximately 10 years (Fuchs, 1999). In many characteristics, tree shrews are closer to primates than they are to rodents. Certain tree shrew genes show a high degree of homology with human genes, such as those for several receptor proteins of neuromodulators and the amyloid-β precursor protein (Meyer et al., 1998; Palchaudhuri et al., 1998, 1999; Pawlik et al., 1999; Meyer et al., 2000). This raises the possibility that tree shrews may become an alternative model for studying age-related brain changes in socially homogenous and stable cohorts (Fuchs and Flügge, 2002).

MATERIALS AND METHODS
Experimental animals

Five young-adult (±1 year of age) and six aged (4–9 years of age) male tree shrews (*Tupaia belangeri*) from the breeding colony at the German Primate Center (Göttingen, Germany) were used (for details about housing see Fuchs, 1999). Tree shrews reach maturity after about 3 months of age. Because the life span of tree shrews under laboratory conditions is approximately 10 years, or about one-eighth of the human life span, the age of our oldest animal corresponded to a human age of 72.

All animal experimentation was conducted in accordance with the "Principles of Laboratory Animal Care" (NIH publication no. 86-23, revised 1985) and the European Communities Council Directive of November 24th, 1986 (86/EEC) and were approved by the Government of Lower Saxony, Germany.

Behavioral testing

The test apparatus consisted of an opaque PVC board (65 × 20 × 1 cm) in which 23 circular holes (2.0 × 0.7 cm) were arranged in three parallel lines (Ohl et al., 1998; Bartolomucci et al., 2002). The holes were covered by self-closing lids in order to avoid visual and olfactory cues of the hidden rewards (small pieces of dried almond). Each animal had its own holeboard.

In a 4-day habituation period, the animals were accustomed to the presence of the holeboard within their home cages. In this period, the animals learned to search for the rewards by opening the lids that covered the holes. The habituation period was followed by a 4-day learning phase, in which the performance of the animals reached a baseline level. Then we started the actual experiment, in which the animals were tested during 4 consecutive days. Two random distributions of five baited holes were presented each day. Three trials were performed for each distribution. In the first two trials (cue-directed), the

baited holes were marked with colored tape on the top of the lids. In the third trial (non-cue-directed), these marks were removed.

The intertrial interval within one distribution was 15 minutes, and the interval between two distributions was 30 minutes. A trial started when the animal voluntarily reached the holeboard. A trial was finished when the animal had retrieved all five rewards. Trials that were not started within 5 minutes, as well as trials that were not finished within 7 minutes, were considered as nonperformed trials, and therefore not analyzed. The number of wrong choices (opening of a nonbaited hole) and repeated choices (reopening of a previously emptied hole) per trial were considered as specific and simultaneous measures of reference and working memory, respectively. These types of memory might reflect different underlying central processes (Van der Staay, 1999). For data analysis, we performed a two-way analysis of variance (ANOVA) and the Tukey HSD post hoc test, with group (young and aged) as the between-groups factor and memory error type (wrong and repeated choices) as the within-subjects factor.

Perfusion and tissue preparation

One day after the last memory test, the animals were anesthetized by an intraperitoneal administration of an overdose of ketamine (Ketavet®, Pharmacia & Upjohn, Erlangen, Germany), xylazine (Rompun®, Bayer, Leverkusen, Germany), and atropine (WDT, Hannover, Germany) 5:1:0.01. The descending aorta was clamped, and the animals were perfused transcardially with cold 0.9% NaCl for 3 minutes, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 12 minutes. To prevent the development of postperfusion artifacts, the heads were postfixed in fresh fixative at 4°C (Cammermeyer, 1978). On the following day, the brains were gently removed and stored in 0.1 M PB at 4°C. For cryoprotection, the brains were immersed in 2% dimethylsulfoxide (DMSO) and 20% glycerol in 0.125 M phosphate-buffered saline (PBS) at 4°C. The right hemispheres were then dissected into blocks that contained the entire hippocampal formation. The tissue blocks were frozen on dry ice and stored at −80°C before serial sectioning at a thickness of 50 μm on a Leica cryostat microtome. A stereotaxic brain atlas of the tree shrew (Tigges and Shantha, 1969) was used for reference during the dissecting and cryosectioning procedures. Because the orientation of the hippocampal formation is almost vertical (see Fig. 1A), we chose to cut horizontal sections, which provided the best condition for subfield delineation and a sensible basis for estimation of neuron number (Keuker et al., 2003b).

A total of 10 systematic series of horizontal sections per hippocampal formation were collected in 0.1 M PBS. One series was chosen for stereological evaluation by selection of a random number between 1 and 10. The sections were mounted on glass slides in a 0.1% gelatin solution. After overnight drying at 37°C, the sections were defatted and stained with cresyl violet. Care was taken during the staining procedure to ensure that all neuronal compartments could be identified. Then the sections were cleared in xylene, mounted with Eukitt, and coverslipped. Photomicrographs were taken using a Zeiss Axiophot II photomicroscope (Carl Zeiss). The original negative films were scanned at the original size with 1,200 dpi resolution. Adobe Photoshop software was used for minor darkness/brightness adjustments. The sharpness of the images was
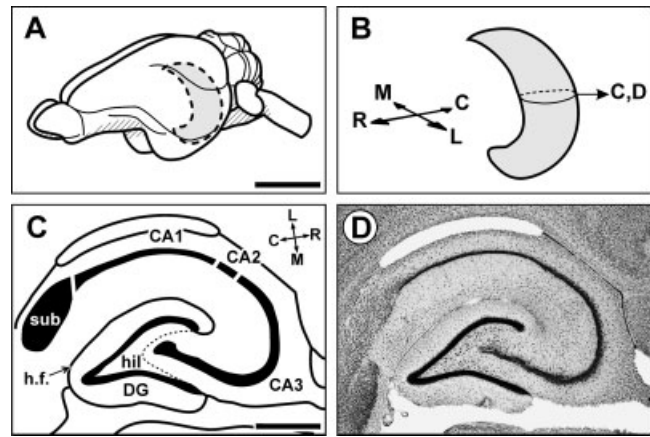

Fig. 1. Macro- and microscopical anatomy of the tree shrew hippocampal formation. **A:** Line drawing of the tree shrew brain showing the general orientation of the hippocampal formation. The lateral view shows the hippocampal formation (gray) for the left hemisphere. The dashed lines indicate that the hippocampal formation is covered by the cortex. **B:** The isolated hippocampal formation shows the level of the images in C and D. **C:** Schematic drawing of the hippocampal subfields through the middle part of the hippocampal formation in tree shrews. **D:** Nissl staining in a section that exactly corresponds to C. C, caudal direction; L, lateral direction; M, medial direction; R, rostral direction; sub, subiculum; CA1, CA2, and CA3, CA1, CA2, and CA3 subfields of the hippocampal formation; DG, dentate gyrus; hil, hilus of the DG; h.f., hippocampal fissure. Scale bar = 1 cm in A; 1 mm in C (also applies to D).

increased slightly. The pictures did not require any modification, such as for artifacts.

Stereological evaluations

Prior to the stereological neuron counting and volumetric measurements, the animal numbers were coded, and the code was only revealed when all data had been collected. All measurements were carried out by one experimenter (J.I.H.K.) on a Zeiss III RS microscope, to which a defined stepping motor in the x,y-axes (LUDL Electronic Products, Hawthorne, NY) and an electronic microcator (Heidenhahn MT 12, Traunreut, Germany) were attached. STEREOINVESTIGATOR 3.16 software (MicroBrightField, Colchester, VT) was used for counting neurons and for measuring the subfield areas (for details on the design, see Keuker et al., 2001).

An overview of the Nissl staining and a schematic drawing of the various subfields of the tree shrew hippocampal formation are given in Figure 1D and C, respectively. The images of Figure 1 are taken from our previous study, which deals extensively with the cytoarchitecture of the tree shrew hippocampal formation (Keuker et al., 2003b). We applied the subfield definitions according to these earlier data. Detailed photomicrographs of the subfields subiculum, CA1, CA3, and hilus are presented in Figure 2.

Neuron numbers. The total unilateral neuron numbers of the tree shrew hippocampal fields of the subiculum, CA1, CA2, CA3, and hilus were estimated by using the modified optical fractionator technique. The optical fractionator is an unbiased counting method, which is independent of the size, shape, and orientation of the cells to be counted and which combines the optical disector (Sterio, 1984) with a fractionator-sampling scheme (Gun-
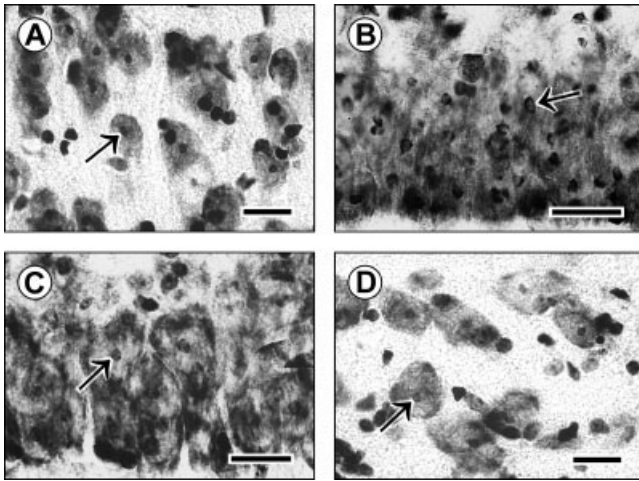
Fig. 2. Representative detailed photomicrographs of Nissl-stained cryosections (50 μm) of the tree shrew hippocampal formation. **A:** The subiculum. **B:** CA1 region. **C:** CA3 region. **D:** Hilus of the dentate gyrus. Arrows in A–D point to nucleoli, which were the counting criterion in the current study. In the cryosections, the outlines of the nuclei were rather difficult to distinguish, especially in the neuron-dense subfields of CA1 and CA3. The nucleoli, however, could always be recognized. Scale bars = 20 μm in A–D.

TABLE 1. Experimental Parameters Used With the Optical Fractionator[1]

	a(frame) (μm^2)	$A(x,y$ step) (μm^2)	asf	tsf	ssf
Subiculum[2]	25×25	150×150	0.0278	1	1/10
CA1[2]	22×22	150×150	0.0215	1	1/10
CA2[2]	22×22	75×75	0.0860	1	1/10
CA3[2]	25×25	250×250	0.0100	1	1/10
Hilus[3]	75×60	200×200	0.1125	1	1/10

[1]Area of the optical disector counting frame, a(frame); x and y step sizes, $A(x,y$ step); asf, area sampling fraction [a(frame)/$A(x,y$ step)]; tsf, thickness sampling fraction, calculated by the height of optical disector, h/section thickness, t (tsf = 1, due to modified optical disector); ssf, section sampling fraction.
[2]All evaluations were performed with a 100× objective lens (N.A. 1.3).
[3]Evaluations were performed with a 40× objective lens (N.A. 0.75).

TABLE 2. Counting Results From the Optical Fractionator[1]

	No. of sections	No. of frames per section	No. of counted neurons per optical disector	ΣQ^-
Subiculum	18 ± 1	26 ± 3	1.12 ± 0.10	514 ± 71
CA1	17 ± 1	27 ± 4	1.68 ± 0.18	779 ± 91
CA2	16 ± 1	13 ± 2	1.16 ± 0.10	240 ± 49
CA3	16 ± 1	16 ± 2	1.15 ± 0.18	279 ± 32
Hilus	15 ± 1	20 ± 4	1.46 ± 0.21	456 ± 89

[1]With settings as in Table 1: number of sections per hippocampal subfield, number of counting frames per section, number of counted neurons per optical disector, and number of total counted neurons per hippocampal subfield, ΣQ^-. Data are presented as mean ± SD.

dersen et al., 1988). Details of this method have been described before (West et al., 1991; Keuker et al., 2001). The experimental parameters for each of the hippocampal regions (Table 1) were established in a pilot experiment, and were uniformly applied to all animals. The optical disector frame area, a(frame), and the area sampling area, $A(x,y$ step), were selected such that approximately 1–2 neurons per optical disector were counted on average (Table 2). The pyramidal neurons of the subiculum and CA regions were identified with a 100× objective lens (N.A. 1.3). The more widely separated neurons in the hilus of the dentate gyrus were counted with a 40× objective lens (N.A. 0.75).

In the current experiment we applied the modified optical disector. This means that the height of the optical disector, h, equals the actual section thickness, t; hence, the thickness sampling fraction equals 1. We used the following optical disector counting rules: neurons were scored only when their nucleolus (or a part thereof) was within the optical disector counting frame, thereby not touching the exclusion lines. When a nucleolus touched both an inclusion line and an exclusion line, the nucleolus was not scored (see also Harding et al., 1994, 1998). If more than one nucleolus per neuron was encountered, which very rarely occurred, we applied the counting rules on the larger nucleolus, neglecting the smaller one.

Individual estimates of the total neuronal number (N) for each subfield of the hippocampal formation were calculated according to the following formula: N $= \Sigma Q^- \times$ 1/ssf $\times$ 1/asf $\times$ 1/tsf, where ΣQ^- = the sum of the counted neurons per subfield per animal, ssf = section sampling fraction, asf = area sampling fraction, and tsf = thickness sampling fraction (Table 1). To test the possibility that aging results in a diffusely distributed pattern of modest neuron loss, we additionally calculated the total neuron number as the sum of the neuron numbers in the subicu-

lum, CA1, CA2, CA3, and hilus. Note, however that, based on anatomical definitions (Keuker et al., 2003b), this is not the total neuron number of the hippocampal formation.

The corresponding individual CEs (coefficients of error) were calculated with the previously described formula (Keuker et al., 2001). This is a modified formula for systematic samples from a continuous brain structure with a relatively high homogeneity of neuron distribution (Gundersen et al., 1999) and is thus suited for systematically sampled sections along the long axis of the hippocampal formation. The group means of the neuronal estimates of each hippocampal subfield in the young and aged tree shrews were tested with the two-tailed unpaired Student's t-test, and the significance level was defined at $\alpha = 0.05$.

Volumetric estimations. The volumes of the hippocampal subfields were assessed in the same sections as those used for counting neurons. The volumes were estimated according to the formula based on the Cavalieri method: $V = \Sigma A \times t_{\text{nom}} \times$ 1/ssf, where ΣA = the summed areas of the delineated subfield (computed with STEREOINVESTIGATOR 3.16), t_{nom} = the nominal section thickness of 50 μm, and ssf = the section sampling section (Table 1). The subfield areas were delineated with a 2.5× objective lens (N.A. 0.08). The volume of the total dentate gyrus was achieved by summing the volumes of its molecular layer and granule cell layer. The volume of the total hippocampal formation was calculated from the volumes of all hippocampal subfields, including the subiculum. The volumes are reported as mm^3. The group means of the estimated volumes of all hippocampal subfields of the young and aged tree shrews were tested with a two-tailed unpaired Student's t-test, with a significance level of $\alpha = 0.05$.

RESULTS
Behavioral testing

The two-way ANOVA revealed a significant interaction between group (young and aged) and memory error type

Fig. 3. Memory performance of young (n = 5) and aged (n = 6) tree shrews on the modified holeboard. **A:** Young and aged tree shrews make approximately the same number of wrong choices, which reflect reference memory performance. **B:** Aged tree shrews make significantly more repeated choices than young animals. Repeated choices are errors due to impaired working memory. Bars represent mean number of choices per trial ± SEM. *, $P < 0.05$.

TABLE 3. Mean Estimated Individual Unilateral neuron numbers (N; $\times$ 10^3) in the Hippocampal Subfields of Young and Aged Male Tree Shrews; Mean Group Numbers (Mean N), SD, and Mean CEs[1]

Age (yr)	Subiculum	CA1	CA2	CA3	Hilus	Total no. of neurons
0.9	169	334	34	258	42	837
1.2	187	360	42	277	54	920
1.3	168	364	25	309	41	907
1.3	240	421	26	223	40	950
1.4	208	352	25	300	39	924
4.6	154	258	26	295	35	768
5.5	154	364	23	284	31	856
5.7	200	410	30	219	27	886
6.3	174	368	23	312	38	915
6.8	185	376	26	288	49	924
9.0	194	374	29	302	49	948
Young						
Mean N	194	366	30	273	43	908
SD	30	33	8	35	6	42
Mean CE[2]	0.051	0.047	0.066	0.065	0.057	
Aged						
Mean N	177	358	26	283	38	883
SD	20	52	3	33	9	65
Mean CE[2]	0.051	0.046	0.070	0.065	0.056	

[1]The total neuron number is the sum of total numbers of the subfields subiculum, CA1, CA2, CA3, and hilus.
[2]The mean CE was calculated as $\sqrt{\text{mean (CE}^2)}$.

TABLE 4. Young vs. Aged Tree Shrews[1]

	Mean N		
	Young (n = 5)	Aged (n = 6)	P value
Subiculum	194	177	0.28
CA1	366	358	0.78
CA2	30	26	0.23
CA3	273	283	0.64
Hilus	43	38	0.32
Total no. of neurons	908	883	0.48

[1]Mean estimated unilateral neuronal numbers (N: $\times 10^3$) and statistical results of the unpaired Student's t-test (two-tailed). The total neuron number is the sum of the total numbers of the subfields subiculum, CA1, CA2, CA3, and hilus.

(wrong and repeated choice) ($F_{1,9} = 9.65$, $P = 0.01$). Post-hoc analysis showed no significant difference in the hippocampus-dependent reference memory performance between young and aged tree shrews ($P = 0.71$, Fig. 3A). However, the working memory performance of the aged tree shrews was significantly reduced ($P = 0.04$), as shown by the higher number of mean repeated choices made by aged tree shrews compared with young animals (Fig. 3B).

Task acquisition, motivation (as measured by the time to reach the holeboard), and motility (time spent per hole) were not affected by age (data not shown).

Stereological evaluation

Neuron numbers. For each individual, estimated unilateral neuron numbers (N) in each hippocampal subfield for the young and aged tree shrews are shown in Table 3, as well as the mean group value of N, the standard deviation, and the mean CE. Statistical analyses of the mean neuron numbers of the young and aged animals did not reveal differences in any evaluated hippocampal subfield, nor were differences observed in the total neuron numbers (Table 4).

Using the experimental parameters for the modified optical fractionator that were established during a pilot experiment, the average number of counted neurons per subfield varied between 240 and 779 (Table 2). These counts were made in 15–18 sections on average per subfield, in 13–26 optical disectors on average per section. Therefore, our settings were in accordance with well-accepted recommendations by Gundersen and Jensen (1987). Moreover, the parameters yielded an adequate estimation precision for the current experiment: 1) the individual CE values (which have to be under 1.0) varied between 0.42 and 0.74 (not shown); and 2) the biological coefficient of variance (BCV)[2] (Keuker et al., 2001) was substantially higher than 50% of the total coefficient of variance (61–95%).

In the current study, we used a modified optical disector, which could have caused a potential bias. In contrast to the unbiased optical disector technique, we did not use guard zones at the top and bottom of the section. If we had used appropriate guard zones in the cryosections, however, we would have to use a relatively low optical disector height. Accordingly, we would need to increase the number of sampling sites, which probably would have caused another bias (Harding et al., 1994). Nonetheless, we are aware that we created a potential bias, because by using the modified optical disector, we did not take into account so-called lost caps and split nuclei. However, we only considered nucleoli in our quantification, and nucleoli are less likely than nuclei to be split by the knife and therefore provide better resolution (Coggeshall and Chung, 1984).

Moreover, all the individual estimates of the CA1 and CA3 subregions in the current study are within the range of the CA1 and CA3 estimates of control and psychosocially stressed tree shrews, as presented in a previous study from our laboratory (Vollmann-Honsdorf et al., 1997; Keuker et al., 2001). In the latter study, unbiased stereology on thick plastic sections was employed. Thus, the potential bias from using cryosections and from the use of the modified optical disector in the present study is expected to be relatively small. This is in line with a study from Harding et al. (1994), who did not find differences in the outcome between the unbiased and the modified optical disector in 50-μm cryosections. Evidence of only a mild, if any, bias from the use of cryosections is given in a calibration study by Hatton and Von Bartheld (1999), who revealed that in plastic and paraffin sections, but not in cryosections, a displacement of nuclei occurs as a result of the sectioning: the plastic and paraffin sections consis-

TABLE 5. Estimated Unilateral Subfield Volumes and Statistical Results of the Unpaired Student's t-test (Two-Tailed) in Young and Aged Tree Shrews

	Volume (mm^3)		
	Young (n = 5)	Aged (n = 6)	P value
Subiculum	8.48 ± 1.05	8.06 ± 0.82	0.47
CA1	18.10 ± 2.85	17.10 ± 2.51	0.55
CA2	2.03 ± 0.35	1.97 ± 0.23	0.74
CA3	16.96 ± 2.64	17.89 ± 2.92	0.60
Hilus	4.42 ± 0.88	4.64 ± 1.30	0.76
Dentate gyrus			
Granule cell layer	4.16 ± 0.61	4.46 ± 1.01	0.57
Molecular layer	11.16 ± 1.80	10.28 ± 1.78	0.44
Total	15.31 ± 2.33	14.74 ± 2.72	0.72
Total hippocampal formation	65.31 ± 8.87	64.39 ± 9.24	0.87

tently showed a higher density of neuronal nuclei in the upper and lower 0–20 % of the tissue section, and a lower density in the core 30–70% of the section.

Volumetric estimations. The volumes of the entire hippocampal subfields and of the total hippocampal formation did not differ significantly between young and aged tree shrews (Table 5). Also, the volumes of the neuron-containing layers of the subiculum, CA1, CA2, and CA3 subfields were not changed by aging (data not shown).

DISCUSSION

In the present study, we used the tree shrew as an animal model of aging. A general question about animal models is whether they mimic aging-related changes in human physiologic mechanisms. It is presumed that during aging the brains of various mammalian species have impaired metabolic processes, because postmitotic cells, such as neurons and muscle cells, have a much higher level of damaged mitochondria during aging than do dividing cells, such as cells in the liver or skin (Cortopassi et al., 1992). Lipofuscin, for example, the end-product of lipid peroxidation in postmitotic cells, is formed in tissues under conditions of high oxidative stress, and its accumulation has been shown in the brains of aged rats (Oenzil et al., 1994), dogs (Dimakopoulos and Mayer, 2002), marmosets (Honavar and Lantos, 1987), rhesus monkeys (Peters et al., 1994; Tigges et al., 1995; Siddiqi and Peters, 1999), and humans (Mrak et al., 1997; Dei et al., 2002). Therefore, we can assume that the brain of an aged tree shrew shows similarities in certain, but probably not all, aging-related processes as they occur in the aging human brain. Some processes during aging probably develop at a rate that is relative to the maximal life span and will leave similar signs in brains of aged tree shrews and humans. However, other aging-associated pathological processes may occur at the same rate in tree shrews and humans; although such processes may cause impairments in aging human brains, they may not yet cause impairments in aged tree shrews, because of their shorter life span.

The current study is the first to combine a memory test with stereological analyses in young and aged tree shrews. The main findings of the present study are: 1) impaired working memory in aged tree shrews but preserved reference memory; 2) unchanged hippocampal neuron numbers in aged tree shrews; and 3) preserved hippocampal subfield volumes in aged tree shrews.

It is well known that the hippocampal formation has a strong role in spatial reference memory (Squire, 1992). Spatial working memory depends mainly on both the hippocampal formation and the prefrontal cortex (Jeltsch et al., 2001; Lee and Kesner, 2003). Reference and working memory can be observed separately, for example, with spatial tasks such as the Morris water maze or the radial arm maze (Decker, 1995). In the present study, we based our behavioral observations on the tree shrews' performance in the spatial holeboard test, which is carried out in the animals' home cages and makes use of the tree shrews' natural foraging behavior. The current holeboard test with tree shrews is based on their voluntary stress-free behavior (Ohl et al., 1998). Although the validity of the holeboard task as an assessment of hippocampal integrity remains to be verified in tree shrews, strong evidence thereof comes from rodent studies.

Both physical (Oades and Isaacson, 1978) and toxic (Jarrard, 1993) lesions of the hippocampal formation produced severe deficits in the performance of spatial tasks. Van der Zee et al. (1992) provided physiological evidence from intact mice that the hippocampus is involved in a spatial holeboard test. In this study, protein kinase C (PKC), a key enzyme for signal transduction and various mechanisms of neuronal plasticity (Nishizuka, 1988), was detected immunocytochemically in the hippocampus after various degrees and forms of training in a spatial holeboard test. The staining intensity in the hippocampus of isozyme PKCγ, which is an indicator of physiologically activated neurons that are involved in the holeboard test, was drastically increased in trained mice, but not in pseudo-trained, habituated, or naive mice (Van der Zee et al., 1992). Moreover, the more neuroethologically relevant a spatial task is to a specific animal species, the more the hippocampus can be shown to play an important role in such a task (Suzuki and Clayton, 2000). In this regard, we can assume that the holeboard task (assessing rats and tree shrews) is more effective in testing spatial memory than, for example, testing rats in the Morris water maze.

The behavioral data from investigations that combined a memory test with stereological and morphometric analyses (West et al., 1993; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Berman et al., 1997; Rapp et al., 1999) are not readily comparable to our behavioral results. The above-mentioned studies did not clearly differentiate between reference memory and working memory, or did not include a particular spatial component. One study with rhesus monkeys used a spatial memory test that was similar to tasks designed for rats (Rapp et al., 1997). The latter experiment showed that aged rhesus monkeys had impaired spatial memory, whereas recognition memory was preserved. However, this report contained no additional anatomical information about the hippocampal formation. A rodent study that differentiated between reference and working memory revealed that, compared with young animals, reference memory was impaired in rats of 11, 17, and 24 months of age, whereas working memory only was impaired in 24-month-old rats (Frick et al., 1995). Another study in rats, also evaluating both reference and working memory, reported impairment of both memory types in a subgroup of aged rats only (Kadar et al., 1994). However, the above-mentioned studies in rats either did not include hippocampal morphology or did not use stereological methods.

There are at least three possible explanations for the fact that we did not find an age-associated hippocampal neuron loss. First, the group sizes in the current study are not as large as can be used in, for example, rat studies. To demonstrate that the current nonsignificant difference in the total neuron number is significantly lower in aged tree shrews than in young animals, we would have needed to include at least 45 tree shrews per group (computed with the Student's t-test formula to calculate the group size on the condition that the variance and range of counts are similar with larger groups). Moreover, our sampling within subjects was sufficient, considering that the BCV^2 was always considerably higher than 50%. Second, most of the aged tree shrews were between 4 and approximately 6 years of age, ages that could be regarded as middle-aged rather than aged or old. Because of this limitation, we might have overlooked the possibility that in later life, i.e., in the oldest of the aged tree shrews, a neuron loss may occur. Nevertheless, should neuron loss in later life be inevitable, our oldest tree shrew, and even our oldest two animals, would have had substantially lower neuron numbers than the mean neuron number of the aged group (Table 3). The third and most probable explanation is that, as in other animal models of aging, there is no neuron loss associated with aging in tree shrews.

Our finding of preserved hippocampal neuron numbers in aged tree shrews is in line with data from aged mice (Calhoun et al., 1998), rats (Rapp and Gallagher, 1996; Rasmussen et al., 1996) and rhesus monkeys (West et al., 1993; Berman et al., 1997; Keuker et al., 2003a). Few of these studies, however, combined the estimation of neuron numbers with memory assessment. Remarkably, a subgroup of aged rats (Rapp and Gallagher, 1996; Rasmussen et al., 1996) or rhesus monkeys (West et al., 1993; Berman et al., 1997) had impaired hippocampus-dependent memory, despite preserved hippocampal neuron numbers. In the current study, we found preserved hippocampal neuron numbers and stable reference memory performance in aged tree shrews. However, we detected impaired working memory in aged tree shrews, which could be indicative of diminished hippocampal integrity. Moreover, as the above-mentioned studies illustrate, preservation of neuron number does not necessary imply intact hippocampus-dependent memory functioning.

We showed that the volumes of the hippocampal formation and its separate subfields were not reduced in aged tree shrews. Here, we only focus on investigations that link hippocampal volume to spatial memory performance. Structural MRIs of licensed London taxi drivers revealed a strong positive correlation between posterior hippocampal volume and spatial navigation experience (Maguire et al., 2000). Adversely, the taxi drivers' anterior hippocampal formation was smaller than that of control subjects. In rats, aging affected mainly the septal part of the hippocampal formation; a smaller volume correlated with impaired spatial memory performance (Rapp et al., 1999). The septal and temporal parts of the rodent hippocampal formation correspond to the posterior and anterior primate hippocampus, respectively (Colombo et al., 1998; Moser and Moser, 1998). Interestingly, the posterior (or septal) hippocampal formation appears to be more important for encoding spatial memory than the anterior (or temporal) hippocampus (Colombo et al., 1998; Moser and Moser, 1998).

As mentioned before, aged rats can be screened in the Morris water maze to differentiate the animals into cognitively impaired and unimpaired groups. Compared with young and unimpaired aged rats, cognitively impaired aged rats showed an increased activity of the hypothalamic-pituitary-adrenal axis (Issa et al., 1990). Moreover, aged rats with defective memory had impaired glucocorticoid negative feedback (Issa et al., 1990; Bizon et al., 2001). Previously, we examined the free urinary cortisol levels of more than 135 tree shrews across the entire life span (Van Kampen and Fuchs, 1998). Cortisol levels increased steadily in young animals (between 3 weeks and 5 months of age). However, at approximately 6 months of age, the cortisol level reached a steady level and did not change throughout life. The highest examined age was 8 years. Data of constant cortisol concentrations in aged tree shrews, therefore, concur with unchanged hippocampal volume and stable spatial reference memory, shown in the current study.

In summary, we demonstrated that hippocampal neuron numbers and subfield volumes are unchanged in aged tree shrews, compared with young animals. Using the holeboard spatial memory test under the current conditions, we could show an age-related impairment of working memory, but we did not observe impaired reference memory. As mentioned before, however, preserved hippocampal neuron numbers do not necessary imply intact hippocampus-dependent memory functioning. Therefore, we cannot exclude the possibility that aged tree shrews have an impaired hippocampus-dependent memory. On the other hand, because proper hippocampal functioning depends on intact intrahippocampal connectivity and appropriate connections from other brain areas, further study of the molecular and structural changes is required to clarify whether the function of the hippocampal formation is impaired in aged tree shrews.

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