Early toothless condition suppresses cell proliferation in the hippocampal dentate gyrus of SAMP8 mice

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Abstract Early toothlessness in senescence-accelerated prone (SAMP8) mice leads to increased plasma corticosterone levels, learning deficits, neuronal death, and increased astroglial responsiveness in the hippocampus. New cells are generated in the hippocampal dentate gyrus (DG) throughout life in animals as well as humans. Neurogenesis in the hippocampal DG is sensitive to glucocorticoid levels and environmental triggers such as learning. Here we investigated the mechanisms underlying impaired hippocampal function resulting from early masticatory dysfunction, by examining the effects of tooth loss soon after tooth eruption on plasma corticosterone levels, learning ability in the Morris water maze test, and cell proliferation in the hippocampal DG of 1-, 5-, and 9-mo-old SAMP8 mice. Bromodeoxyuridine, a marker of newborn cells, was injected, and BrdU-positive cells were quantitatively analyzed to detect cell proliferation in the hippocampal DG using immunohistochemical techniques. Early toothlessness enhanced the age-related increase in plasma corticosterone levels and learning deficits, and led to a decrease in the number of BrdU-positive cells in the hippocampal DG. Plasma corticosterone levels, learning deficits, and the number of BrdU-positive cells in the hippocampal DG was significantly different between in 5- and 9-mo-old early toothless mice and age-matched control mice, but not between 1-mo-old early toothless mice and controls. These findings suggest that early toothlessness leads to increased plasma corticosterone levels and a decrease in cell proliferation in the hippocampal DG, thereby leading to learning deficits.

Introduction Extensive evidence suggests that reduced mastication is an epidemiologic risk factor for Alzheimer’s disease1), physical and mental impairment, and mortality2). Findings from recent studies using senescence-accelerated prone (SAMP8) mice indicate that tooth loss soon after tooth eruption results in increased plasma corticosterone levels, spatial learning deficits in the Morris water maze test, neuronal death, and increased astroglial responsiveness in the hippocampus3–5). Moreover, removing the upper molars in 2-mo-old mice accelerates the aging process and shortens life span6). Removing the molars in 11-wk-old rats impairs both spatial memory and induces cholinergic neuronal system dysfunction7). Long-term edentulousness causes dementia8). Together, these findings suggest that loss of molars early in life accelerates the hippocampal

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aging process. New cells are generated in the subgranular cell layer in the dentate gyrus (DG) of the hippocampus, and in the subventricular zone lining the wall of the lateral ventricle throughout the lifetime in animals, including humans. The axons of adult-generated hippocampal neurons extend into the hippocampal CA3 regions, and newborn cells mature into functional neurons in the hippocampus. Hippocampal-mediated learning might be related to the generation of new neurons in adult DG. This cell generation is highly sensitive to various hormonal and environmental stimuli; for example, stress and/or increased plasma glucocorticoid level reduces new cell birth and aging suppresses cell birth in the DG. In contrast, exposure to an enriched environment or learning, e.g., water maze test tasks, stimulates neurogenesis. Aoki et al. reported that soft diet feeding suppressed cell proliferation in the DG of rat hippocampus, suggesting that decreased masticatory function suppresses cell proliferation in the hippocampal DG.

Here we examined whether removing teeth soon after tooth eruption impairs of cell generation in the hippocampal DG later in life, leading to learning deficits.

Methods

Male SAMP8 mice (1-mo-old; n = 40) were used. The features of the SAMP8 strain were described previously. Briefly, SAMP8 mice undergo normal maturation up to the age of 6 mo, but then exhibit accelerated aging (median life span 12 mo compared with 2–3 y for the parent strains). The animals were bred and maintained under conventional conditions: housed in groups of 5 in plastic cages under temperature-(23 ± 1°C), humidity-(55% ± 2%), and light-(12 h: light period, 0600–1800; dark period, 1800–0600) controlled conditions; and allowed free access to food and water. The mice were treated in accordance with the principles approved by the Council of the Japanese Neuroscience Society.

Mice at 1-mo of age were anesthetized with sodium pentobarbital (35 mg/kg), and their upper molars were extracted (toothless condition) as described previously. Following surgery, the mice were maintained for 1 week (young group), 4 months (mature group), or 8 months (aged group). Control animals underwent the same anesthetic procedure, but their molars were not removed.

Corticosterone levels have a circadian variation, peaking at the beginning of the dark period when activity is generally greatest, and reaching the trough levels near the end of the dark period or the beginning of the light period, when the mice are least active. Because the peak plasma corticosterone levels occur at 20:00, blood was sampled at 20:00 as previously described. Animals were decapitated, and blood was collected in 2.0 ml micro centrifuge tubes that did not contain anticoagulant. Serum was collected by immediately centrifuging the blood samples 3,500 × g for 10 min at 4°C and decanting the serum. The serum was stored at −80°C until assayed. Corticosterone was measured by radioimmunoassay at the SRL Biochemistry Lab (Tokyo, Japan).

To determine the effect of the tooth loss on cell proliferation, 14 days (young group), 4 months (mature group), or 8 months (prolonged toothless condition) after molar removal, the mice were injected with bromodeoxyuridine (BrdU: Sigma, St. Louis; 10 mg/ml dissolved in 0.9% NaCl, i.p.) 5 times a day at 3-h intervals at a dose 50 mg/kg, as described previously. The next day the mice were killed and their brains were collected.

To assess the effects of the early toothless condition on learning and memory, performance in the Morris water maze learning task was tested, as previously described, beginning at either 8 days (young group), 4 months (mature group), or 8 months (prolonged toothless condition) after surgery to remove the molars. Briefly, a stainless steel tank (diameter: 90 cm, height: 30 cm) was filled with water (~28°C) to a height of 22 cm, and the water surface was covered with floating 2-mm diameter polystyrene foam granules. A platform 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. The mice were placed in the water at 1 of 4 points around the perimeter of the tank and given 28 training trials over 7 consecutive days (4 trials/d). The sequence of starting points was changed randomly each day. The swim paths and latencies to reach the platform were monitored using a CCD video camera linked to a computer system (Mover-tr/2D, Library Co., Ltd., Tokyo, Japan). On day 6, 2 h after the water maze test, each animal received a visible probe test (4 trials/animal), using a black platform that extended 1 cm above the surface of the water and had a small flag with an orange circle instead of the hidden platform.
On days 5, 6, and 7 of the Morris water maze test, bromodeoxyuridine (BrdU: Sigma, St. Louise; 10 mg/ml dissolved in 0.9% NaCl, 50 mg/kg, i.p.) was injected as described previously\textsuperscript{24}. The brains of the mice were collected the next day after completing the Morris water maze test.

After being anesthetized with pentobarbital sodium (40 mg/kg), the mice were transcardially perfused with 30 ml of saline at 37°C, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were then removed and placed in 2% paraformaldehyde fixative overnight at 4°C.

Tissue sections (40-μm thick) were prepared on a microslicer (DTK-1000W, Dosaka, Kyoto, Japan). To denature the DNA, the sections were incubated for 2 h in 50% formamide/2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate) at 65°C, incubated for 30 min in 2 N HCl at 37°C, and neutralized for 10 min in 0.05 M TBS, pH 8.5. Floating sections were then 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, rinsed with PBS, and incubated for 60 min at room temperature with 5% normal goat serum. After rinsing again with PBS, the sections were incubated with rabbit polyclonal anti-BrdU antiserum (Abcam PLC, Cambridge, UK) distilled 1:200 in PBS containing 0.3% 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 PBS for 2 h at room temperature, rinsed with PBS and 0.05 M Tris-HCl buffer, pH 7.6 (TBS), and incubated with peroxidase-conjugated streptavidin (Dako Cytomation) diluted 1:500 with PBS for 1 h at room temperature. Bound complex was visualized using 3,3’-diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.01%) in TBS. Control sections were treated with non-immature rabbit immunoglobulin instead of primary antibody.

All BrdU-labeled cells including individual cells within a cluster, were counted unilaterally (left side) regardless of size or shape in every 12th section throughout the hippocampus (bregma -2.12 mm to -6.30 mm)\textsuperscript{25}. Analysis of each group of animals was performed with the investigator blind to the treatment group of the animal.

Two-way repeated or factorial analysis of variance (ANOVA) followed by Tukey’s multiple comparison analysis was used to evaluate the statistical significance of the differences in the biochemical, behavioral, and morphologic results between groups.

**Results**

The plasma corticosterone levels for both the control and early toothless mice are shown in Fig. 1. The plasma corticosterone levels in 5-mo and 9-mo-old early toothless mice were 185% and 140% that of age-matched control mice, respectively. The plasma corticosterone levels increased with age \[F(2,29) = 53.65, P<0.01\], and was significantly different between control mice and early toothless mice \[F(1,29) = 24.63, P<0.01\]. Age significantly affected plasma corticosterone levels in both the control and early toothless condition \[F(2,29) = 5.94, P<0.01\]. Plasma corticosterone levels significantly increased in the early toothless conditions compared to the controls at both 5 and 9 months (5-mo-old, \(P<0.05\); 9-mo-old, \(P<0.05\)).

The results of Morris water maze test were significantly different between the control and early toothless conditions \[F(1,204) = 14.382, P<0.01\], and ages \[F(2,204) = 59.581, P<0.01\] (Fig. 2A). The interaction between the control or early toothless condition and age was no significant. Performance in the visible probe test did not differ significantly between groups (Fig. 2B).
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Fig. 2 Spatial learning in the Morris water maze test (A) and visible probe test (B). The results are expressed as the mean score (mean ± SD, n = 5 for each group) of four trials per day. Note that the early toothless mice required a longer time to reach the platform (A), but there was no significant difference in latency among groups in the visible probe test (B).

Fig. 3 Representative photomicrographs showing BrdU-positive cells in the hippocampal DG (3A) and effect of the early toothless condition on number of BrdU-positive cells in the DG. The results are expressed the mean number of neurons/mm² (mean ± SD, n = 5 for each group). ※: P < 0.05. Note the greater reduction in the number of BrdU-positive cells in the DG of 5-mo and 9-mo-old early toothless mice.
Representative photomicrographs of BrdU-positive cells in the hippocampal DG of control and early toothless mice and the number of BrdU-positive cells in the DG of control and early toothless mice are shown in Figs. 3A and 3B, respectively. The number of BrdU-positive cells in the DG decreased with age \( F(2,29) = 124.18, \ P < 0.01 \) and differed significantly between control and early toothless mice \( F(1,29) = 788.6, \ P < 0.01 \). The interaction between the control or early toothless conditions, and age in the number of BrdU-positive cells in the DG was significant \( F(2,29) = 365.640, \ P = 0.0443 \). In particular, the number of BrdU-positive cells in the DG was a significantly different between control and early toothless mice in the mature and aged mice (5-mo-old, \( P < 0.05 \); 9-mo-old, \( P < 0.05 \)).

**Discussion**

In the present study, we used biochemical, behavioral, and morphologic techniques, to examine whether early removal of molars enhances the age-dependent increase in plasma corticosterone levels, deficits in learning and memory, and decrease in cell proliferation in the hippocampal DG. The morphologic findings were similar to previously reported findings that soft diet feeding suppresses cell proliferation in the DG of the rat hippocampus\(^1\), indicating that masticatory dysfunction leads to suppressed cell proliferation in the hippocampal DG. The mechanism induced by the early toothless condition is purely speculative at the present time, but we propose two possible explanations.

One possible explanation is that the early molarless condition affects the aging process in hippocampus. Onozuka et al., reported that aged mice in the molarless condition exhibited learning deficits in the Morris water maze test associated with hippocampal changes, *e.g.*, increased plasma corticosterone levels, decreased cell generation, increased neuronal death, and hypertrophied glial fibrillary acidic protein positive cells\(^2,22,26,27\). These changes in hippocampus induced by the toothless condition are very similar to the changes observed with advancing age\(^28–30\). Therefore, the molarless condition appears to accelerate the aging process in the hippocampus. With respect to Alzheimer’s disease and aging, the most vulnerable circuit in the cerebral cortex is the projection referred to as the perforant pathway\(^31\), which originates in the entorhinal cortex and terminates in the hippocampal DG, thus providing the key interconnection between the neocortex and the hippocampus\(^32,33\). The entorhinal cortex is a region of extraordinary convergence of inputs from the association cortex, essentially funneling highly processed information from the neocortex to hippocampal DG and playing a crucial role in learning and memory\(^34,35\). Aged SAMP8 mice in the molarless condition show attenuated hippocampal Fos induction in associated with impaired water maze performance\(^36\), decreased dendritic spine number in hippocampal pyramidal neurons\(^21\), and septohippocampal cholinergic system dysfunction\(^37\). Trigeminal input has a facilitatory effect on synaptic transmission in various regions of the cerebral cortex\(^38,39\), and therefore the behavioral and morphologic changes in aged toothless mice may be largely due to reduced afferent input from the jaw to the cerebral cortex due to the lack of mastication and mastication-associated face and jaw movements. The hippocampal formation, especially the DG, is particularly vulnerable to aging, which might explain the cognitive decline during aging\(^40\). Aging severely attenuates adult neurogenesis\(^18\). In particular, the proliferation process is severely influenced by aging, and might be the cause of age-dependent cognitive dysfunction\(^41\). Indeed, the early toothless condition induces same behavioral and morphologic changes in the hippocampus\(^3–5\), and thus appears to accelerate the aging process in the hippocampus.

Another possibility is that, the early toothless condition affects plasma corticosterone levels. The proposal by Sapolsky et al.\(^42,43\) that glucocorticoids contribute to brain aging is now widely accepted. Elevated glucocorticoid levels are frequently detected in aged rats and may contribute to age-related memory and learning deficits\(^44,45\). In addition, stress and corticosterone can reduce adult progenitor proliferation, and may relate to the decline in neurogenesis during aging. Following adrenalectomy, neurogenesis increases in old rodents, suggesting that reduced neurogenesis during aging\(^46\) might be causally related to increased glucocorticoid levels\(^47\). Stress and increased corticosterone impair learning and memory\(^48,49\). Indeed, in the present study, 5-mo, and 9-mo-old toothless mice had increased plasma corticosterone levels, deficits in spatial memory, and suppressed of cell proliferation, suggesting that the toothless condition induces an increase in circulating corticosterone via chronic stress, which might exacerbate the age-related suppression of cell birth in hippocampal DG.
Conclusion

An early toothless condition age-dependently suppresses cell generation in the hippocampal DG of SAMP8 mice, leading to impaired of learning and memory.

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