

Changes in extracellular space size and geometry in APP23 transgenic mice: A model of Alzheimer's disease

Eva Syková^{*†‡}, Ivan Voříšek^{*†}, Tatiana Antonova^{*†}, Tomáš Mazel^{*†}, Melanie Meyer-Luehmann[§], Mathias Jucker[§], Milan Hájek^{†¶}, Michael Or^{¶**}, and Jan Bureš^{||}

^{*}Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic; [†]Department of Neuroscience and Center for Cell Therapy and Tissue Repair, Charles University, Second Medical Faculty, Vúvalu 84, 150 06 Prague 5, Czech Republic; [§]Department of Cellular Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Otfried-Müller Strasse 27, D-72076 Tübingen, Germany; [¶]Department of Radiology, Institute for Clinical and Experimental Medicine, Vídeňská 1958, 140 21 Prague 4, Czech Republic; ^{||}Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic; and ^{**}Department of Psychiatry, Charles University, First Medical Faculty, Ke Karlovu 2, 128 08 Prague 2, Czech Republic

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Diffusion parameters of the extracellular space (ECS) are changed in many brain pathologies, disturbing synaptic as well as extrasynaptic "volume" transmission, which is based on the diffusion of neuroactive substances in the ECS. Amyloid deposition, neuronal loss, and disturbed synaptic transmission are considered to be the main causes of Alzheimer's disease dementia. We studied diffusion parameters in the cerebral cortex of transgenic APP23 mice, which develop a pathology similar to Alzheimer's disease. The real-time tetramethylammonium (TMA) method and diffusion-weighted MRI were used to measure the ECS volume fraction (α = ECS volume/total tissue volume) and the apparent diffusion coefficients (ADCs) of TMA (ADC_{TMA}), diffusing exclusively in the ECS and of water (ADC_W). Measurements were performed *in vivo* in 6-, 8-, and 17- to 25-month-old hemizygous APP23 male and female mice and age-matched controls. In all 6- to 8-month-old APP23 mice, the mean ECS volume fraction, ADC_{TMA} , and ADC_W were not significantly different from age-matched controls (α = 0.20 ± 0.01 ; ADC_{TMA} , $580 \pm 16 \mu m^2 s^{-1}$; ADC_W , $618 \pm 19 \mu m^2 s^{-1}$). Aging in 17- to 25-month-old controls was accompanied by a decrease in ECS volume fraction and ADC_W , significantly greater in females than in males, but no changes in ADC_{TMA} . ECS volume fraction increased (0.22 ± 0.01) and ADC_{TMA} decreased ($560 \pm 7 \mu m^2 s^{-1}$) in aged APP23 mice. The impaired navigation observed in these animals in the Morris water maze correlated with their plaque load, which was twice as high in females (20%) as in males (10%) and may, together with changed ECS diffusion properties, account for the impaired extrasynaptic transmission and spatial cognition observed in old transgenic females.

aging | cortex | diffusion | ion-selective microelectrodes | magnetic resonance

Alzheimer's disease is the most common form of cerebral degeneration leading to dementia. A key neuropathological feature of Alzheimer's disease is the deposition of amyloid fibrils within the neuropil as senile plaques and in the walls of cerebral and meningeal blood vessels (1). The major component of the extracellular amyloid deposits is a 40- to 42-residue protein [termed amyloid β -protein (2, 3)], which is derived from the endoproteolysis of the integral membrane amyloid precursor protein (APP) (4). Normally, rodents do not develop amyloid plaques; however, APP23 transgenic mice with neuron-specific overproduction of mutated human APP751 (Swedish double mutation) show their first amyloid plaques as early as 6 months of age (5). Recent experiments (6) revealed that amyloid plaques may also form in wild-type grafts implanted into the brain of APP23 transgenic animals, showing that the formation of β -amyloid plaques is possible even in the absence of the intracellular production of the amyloid protein.

The extracellular space (ECS) forms the microenvironment of cells, and its size and composition are important for their function. Neurons and glial cells release neuroactive substances, which then diffuse in the ECS to reach their target receptors (7, 8). This mode of extrasynaptic communication is called "volume" transmission (9, 10). Investigators have hypothesized that volume transmission provides a mechanism of long-term information processing in functions such as vigilance, sleep, depression, memory formation, and CNS plasticity (7, 11). Extrasynaptic transmission is strongly modulated by the ability of neurotransmitters to diffuse in the ECS (i.e., by their diffusion coefficient in the ECS) and by the space available for diffusion (i.e., the size of the ECS), which are parameters that are altered during many pathological as well as physiological states (11), including development and aging (12, 13). The common symptoms of Alzheimer's disease are forgetfulness, sleeplessness, anxiety, and depression, which are impaired functions in which extrasynaptic transmission could also be involved (10).

The aim of our study was to investigate diffusion changes in the cerebral cortex of transgenic APP23 mice, which develop amyloid plaques during aging. The extracellular diffusion parameters were studied by the real-time tetramethylammonium (TMA) method (14). This method can estimate the ECS volume fraction α that describes the size of the ECS (α = ECS volume/total tissue volume) and the apparent diffusion coefficient (ADC) of TMA (ADC_{TMA}) in the ECS by monitoring the diffusion of TMA introduced into the ECS (15). Although the nonspecific loss of TMA from the ECS is very low, it is described by a third parameter: the nonspecific cellular uptake of TMA (k'). The ADC_{TMA} in the ECS is always less than its free diffusion coefficient because of the complexity of the ECS, which introduces obstacles into the diffusion pathway of TMA (e.g., cellular structures or extracellular matrix molecules) and, thus, obstructs the diffusion of TMA as compared with a free medium. Therefore, the ADC in the ECS does not depend only on the physical properties of the diffusing compound (as is the case with the free diffusion coefficient) but also on the composition and structure of the ECS (16, 17). Although the TMA method is the only one that yields absolute values of α , ADC_{TMA} , and k' , it cannot be used in humans because of its invasiveness. This fact makes it difficult to compare TMA results obtained in APP23 mice to data obtained in Alzheimer's disease patients. To supplement our study, we used diffusion-weighted MRI (DW-MRI), which is widely available as an adjunct to MRI scanners in regular clinical use. By using DW-MRI, we measured the ADC of water (ADC_W) in the

Abbreviations: ADC, apparent diffusion coefficient; MR, magnetic resonance; TMA, tetramethylammonium; ADC_{TMA} , ADC of TMA; ADC_W , ADC of water; ECS, extracellular space; ISM, ion-selective microelectrode; DW-MRI, diffusion-weighted MRI.

[†]To whom correspondence should be sent at the * address. E-mail: sykova@biomed.cas.cz.

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tissue. Our aim was to correlate the changes in ECS diffusion parameters with the results of DW-MRI measurements, amyloid plaque load, and behavioral deficits.

Materials and Methods

Transgenic Mice. The generation of APP23 transgenic mice has been described (5). Briefly, a murine Thy-1 promoter element was used to drive the neuron-specific expression of mutated human APP751 (Swedish double mutation) in B6D2 mice. Hemizygous APP23 mice (6- to 8-months old and 17- to 25-months old) and age-matched nontransgenic controls were used. The mice were from the F₈-F₁₀ generations of backcrossing to B6 mice.

Experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize both the suffering and the number of animals used.

The Real-Time Ionophoretic TMA Method. The ECS diffusion parameters (i.e., α , ADC_{TMA} , and k') were measured by using the real-time ionophoretic method, as described in refs. 14 and 18. In summary, TMA (a substance to which cell membranes are relatively impermeable) was released by ionophoresis from a micropipette, and its local concentration was measured with TMA⁺ ion-selective microelectrodes (ISMs) located ≈ 100 – 150 μm from the release site (see Fig. 1a). Ionophoretic electrodes were made from theta-glass tubing (Clark Electromedical Instruments, Pangbourne, U.K.) and filled with 150 mM TMA⁺. The concentration of TMA⁺ was then measured by means of a double-barreled TMA⁺-ISM at a fixed distance from the tip of the ionophoretic electrode. TMA⁺ ISMs were prepared by a procedure described in ref. 18. We used a Corning 477317 ion exchanger, and the ion-sensitive barrel was back-filled with 150 mM TMA⁺ chloride. The reference barrel contained 150 mM NaCl. The shank of the ionophoretic electrode was bent so that it could be aligned parallel to the TMA⁺ ISM. The bent ionophoresis microelectrode and ISM were then glued together in a fixed array, and the spacing of the tips was measured before and after each experiment by using a microscope equipped with a graticule. The time-dependent rise and fall of the extracellular TMA⁺ concentration during and after an ionophoretic pulse (TMA⁺ diffusion curves; Fig. 1b) were fitted to a radial diffusion equation modified to account for extracellular volume fraction α , the ADC_{TMA} , and the nonspecific TMA⁺ uptake k' (14). Diffusion curves were first recorded in 0.3% agar gel in 150 mM NaCl/3 mM KCl/1 mM TMA chloride to calibrate the microelectrode array (in agar $\alpha = 1$, $k' = 0$ and $ADC_{TMA} = 1,043 \mu\text{m}^2\text{s}^{-1}$; free diffusion coefficient, 25°C). Similar recordings repeated in layers III–VI of the cerebral cortex were analyzed to yield the ECS diffusion parameters α , ADC_{TMA} , and k' . Measurements of ECS diffusion parameters were performed at several depths (in 100- μm steps from a depth of 200–300 μm to 600–900 μm below the brain surface) in one to three tracks (70 tracks in 39 animals) within the somatosensory cortex (1.5 mm posterior to bregma, 1.5 mm lateral to midline). All measurements within one track were averaged, and the averages were used for final statistics.

Animals were anesthetized with sodium pentobarbital (initial dose, 90 mg/kg; dose each subsequent 1 h, 30 mg/kg). Their cortex was exposed by 1-mm-diameter trephination holes (1.5 mm lateral and either at bregma or 1.5 mm caudal to bregma), and the dura was removed from this area. During measurements, the animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The body temperature was maintained at 37°C, and breathing was spontaneous.

DW-MRI. DW-MRI measurements were performed by using an experimental magnetic resonance (MR) spectrometer Biospec 4.7 T system (Bruker, Ettlingen, Germany) equipped with a 200 mT/m gradient system and a head surface coil. For diffusion-weighted measurements, four coronal slices were selected (thickness, 0.8 mm;

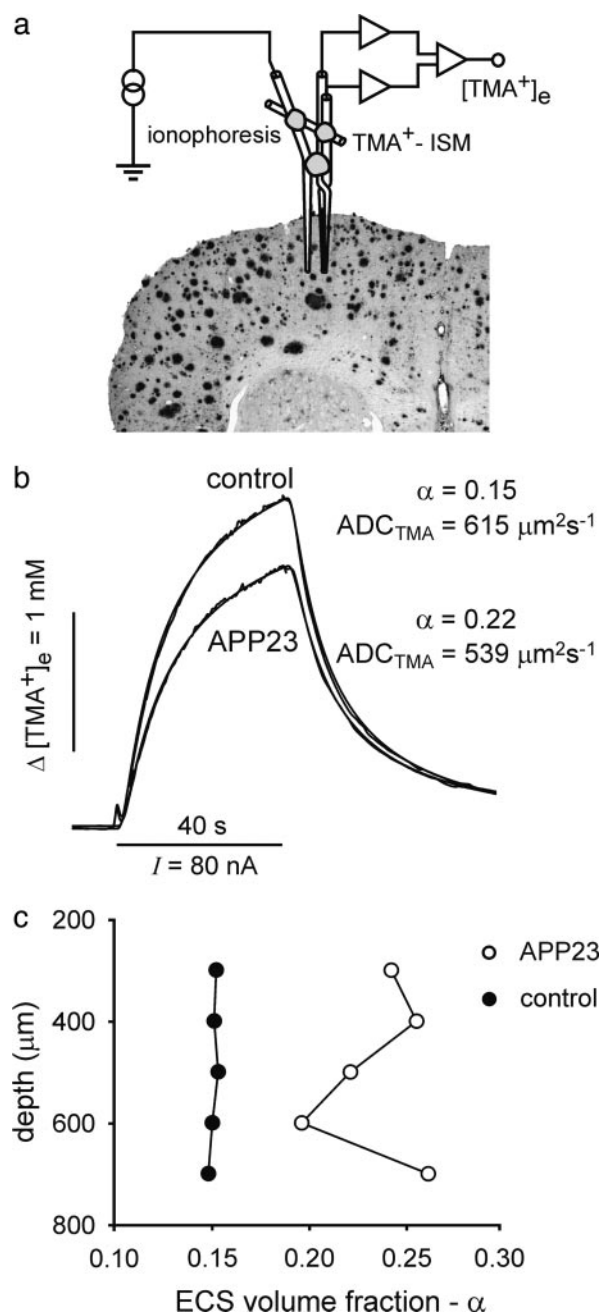


Fig. 1. TMA⁺ measurements and typical diffusion curves in control and APP23 mice. (a) A double-barreled TMA⁺-selective microelectrode (TMA⁺ ISM) and a micropipette for TMA⁺ ionophoresis were glued together with dental cement to stabilize the intertip distance at 100–150 μm , and this microelectrode array was introduced into the dorsal brain cortex. (b) Comparison of TMA⁺ diffusion curves obtained in 17- to 25-month-old transgenic and control mice. The diffusion parameters can be determined from the shape and magnitude of the curves. The ECS was larger, and ADC_{TMA} was lower, in APP23 animals than in controls. (c) ECS volume fraction (α) plotted against the depth of measurements (the zero level corresponds to the brain surface); α was changed in 17- to 25-month-old APP23 mice compared with age-matched controls.

interslice distance, 1.2 mm; field of view, $1.92 \times 1.92 \text{ cm}^2$; matrix size, 256×128). DW images were measured by using the stimulated echo sequence and the following parameters: b-factors, 136, 329, 675, 1,035, 1,481, and 1,825 s/mm^2 ; $\Delta = 30 \text{ ms}$; echo time, 46 ms; repetition time, 1.2 s. The diffusion gradient pointed along the

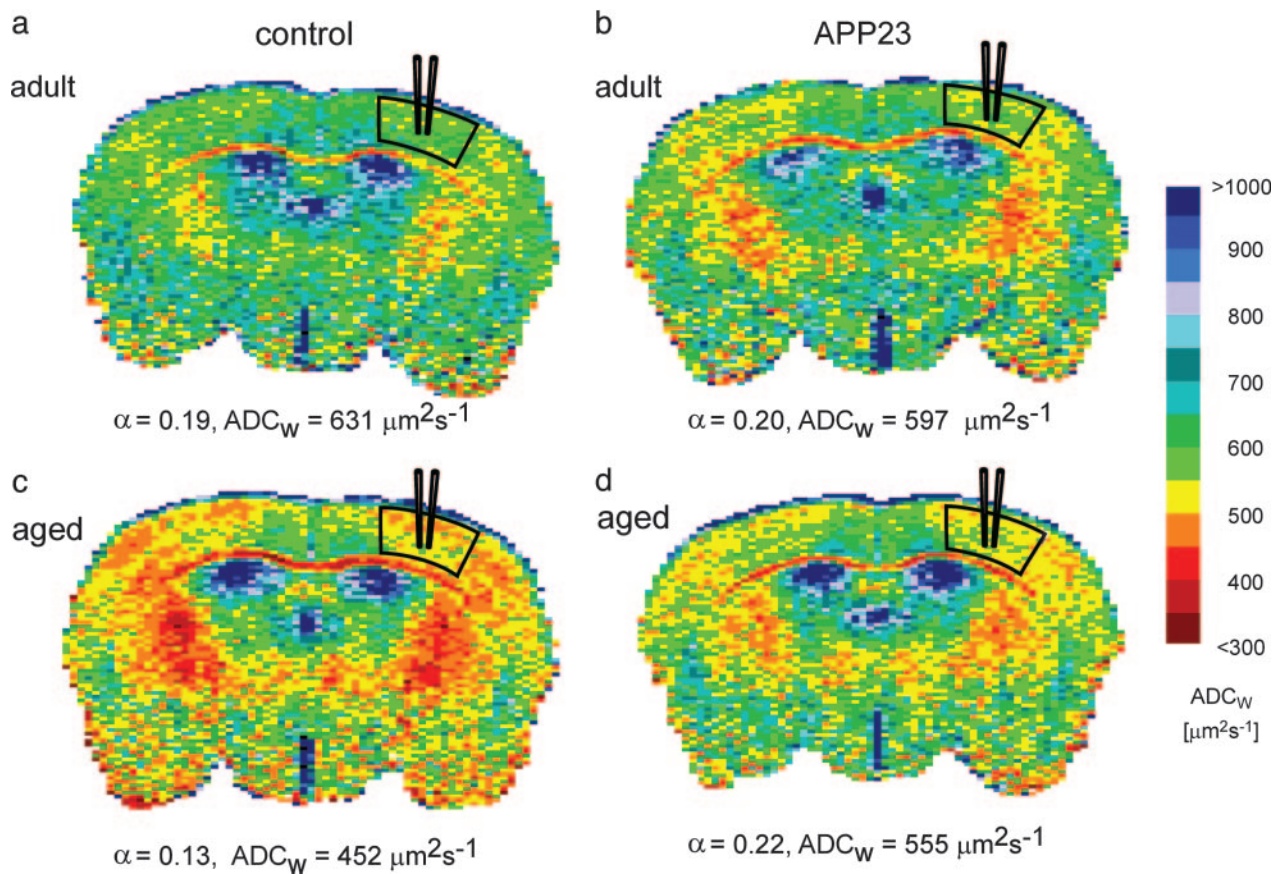


Fig. 2. ADC_w maps acquired in the brain of control and APP23 female mice. The mean value of ADC_w was calculated in the areas indicated. In the corresponding region, TMA⁺ measurements were performed on the same animals a few days later. The mean values of ADC_w and ECS volume fraction (α) are given below each map. There were no differences between adult control (a) and adult transgenic (b) mice. (a and c) A decrease in ADC_w and α was found during aging in control mice. In aged APP23 mice (d), there was an increase in both ADC_w and α when compared with age-matched control mice (c).

differences observed between individual cortical layers. Interestingly, the decrease was more pronounced in females, with α being significantly lower in aged females than in aged males ($P = 0.0095$).

In both male and female 17- to 25-month-old APP23 mice, the ECS volume fraction increased significantly in comparison with both their age-matched controls and also adult (6- to 8-month-old) control animals. ADC_{TMA} significantly decreased only in 17- to 25-month-old APP23 females when compared with age-matched controls. Typical diffusion curves are shown in Fig. 1*b*. Fig. 1*c* shows the typical variations of ECS volume fraction at different depths in the cortex of an APP23 aged female compared with the uniform values in a control aged female. The larger amplitude of the diffusion curve in Fig. 1*b* indicates a smaller ECS volume fraction, whereas the slower increase and decrease of the diffusion curves reflects a lower ADC_{TMA} . Nonspecific TMA⁺ uptake was significantly higher in both control and APP23 aged groups [$k' = (5.0 \pm 0.4) \times 10^{-3} \text{ s}^{-1}$] compared with the respective groups of 6- to 8-month-old animals [$k' = (3.0 \pm 0.5) \times 10^{-3} \text{ s}^{-1}$].

MR Measurements of ADC_w . ADC_w maps of coronal brain slices were evaluated in the primary somatosensory cortex in the location that corresponded to the site of TMA measurements (see Fig. 2). Details about the evaluation of ADC_w maps are given in *Materials and Methods*. Fig. 2 shows typical ADC_w maps measured in female mice. To visualize the differences between animals, ADC_w maps were converted to pseudocolor images.

During aging, ADC_w decreased significantly in control female mice (Fig. 2*a* and *c*). We compared the mean values of ADC_w in the somatosensory cortex of APP23 transgenic mice with those

measured in age-matched controls. In 6- to 8-month-old transgenic APP23 animals, the values of ADC_w were not significantly different from controls (Fig. 2*b*). Female 17- to 25-month-old APP23 mice showed a higher ADC_w than age-matched controls (Fig. 2*d*). In males, we did not observe significant differences in ADC_w between young and aged animals or between APP23 mice and controls.

The differences in diffusion coefficients between any two individual measurements of the phantoms were <5%, and there were no significant differences in the diffusion coefficients of the same compound measured in conjunction with different groups of mice. These findings demonstrate the good reproducibility of our ADC_w measurements and confirm that no systematic errors occurred.

Behavioral Testing. The main results of the behavioral tests are summarized by the learning curves shown in Fig. 3*a* and *b*. Two-way ANOVA (8 groups \times 9 days) with repeated measures on days revealed significant main effects of groups ($F_{7,34} = 5.91$, $P < 0.01$) and days ($F_{8,272} = 60.02$, $P < 0.01$) as well as significant interaction ($F_{56,272} = 1.46$, $P < 0.05$). Newman-Keuls post hoc tests showed that all groups except the old transgenic females decreased their escape latencies from 35–55 s on day 1 to asymptotic values of 7–17 s on days 7–9. The aged APP23 females started to differ from the other groups on day 4 ($P < 0.05$) and continued to perform poorly until the end of the experiment ($P < 0.01$).

In the probe-trial experiment, the old female transgenic mice showed no preference for the target quadrant and made fewer crossings of the target annulus than the mice of the other age-matched groups (Fig. 3*c*). These groups, as well as all of the adult

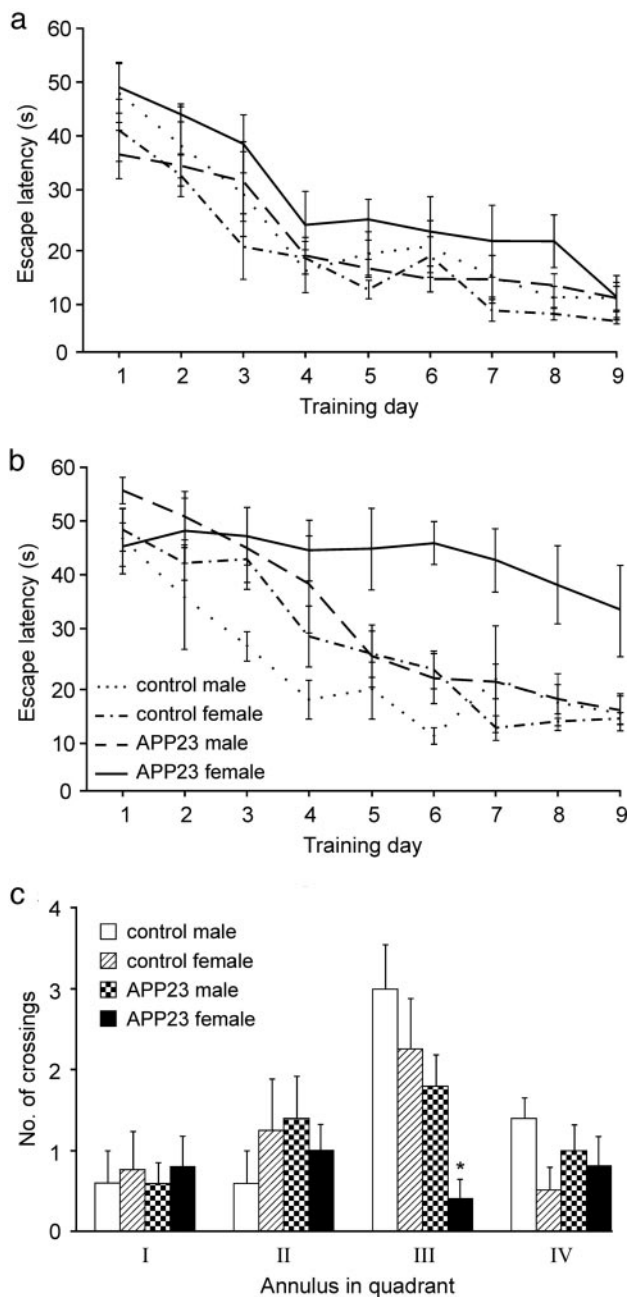


Fig. 3. Results of behavioral testing of control and APP23 mice. (a) Adult mice. (b) Aged mice. (a and b) No between-group differences in learning performance could be demonstrated on days 1 or 2. The escape latencies of the old female transgenic mice started to differ significantly from the other groups on day 3, and their performance did not improve throughout the duration of the experiment. However, learning of the old male transgenic mice did not differ from their respective controls. (c) Aged mice. In the probe trial on training day 6, the old female transgenic mice showed no preference for the target quadrant, made fewer annuli crossings, and visited the target position significantly less frequently than the other groups.

mouse groups, clearly preferred the target quadrant and especially the area corresponding to the target location, and there were no significant differences among the groups.

The β -Amyloid Plaque Load. Histological sections stained for amyloid plaque load were analyzed in the cortex of 6- to 25-month-old APP23 mice. The plaque load increased with the increasing age of

the animals. In 6- to 8-month-old mice, the amyloid plaque load was $<1.6\%$ in both males and females. In animals older than 17 months, the amyloid plaque load was significantly higher in females ($20.0 \pm 1.6\%$, $n = 13$) than in males ($10.4 \pm 2.5\%$, $n = 8$). However, the amyloid plaque load varied greatly (in females, the plaque load ranged 9–26%; in males, the plaque load ranged 3–18%).

Discussion

The concentration of β -amyloid is one of the key factors in its transition from a monomeric to a fibrous form and its aggregation into amyloid plaques (26). The β -amyloid concentration can be influenced substantially by changes in interstitial fluid diffusion and bulk flow in the perivascular spaces (27). In our study, we investigated extracellular diffusion in the cerebral cortex of transgenic APP23 mice and age-matched controls. The following two age groups were selected: adult 6- to 8-month-old mice that were just starting to develop amyloid plaques, and old 17- to 25-month-old mice with a mean amyloid plaque load of 15.2%.

The values of the ECS diffusion parameters obtained in 6- to 8-month-old control animals did not significantly differ from published data (11, 15, 16). In age-matched APP23 mice, we did not detect any significant differences. This observation would imply that the ECS diffusion parameters are not changed substantially in young animals before amyloid deposition.

Aging in control mice was associated with a decrease in ECS size, in agreement with our previous measurements in aged rats (13, 28). Our study shows that the decrease found in the mice is even more pronounced than it is in rats and, furthermore, that it is greater in aged females than in males. The decrease in ECS volume fraction during aging might be attributed to the loss of extracellular matrix molecules such as chondroitin sulfate proteoglycans (CSPG), fibronectin (13), or highly sialylated cell-adhesion molecules, such as polysialic acid neural cell-adhesion molecule (NCAM) (29). Recently, we found that in knock-out mice lacking tenascin-R (a glycoprotein that is an important component of the extracellular matrix), the ECS is significantly smaller than in wild-type mice (30). Therefore, extracellular matrix molecules may act like a sponge by binding a large number of water molecules, and because of the mutual repulsion of their numerous negatively charged residues, they tend to occupy a lot of space. Thus, their loss during aging might lead to a decrease in the ECS volume. ECS shrinkage might also be related to a general decrease in hydration during aging. Dehydration is associated with an increase in colloid density (31), resulting in a decrease in catalytic enzyme activity and possibly contributing to the accumulation and deposition of certain substances intracellularly and extracellularly. In particular, the decrease in ECS volume during aging might contribute to the early stages of amyloid plaque formation.

Also, we have found a significant difference in the ECS volume fraction between aged control females and age-matched control males. An even more pronounced difference was found for ADC_w . This sex difference is probably the first observation of a gender difference in these two parameters. However, other differences have been described by Mouton *et al.* (32), who reported that female mice have a greater number of astrocytes and microglia in the hippocampus than males and that this difference increases with age. Mouton *et al.* suggested that these changes might be related to the decrease in 17 β -estradiol after estropause; however, there is no direct evidence. In aged APP23 mice, we found a significant increase in ECS volume fraction and a decrease in the ADC_{TMA} , closely related to plaque deposition. The simplest explanation is that the extracellular deposition of amyloid directly results in ECS enlargement, representing at the same time an additional diffusion barrier and, thus, leading to a decrease in ADC_{TMA} . An alternative explanation could be that an increased concentration of soluble amyloid attracts water into the ECS.

Unlike TMA, water crosses cell membranes relatively easily; for that reason, ADC_w does not characterize only the diffusion prop-

erties of the ECS but also the diffusion properties of the intracellular space and the permeability of cell membranes for water (33). Our MRI measurements revealed that ADC_W changes in APP23 mice predominantly corresponded to variations in ECS volume. In the cortex of aged animals, there was no significant difference in ADC_{TMA} among the measured groups of animals, although ADC_W decreased. This result could be related to the fact that ADC_W is composed of many components (34, 35). In contrast to the TMA method, which measures ADC_{TMA} exclusively in the ECS, the MR method measures a weighted average ADC_W across multiple subcompartments that differ in their diffusion properties, e.g., in the extracellular and intracellular spaces. If the volume ratio between the subcompartments changes in the tissue, then the resulting ADC_W is affected also (30).

Is there a link among ECS volume, diffusion changes, and the behavioral deficits that we have found in aged female APP23 mice? A similar gender-dependent impairment of water-maze performance in aged APP23 mice (36) was attributed to the observation that the plaque load was three times higher in females than in males (37). In the experiments that we describe here, the plaque load was only 1.9 times higher in females than in males, but the behavioral deficit was well expressed. This observation suggests that the navigation failure observed in aged female APP23 mice could not only be due to the accumulation of amyloid plaques but also to concomitant changes in ECS volume and other diffusion parameters, which may contribute to a decrease in the extracellular concentration of many important neuroactive substances, such as acetylcholine, dopamine and serotonin, and to a decreased efficiency of volume transmission. The functional radius of protein and peptide molecules, including hormones and growth factors, would also be compromised. An increase in ECS volume leads to diminished extracellular-field potentials and reduced excitability in the

neocortex (38). Glutamate and γ -aminobutyric acid spillover and crosstalk between synapses, which depend critically on diffusion parameters, have been suggested to play a role in long-term potentiation and long-term depression (39, 40). Therefore, the changes in ECS volume and ADC_{TMA} observed in our study, the rearrangement of astrocytic processes, and amyloid plaques can contribute to behavioral deficits in severely affected APP23 females.

The volume of the ECS measured by the TMA method is only that part of the ECS that is available for the diffusion of TMA^+ . Obviously, the volume of amyloid plaques, which are located extracellularly, is not included in the ECS volume fraction as measured by the TMA method. The amyloid plaque load in old female transgenic mice is $\approx 20\%$ (i.e., one-fifth of the tissue volume is occupied by amyloid). The real ECS volume is the sum of both these volumes; in old female transgenic mice, it is $\approx 40\%$ of the tissue volume. The possible consequences are cell shrinkage/death and/or the loss of their processes because of the greatly reduced intracellular compartment. Because the only way that neurons and glial cells can communicate is by extracellular diffusion, we can expect changes in neuron–glia interactions, synaptic efficacy, and synaptic plasticity (16, 17, 41) in the APP23 mouse model of Alzheimer's disease.

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