Effects of early tooth extractions on hippocampal GFAP-positive cells in aged senescence-accelerated mice

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Abstract The effects of early tooth extractions on age-associated changes in cognitive function were evaluated. Specifically, teeth were extracted at an early age in senescence-accelerated mice (SAM)P8, and the number of glial fibrillary acid protein (GFAP)-positive astrocytes and spatial perception were evaluated at young, mature, and old ages. The following results were obtained:

1. Concerning spatial perception evaluated by the Morris water maze test, the shortening of the time until the animals reached the platform was significantly slower in mature or old mice of the tooth extraction group compared with age-matched controls. However, no significant difference was noted in the rate of shortening in young mice between the tooth extraction and control groups.
2. The number of GFAP-positive cells was significantly higher in the CA3 region of the hippocampus in the mature or old mice of the tooth extraction group compared with age-matched controls. However, no significant difference was observed in the CA1 and dentate gyrus (DG) region of the hippocampus in the mature or old mice and in the CA1, CA3, and DG region in the young mice between the two groups.

These results suggest that the loss of teeth at an early age accelerated aging and that GFAP-positive astrocytes increased to compensate for a decrease in pyramidal cells.

Key words

Early tooth extraction,
Glial fibrillary acid protein
(GFAP)-positive astrocytes,
Hippocampus,
Morris water maze test,
Senescence-accelerated mice
(SAMP8)

Introduction

Some studies have indicated that aging and tooth loss promote not only changes in oral structures such as the jaw and temporomandibular joint¹⁾ but also Alzheimer's disease²⁾ and dementia³⁾, and that such a loss of teeth is a risk factor for these disorders. Also, changes in the hippocampus induced by the loss of teeth resemble those associated with aging, and an elevation of the plasma glucocorticoid (GC) level⁴⁾, a decline in spatial memory^{5,6)}, and decreases in the number of neurons in the hippocampus^{4,7)},

acetylcholine release³⁾, and number of c-Fos protein-positive cells linked to learning tests⁸⁾ have been reported. In addition, astrocytes positive for glial fibrillary acid protein (GFAP), which is produced as an intermediate filament protein, have been reported to show a compensatory increase with a decrease in pyramidal cells and to be an index of aging^{9–11)}. However, these findings have been obtained by extracted teeth at young, mature, or old ages to evaluate its effects after extractions soon. In this study, therefore, we extracted teeth from young senescence-accelerated mice (SAM)P8 shortly after eruption, maintained the animals until mature or old ages, and examined whether the long-term absence of teeth induces functional and morphological changes in the

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hippocampus. Specifically, the Morris water maze test was performed for functional evaluation to examine the effect of an early loss of teeth on spatial memory. Also, the number of GFAP-positive astrocytes in the hippocampus was examined to evaluate the morphological effects of tooth extractions.

Materials and Methods

Sixty male SAMP8 were used in the study. The strain was kindly donated by the institute of Frontier Medical Sciences, Kyoto University. For breeding, brother-sister mating was performed in the animal room of the Central Research Division, School of Dentistry, Asahi University. The SAMP8 mouse has a median life span of 12 months and begins to show deficits in learning and memory 6 months after birth. A detailed description of the features of this strain has been given by Hosokawa *et al.*¹²⁾ The animals were bred and maintained under conventional conditions; housed in groups of 5 in plastic cages (175W × 245D × 125H mm) under temperature (23 ± 1°C), humidity (55 ± 2%), and light (12 h; light period, 6:00–18:00; dark period, 18:00–6:00) controlled conditions; and water were given *ad libitum*. This experiment was conducted according to the Animal Experiment Guidelines established by Asahi University.

Tooth extraction procedure

The mice were separated from the dams 4 weeks after birth. At 1 month after birth, 30 mice were anesthetized with sodium pentobarbital (Nembutal®, Dainihonseiyaku Co., Ltd., Osaka, 30 mg/kg intraperitoneal injection), fixed in the supine position, and the upper molar teeth on both sides were extracted using an excavator as carefully as possible to minimize bleeding (extraction group). The other 30 mice underwent the same anesthesia without surgery and served as controls (control group). Following surgery solid food (CE-2®, Japan Clea Co., Ltd.) was given and the mice were maintained for 1 week (young group), 4 months (mature group), and 8 months (old group).

Morris water maze test

The Morris water maze test is a sensitive behavioral assay for hippocampal abnormalities^{5,6)}. At 1 week (young group), 4 months (mature group), and 8 months (old group) after surgery, Morris water maze test was performed as described previously^{13–15)}.

Briefly, a stainless steel tank (90 cm in diameter, 30 cm deep) was filled with water (approximately 28°C) to a height of 22 cm and the water surface was covered with floating polystyrene foam granules (approximately 2 mm in diameter). A platform (12 cm in diameter) was submerged 1 cm under the water surface and located at a constant position near the center of one of the four quadrants of the pool. Training consisted of 28 trials over 7 days (4 trials per day, approximately 5-min intertrial interval). For each trial, the mice were placed into the water from one of four points at the perimeter of the tank. The sequence of the starting positions was randomized daily. A CCD video camera linked to a computer system (Move-tr/2D, Library Co., Ltd., Tokyo, Japan) was used to measure the latency of each mouse to reach the platform. On the test day 6, 2 h after the maze test, a visible probe test (four trials per animal) was also carried out on all animals, as described previously¹⁰⁾, in which the hidden platform was replaced by a block platform extending 1 cm above the water and bearing a small flag with a painting of an orange ball.

Morphological analysis

For immunohistochemical analysis of GFAP induction in the hippocampal formation, all mice were subjected to the normal water maze learning test on the final test day (day 7). Each mouse was then anesthetized with sodium pentobarbital (30 mg/kg) and perfused transcardially with 30 ml of saline at 37°C, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 and 0.1% glutaraldehyde. The brain was then removed and placed in 2% paraformaldehyde fixative overnight at 4°C. After rinsing with PB for 72 h at 4°C, tissue sections (40 µm thick) were prepared on a micro slicer (DTK-1000W, Dosaka, Kyoto, Japan), and floating sections were processed through a standard immunohistochemical procedure using the ABC method. The slices were first rinsed with phosphate-buffered saline (PBS), then incubated with 1% H₂O₂ for 10 min at room temperature (RT), rinsed with PBS, and incubated for 60 min at RT with 5% normal serum. After rinsing with PBS, the sections were incubated with rabbit polyclonal anti-GFAP anti-serum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in PBS containing 0.1% Triton X-100 (PBS-T) for 48 h at 4°C, rinsed with PBS, incubated with biotinylated goat anti-rabbit IgG (Dako Cytomation, Glostrup, Denmark) diluted 1:500 in

Fig. 1 (A) Spatial learning in the water maze test. The results are expressed as the mean score (mean $\pm$ SE, $n=5$ for each group) of four trials per day. Note the decrease in the time to reach the platform was significantly smaller in the mature or old tooth extraction groups than in their age-matched controls, but there was no significant difference between the young tooth extraction and control groups. (B) Visible probe test. (mean $\pm$ SE, $n=5$ for each group). Note there was no significant difference between all groups.

PBS for 2 h at RT, rinsed with PBS and 0.05 M Tris-HCl buffer, pH 7.6 (TBS) and incubated with peroxidase-conjugated streptavidin (Dako Cytomation Glostrup, Denmark) diluted 1:500 with TBS for 1 h at RT. Bound complex was visualized using 3, 3'-diaminobenzidine (0.5 mg/ml), nickel sulfate (5 mg/ml) and hydrogen peroxide (0.01%) in TBS. Control sections were treated with non-immune rabbit immunoglobulin instead of primary antibody.

Quantitative analysis of the staining of GFAP-positive cells was performed, as previously described¹⁶.

Briefly, GFAP-positive cells were counted in the left hippocampus (Bregma: -2.18 mm, using the atlas of Franklin and Paxinos¹⁷), on five sections per animal using a $20\times$ microscope objective. For each section, in a double-blind comparison, all labeled cells were counted on a stratum pyramidal length of $280\mu\text{m}$ (diagonal of a $200\times 200\mu\text{m}^2$) in two zones each of the CA1, CA3 and dentate gyrus (DG). The data from these zones were pooled and the mean value for each subfield estimated and expressed as number of cells/ mm^2 .

Fig. 2 Photomicrographs showing GFAP-positive astrocytes in the hippocampal CA1, CA3 and dentate gyrus (DG) subfield of control and extraction mice. Bar 200 μ m. Note in young control and tooth extraction mice, the most frequently observed type of GFAP-positive cell had long, fine, delicate processes. In contrast, in mature and old tooth extraction mice, cellular hypertrophy of GFAP-positive cells was seen which increased with age.

Statistical procedure

The data were statistically processed by analysis of variance and, then, by Scheffe's or Turkey's multiple comparison procedure. A *P* value of less than 0.05 was considered statistically significant.

Results

Morris water maze test

The results of the Morris water maze test for the tooth extraction and control groups are shown in Fig. 1. The time required to reach the platform decreased with repeated trials regardless of age or

Fig. 3 Effects of the extraction condition on the number of GFAP-positive astrocytes in the hippocampal CA1, CA3 and dentate gyrus (DG) subfield. Note GFAP-positive astrocytes were significantly increased in the CA3 region of the hippocampus in the mature or old mice of the tooth extraction group, compared with the age-matched controls. No significant difference was noted in the CA1 or DG region. In the young mice, no significant difference was observed in any region between the tooth extraction and control groups.

group [$F(144,6) = 27.939$, $P < 0.0001$], and significantly increased with an increase in age [$F(144,2) = 23.057$, $P < 0.0001$]. The decrease in the time to reach the platform was significantly smaller in the mature or old tooth extraction groups than in their age-matched controls [$F(6,144) = 27.886$, $P < 0.05$], but there was no significant difference between the young tooth extraction and control groups. There was no significant difference between all groups in the visible probe test.

Immunohistochemical staining

Figure 2 shows the results of immunohistochemical staining for GFAP-positive astrocytes in the tooth extraction and control groups. In young control and tooth extraction mice, the most frequently observed type of GFAP-positive cell had long, fine, delicate processes. In contrast, in mature and old tooth extraction mice, cellular hypertrophy of GFAP-positive cells was seen which increased with age. Figure 3 shows the numbers of GFAP-positive astrocytes in three different subfields. The number of GFAP-positive astrocytes increased with age in the 3 hippocampal regions in both the tooth extraction and control groups [CA1: $F(2,294) = 107.6687$,

$P < 0.0001$, CA3: $F(2,294) = 6.5999$, $P < 0.0001$, DG: $F(2,294) = 174.4385$, $P < 0.0001$]. On turkey's multiple comparisons, GFAP-positive astrocytes were significantly increased in the CA3 region of the hippocampus in the mature or old mice of the tooth extraction group, being 88% and 84%, respectively, compared with the age-matched controls ($P < 0.01$). No significant difference was noted in the CA1 or DG region. In the young mice, no significant difference was observed in any region between the tooth extraction and control groups.

Discussion

In our previous study¹⁸⁾, pyramidal cells were markedly reduced in the CA3 region in old mice of the tooth extraction group compared with age-matched controls. If neurons are reduced due to nerve injury in the central nervous system including the hippocampus, astrocytes repair and supplement cells to compensate^{19,20)}. We, therefore, counted astrocytes positive for GFAP, which is an astrocyte-specific protein used as an index of age-associated change^{21–23)}. GFAP is an intermediate filament protein²⁴⁾ that constitutes the cytoskeleton of glia cells

such as astrocytes and is a protein that usually exists in cytoplasm as soluble polymers²⁵). Stress and aging have been shown to induce gliosis in the central nervous system, particularly the hippocampus^{9,11,16}. In normal aging, also, glial activation is observed as an important physiologic reaction related to the repair of damaged brain tissues^{26,27}. The increase in GFAP-positive astrocytes observed in this study is considered to be a mechanism to compensate for the loss of hippocampal neurons. The increase in astrocytes was also in agreement with the decline of spatial perception observed in the Morris water maze test, and the early loss of teeth was suggested to have accelerated age-associated changes. We have also confirmed a similar marked increase in GFAP-positive astrocytes in the hippocampus of bite-raised mice¹¹). Although we did not perform morphological evaluation in this study, the hypertrophy and hyperplasia of astrocytes have been shown to be induced by nerve injury of the central nervous system, so that astrocytes may compensate for the loss of neurons in size as well as number¹⁰). Candy²⁸) also reported a marked increase in astrocyte activity by blocking action potentials in the afferent nerves. Therefore, the increase in GFAP-positive astrocytes may be explained by a decrease in c-Fos protein-positive cells due to the early loss of teeth, causing a decrease in afferent action potentials and a consequent decrease in the electrical activities of neurons. The c-fos gene and the corresponding protein Fos, which are thought to be involved in processes related to neural plasticity, and considered markers of neuronal plasticity, are considered markers of neuronal activity generated in the brain following different stimuli (spatial learning, pain, occlusion *et al.*)²⁹). The expression of c-fos is rapidly and transiently induced in the hippocampus formation following training on an odor discrimination task³⁰).

The effect of tooth extraction on GFAP-positive astrocytes number was observed only in the CA3 region and not in the CA1 and DG regions. Glutamic acid, an excitatory neurotransmitter released from mossy fiber terminals projecting from DG to CA3, is reportedly involved in changes in the GFAP-positive astrocytes in the CA3 region³¹). Glucocorticoid promotes the release of glutamic acid. Increases in glucocorticoid levels and in the release of excitatory amino acids are speculated to act synergistically as neurotoxins to induce neuronal death in the CA3 region³²).

Conclusion

In senescence-accelerated mice, early tooth extractions induced a decline in spatial memory and an increase in GFAP-positive astrocytes at mature and old ages. These results suggest that the early loss of teeth promotes aging.

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