Changes in the septohippocampal cholinergic system following removal of molar teeth in the aged SAMP8 mouse

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Abstract

We investigated the effect of dysfunctional teeth on age-related changes in the septohippocampal cholinergic system by assessing acetylcholine (ACh) release and choline acetyltransferase (ChAT) activity in the hippocampus and ChAT immunohistochemistry in the medial septal nucleus and the vertical limb of the diagonal band in young-adult and aged SAMP8 mice after removal of their upper molar teeth (molarless condition). Aged molarless mice showed decreased ACh release and ChAT activity in the hippocampus and a reduced number of ChAT-immunopositive neurons in the medial septal nucleus compared to age-matched control mice, whereas these effects were not seen in young-adult mice. The results suggest that the molarless condition in aged SAMP8 mice may enhance an age-related decline in the septohippocampal cholinergic system.

1. Introduction

In recent years, dysfunctional teeth, e.g. as a result of root caries or a high number of missing teeth, have been suggested to be related to the development of senile dementia [4,22]. A direct relationship between the progress of dementia and a reduced ability to masticate (decreased number of residual teeth, use of dentures, or maximal biting force) has been reported in old human subjects with dementia [30]. Shigetomi et al. [36] showed not only that patients with Alzheimer’s disease have fewer teeth than patients with vascular dementia, but also that there is a significant dose–response relationship between the risk of dementia and the number of teeth in Alzheimer’s disease, but not in vascular dementia, and suggested that a systemic effect of tooth loss may be a potential risk factor for Alzheimer’s disease.

In a recent study in which rats had teeth extracted when young and were then tested when old, Kato et al. [23] demonstrated impairment of performance in a radial arm maze task. In the aged accelerated senescence-prone mouse (SAMP8), which has been suggested as a murine model for human senile dementia [17,18], we recently found that the cutting off [31] or removal [32] of the upper molar teeth resulted in reduced spatial learning ability in a water maze test, suggesting that the loss of teeth was involved in the senile process in the hippocampus. However, the central mechanism underlying the relationship between tooth loss and impairment of spatial cognition is not well understood.

The cholinergic neuronal system in the hippocampus plays an important role in spatial cognition and undergoes a variety of age-dependent changes (reviewed in Refs. [2,8]). In rodents, hippocampal acetylcholine (ACh) release declines with aging [40] and there is an age-dependent decline in memory function [15]. Furthermore, levels of the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT), in the hippocampus decrease with age [39], and cholinergic neurons within the medial septal nucleus, projecting to the hippocampal...
formation [1], degenerate during aging [43]. Given that these septal neurons are activated during learning and memory [42], we hypothesized that dysfunctional teeth may enhance the normal age-related deterioration in the septohippocampal cholinergic system in SAMP8 mice, leading to deficits in spatial memory. In the present study, we tested this hypothesis by the use of microdialysis, biochemical, and immunohistochemical techniques to assess the effect of removal of the upper molar teeth (molarless condition) on ACh release and ChAT activity in the hippocampus and on the number of cholinergic neurons in the basal forebrain in young-adult and aged SAMP8 mice.

2. Materials and methods

2.1. Animals

Young-adult (3- to 4-month-old, n = 56) and aged (10- to 11-month-old, n = 61) male SAMP8 mice were used. A detailed description of the features of this strain can be found elsewhere [17,18]. Briefly, the SAMP8 mouse has a median life span of 13 months and begins to show significant age-related deficits in learning and memory 6 months after birth. The strain was kindly donated by Dr M. Hosokawa (Department of Senescence Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto) and was maintained by brother-sister mating in our laboratory. The animals, treated in accordance with the principles approved by the Council of the Physiological Society of Japan, were bred and maintained under conventional conditions housed in groups of five in plastic cages under temperature- and humidity-controlled conditions (23 ± 1 °C, 55 ± 2%) with free access to food and water, the light–dark cycle being set at 12 h, as described previously [32].

2.2. Surgery

The mice were deeply anesthetized with sodium pentobarbital (40 mg kg⁻¹, i.p.) and their upper molar teeth (maxillary molars) extracted [32]. Control animals underwent the same surgical procedure except for molar removal. Following surgery, the mice were fed pelleted food. A slight decrease in food intake [32] in the molarless mice was seen for a few days after the operation, but this parameter was completely restored to pre-molarless levels before the experiments were performed. The time-course of body-weight changes in the young-adult and aged control and molarless groups is shown in Fig. 1. Body weights of all mice were slightly decreased for a few days after the operation and thereafter were the pre-operation level. All experiments were carried out 3 weeks after the operation.

Fig. 1. Time-course of body-weights of control and molarless young-adult or aged mice. (a) Young-adult mice; (b) aged mice. Values are the mean ± S.E. (n = 10 for each group).

2.3. Measurement of extracellular ACh levels

Hippocampal ACh release was measured using an in vivo microdialysis technique [20]. Briefly, a guide cannula was implanted under pentobarbital anesthesia using the coordinates: A = −1.8 mm and L = 1.5 mm (both from the bregma) and V = 1.5 mm (from the skull) [11]. The mice were allowed at least 24 h to recover after implantation of the guide cannula, and the localization of the dialysis probe was verified at the end of each experiment using a microtome cryostat and magnifying lens. A microdialysis probe (membrane 1.0 mm in length and 0.37 mm in diameter; Eicom, Kyoto) was slowly inserted into the dentate gyrus-CA1 area of the hippocampal formation through the guide cannula. The probe was continuously perfused at a constant flow rate of 2 μl/min with Ringer solution (147 mM NaCl, 4 mM KCl, and 2.3 mM CaCl₂) containing 50 μM eserine sulfate, and samples (30 μl) were collected every 15 min. Immediately after sampling, ACh levels were determined using high performance liquid chromatography with electrochemical detection. The recovery of ACh in vitro was 9.8 ± 0.7% (mean ± S.E., n = 7). To assess functional changes in the cholinergic neuronal system of the mice, 100 mM KCl was added to the perfusion medium for 15 min after stabilization of the basal level of extracellular ACh and the amount of ACh released during the 15 min period expressed as a percentage of the basal level, defined as the mean of the four samples taken immediately before the start of KCl perfusion.
2.4. Measurement of ChAT activity

Hippocampal ChAT activity was assayed by the method of Fonnum [10]. Briefly, after decapitation, the brains were rapidly removed and the hippocampi dissected on an ice-chilled plate and immediately frozen in dry-ice. The frozen tissue was weighed and homogenized in 0.32 M sucrose, then ChAT activity was measured by the formation of [14C]ACh from [14C]acetyl coenzyme-A and choline, the product being separated from the labeled substrate by an highly efficient extraction process which gave a low blank and ensured that only [14C]ACh was measured. The incubation mixture consisted of 200 mM sodium chloride, 50 mM sodium phosphate (pH 7.0), 0.075 mM eserine, 6 mM choline chloride, 0.1 mM [14C]acetyl coenzyme-A, and 0.5 mg/ml of bovine serum albumin. A 40 μl aliquot of homogenized tissue was mixed with 10 μl of 2% Triton-X 100, 50 mM ethylenediaminetetraacetic acid (pH 7.4), then the mixture was added to 100 μl of incubation mixture, and incubated for 15 min at 37 °C. The [14C]ACh formed was extracted into a hydrophobic mixture containing sodium tetraphenyl boron. The protein concentration of the tissue was measured using the Bradford method [5] with bovine serum albumin as the standard.

2.5. Immunohistochemistry

For ChAT immunohistochemistry, the animals were anesthetized with pentobarbital sodium, then perfused transcardially with 30 ml of phosphate-buffered saline, pH 7.4, (PBS) containing 50 units/ml of heparin, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, (PB) containing 0.08% glutaraldehyde and 15% saturated picric acid. The brain was then removed and incubated for 24 h at 4 °C in PB containing 2% paraformaldehyde and 7.5% saturated picric acid. Frontal sections (40 μm thick) from the level of the medial septal/vertical limb of the diagonal band of Broca (septum/vDBB) were then cut serially on a microslicer (DTK-2000W, DSK, Kyoto, Japan) and the free-floating sections processed by an avidin–biotin–peroxidase complex (ABC) method using Vectastain (Vector Laboratories) [19]. To quench endogenous peroxidase activity, the sections were incubated for 20 min at room temperature in PBS containing 0.08% glutaraldehyde and 15% saturated picric acid. After a 15 min rinse with PBS, they were incubated for 30 min at room temperature in PBS containing 0.25% Triton X-100, rinsed with PBS, then incubated for 1 h at room temperature in 3% hydrogen peroxide in PBS (PBS-NGS), followed by incubation for 72 h at 4 °C with rabbit anti-ChAT antibody (DAKO), diluted 1:1,000 in PBS-NGS (first step). The sections were then incubated for 24 h at 4 °C with biotinylated secondary antibody (goat anti-rabbit IgG antiserum, Vector Laboratories; diluted 1:100 in PBS-NGS) (second step), then for 1 h at room temperature with ABC reagent (Elite ABC reagent, Vectastain) in PBS (third step), before being treated for 10 min at room temperature with 0.02% diaminobenzidine tetrachloride and 0.01% H2O2 in 0.05 M Tris–HCl buffer (pH 7.6) (fourth step). Sections were washed three times (10 min each) with PBS between each step. After the final PBS wash, the sections were mounted on slides in Entellan (Merck) and processed using the standard procedure for light microscopy.

Negative controls, performed by either omission of primary antibody or substitution of primary antibody with non-immune rabbit immunoglobulin, showed no staining.

2.6. Quantitative evaluation of staining

Using the method of Gunderson et al. [14], a stereological analysis was conducted on ChAT-stained sections in order to quantify the effects of the molarless condition on the ChAT-positive cholinergic population in the septum/vDBB. The selection criteria used were those described by Fischer et al. [6]. The septum/vDBB was defined as extending rostrally from the genu of the corpus callosum and caudally to the crossing of the anterior commissure, and the lateral edge of the vDBB was taken at the medial border of the olfactory tubercle. Cell counting was performed on both sides of the septum/vDBB. Unambiguously positive cells were identified at 100 × magnification using an Olympus BH-2 microscope interfaced with a color video camera (Hitachi) and an Amiga 2000 computer, both connected to a color monitor (Trinitron, Sony). GRID® software (Interactivision, Silkeborg, Denmark), commanding an X–Y step motor on the microscope stage, was used to generate sampling frames in the selected areas, which appeared as an overlay on the microscopic image on the monitor. For further details of the sampling procedure and data analysis, see Gunderson et al. [14].

2.7. Statistical analysis

Unless otherwise stated, the data were analyzed by analysis of variance (ANOVA), the Scheffé post hoc test being performed when appropriate.

3. Results

3.1. Hippocampal ACh release

In previous studies using SAMP8 mice, we showed that aged molarless mice take a longer time thanagematched molar-intact mice to find the submerged platform in a water maze test [31,32]. Furthermore, it is
known that the hippocampal cholinergic system plays a crucial role in spatial learning [41]. Accordingly, in order to test for a relationship between impaired performance in the water maze task and the functional hippocampal cholinergic system in molarless aged mice, the in vivo microdialysis technique was used to compare hippocampal KCl-evoked ACh release in control and molarless mice. The basal level of hippocampal ACh release was 2.3 ± 0.4 pmol/15 min (mean and S.E., n = 5) in young-adult controls and 2.2 ± 0.3 pmol/15 min (n = 5) in young-adult molarless mice (Fig. 2a) and 2.9 ± 0.3 pmol/15 min (n = 5) in aged controls and 2.8 ± 0.4 pmol/15 min (n = 5) in aged molarless mice (Fig. 2b). The trend towards a higher basal level in aged mice was not statistically significant (F3,16 = 0.99, NS). Fifteen min after KCl perfusion, hippocampal ACh release increased to about 456% of the basal level in young-adult controls, 417% in young-adult molarless mice, 305% in aged controls, and 201% in aged molarless mice. There were significant differences between groups, F3,24 = 13.67, P < 0.0001. Significantly less KCl-evoked ACh release was seen in aged molarless mice compared with aged controls (P < 0.05), whereas the molarless condition had no effect in young mice. In the control groups, the KCl-evoked ACh release decreased with aging, but the difference was not statistically significant. These results show that, in molarless aged mice, decreased hippocampal ACh release was positively correlated with spatial learning deficits [31,32].

3.2. ChAT activity in the hippocampus

When we measured hippocampal ChAT activity by the formation of [14C]ACh from [14C]acetyl coenzyme-A and choline, significant differences in ChAT activity were seen between the groups (F3,24 = 13.67, P < 0.0001). In control mice, ChAT activity was higher in the young-adult group than in the aged mice (Table 1), indicating an age-dependent decrease in hippocampal ChAT activity in SAMP8 mice. In addition, aged molarless mice showed a greater reduction (of about 27%) in ChAT activity than age-matched control mice, whereas the molarless condition had little effect in young-adult mice.

3.3. Septum/vDBB ChAT immunohistochemistry

Since most cholinergic innervation of the hippocampus is derived from the medial septal nucleus and the vDBB [1,28], immunohistochemical analysis using anti-ChAT antibody was performed in these regions. In all groups, ChAT-positive cells were scattered throughout the medial septal region (Fig. 3a–d). At a higher magnification, immunoreactivity was often seen in the

Table 1

<table>
<thead>
<tr>
<th>ChAT activity (nmol/h/mg protein)</th>
<th>Young-adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>38.4 ± 1.5</td>
<td>32.6 ± 1.9*</td>
</tr>
<tr>
<td>Molarless (n = 7)</td>
<td>36.1 ± 2.6</td>
<td>23.9 ± 1.3**</td>
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Each value represents the mean ± S.E. The difference between the groups was evaluated by ANOVA, followed by Scheffe post hoc analysis.

* P < 0.05 compared to young-adult control mice.

** P < 0.05 compared to aged control mice.

Fig. 2. Effect of the molarless condition on hippocampal ACh release. (a) Young-adult; (b) aged mice. Values are the mean (% of basal level) ± S.E. (n = 5 for each group). Basal levels were defined as the average value for the four samples taken before KCl perfusion.
cell processes (Fig. 3e–h). An age-dependent decrease in the number of ChAT-positive cells was seen, this effect being greater in molarless mice than in aged control mice. Although a similar age-related decrease in the number of ChAT-positive cells was seen in the vDBB, there was no effect of the molarless condition in this region.

To further evaluate these changes, ChAT-positive cells in both the medial septal region and the vDBB were counted. As shown in Fig. 4a, in the medial septal region, differences were seen between the groups ($F_{3,24} = 18.92, P < 0.0001$). The number of ChAT-positive cells in aged molarless mice was about 73% of that in age-matched control mice and about 59% of that in young-adult control mice, whereas, in young-adult mice, no significant difference was seen between the control and molarless groups. In the vDBB, in contrast, although there was an age-related decrease in ChAT-

![Fig. 3. ChAT-positive cells within the basal forebrain. (a and e) Young-adult control, (b and f) young-adult molarless mouse, (c and g) aged control, (d and h) aged molarless mouse. Scale bars: 200 μm in (a–d), 50 μm in (e–h).](image)

![Fig. 4. Stereological analysis of the effect of the molarless condition on the number of ChAT-positive cells in the medial septal nucleus and the vDBB. (a): medial septal nucleus; (b) vDBB. The y-axis shows the number of ChAT-positive cells/mm$^2$. Each column represents the mean ± S.E. ($n = 7$ for each group). *$P < 0.05$ compared to young-adult control mice. **$P < 0.05$ compared to aged control mice. #*$P < 0.001$ compared to young-adult control mice.](image)
positive cell numbers ($F_{3,24} = 28.85$, $P < 0.0001$), there was no significant difference between the control and molarless groups (Fig. 4b).

4. Discussion

Although age-associated impairments in many neurotransmitter systems have been correlated with impaired memory, in the present study, we focused on possible molarless condition-induced changes in septohippocampal cholinergic function, since the hippocampal cholinergic neuronal system is well documented as undergoing a variety of age-related changes (reviewed in Refs. [2,8]).

The present study using microdialysis, biochemical, and immunohistochemical approaches in young-adult and aged SAMP8 mice provides support for the hypothesis that the molarless condition enhances a normal age-related decrease in the functioning of the septohippocampal cholinergic system which may be linked to impaired learning ability measured in a water maze [31,32]. In the present study, KCl-evoked ACh release in the hippocampus of the aged molarless group was significantly smaller than that in age-matched molar-intact controls, implying that the molarless condition suppresses hippocampal ACh release in aged SAMP8 mice. In agreement with our results, it has been reported that hippocampal ACh release in response to depolarizing stimulation using high potassium concentrations is decreased in aged rats [40]. We therefore suggest that the molarless condition in aged SAMP8 mice may be involved in the development of age-related functional impairment of the cholinergic system in the hippocampus. However, Kato et al. [23] showed, in aged rats, that both the performance in a radial arm maze task and ACh release in the parietal cortex are impaired after extraction of teeth, suggesting that the impairment in spatial memory induced by removal of molar teeth may be due to dysfunction of the cortical cholinergic system. We therefore measured KCl-evoked ACh release in the parietal cortical region of molarless and molar-intact aged SAMP8 mice, but failed to detect any significant difference in the KCl-evoked ACh release (Onozuka et al., Manuscript in preparation). This discrepancy between these two sets of observations may be due to differences in the species or experimental conditions used.

The present data, showing an age-dependent decrease in hippocampal ChAT activity, are in agreement with observed age-related changes in the rat hippocampus [39], and also suggest that the molarless condition enhances this age-related decrease in hippocampal ChAT activity. Previous studies on age-related changes in hippocampal cholinergic neurotransmission have reported decreased, unaltered, or increased ChAT activity (reviewed in Refs. [2,8]). The reasons for these inconsistent results may be differences in the species, strain, sampling, and methods used [2]. Thus, we cannot completely rule out the possibility that the molarless condition-induced decrease in hippocampal ChAT activity may be SAMP8 mouse-specific.

In the present study, aged SAMP8 mice lacking molar teeth showed a significant reduction in the number of ChAT-positive neurons in the septal nucleus compared to age-matched molar-intact SAMP8 mice. In contrast, the molarless condition had no effect on the number of ChAT-positive neurons in the vDBB. During aging, various degenerative changes in basal forebrain cholinergic neurons have been shown in experimental animals [21,29,38]. Lee et al. [27] reported a significant decrease in the number of septohippocampal cholinergic neurons in aged animals. The hippocampus is supplied by cholinergic fibers arising from the medial septal nucleus and ending on pyramidal neurons of the hippocampus and granule neurons of the dentate gyrus [44]. Taken together with the fact that an age-related impairment of cholinergic neurons in the medial septal nucleus is associated with cognitive impairment [9], it is likely that, in molarless aged SAMP8 mice, the reduced number of cholinergic neurons in the medial septal is related to impaired spatial memory [31,32].

It has previously been shown that selective lesions of the septohippocampal cholinergic system do not impair water maze performance [6,34,35]. In order to determine whether the molarless condition-induced reduction in cholinergic neurons in the septum of aged molarless mice was involved in the impairment of spatial memory, we tested the effect on Morris water maze performance [6,34,35]. To determine whether the molarless condition-induced reduction in cholinergic neurons in the septum of aged molarless mice was involved in the impairment of spatial memory, we tested the effect on Morris water maze performance [6,34,35]. To determine whether the molarless condition-induced reduction in cholinergic neurons in the septum of aged molarless mice was involved in the impairment of spatial memory, we tested the effect on Morris water maze performance [6,34,35].

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increase in errors in an eight-arm radial maze, compared to molar-intact control mice. Thus, we suggest that a reduction in hippocampal ACh release in aged mice may reflect a loss of cholinergic neurons in the medial septal nucleus, resulting in alterations of cholinergic septo-hippocampal projection and/or in septal-hippocampal axons or synapses.

In a previous study, we found that the adrenals of molarless mice were heavier than those of control mice, although the difference was not significant [32]. In addition, we have recently found that plasma corticosterone levels in aged molarless SAMP8 mice are significantly greater than those in age-matched sham-operated mice, and that pretreatment with metyrapone, which suppresses the stress-induced rise in plasma corticosterone levels [24,37], attenuates not only the molarless condition-induced increase in plasma corticosterone levels, but also the impairment of spatial learning and the reduction in neuronal density in the CA1 region [33]. Thus, it seems that stress might be involved in the mechanism by which removal of teeth could lead to a decline in cholinergic function. Indeed, it has been reported that stress accelerates the age-dependent degeneration of the septohippocampal cholinergic system [12,13].

In conclusion, we suggest that an age-dependent decline in the septohippocampal cholinergic system of the SAMP8 mouse is enhanced by the molarless condition.

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