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# Tooth loss early in life suppresses neurogenesis and synaptophysin expression in the hippocampus and impairs learning in mice

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ABSTRACT

*Objective:* Tooth loss induced neurological alterations through activation of a stress hormone, corticosterone. Age-related hippocampal morphological and functional changes were accelerated by early tooth loss in senescence-accelerated mouse prone 8 (SAMP8). In order to explore the mechanism underlying the impaired hippocampal function resulting from early masticatory dysfunction due to tooth loss, we investigated the effects of early tooth loss on plasma corticosterone levels, learning ability, neurogenesis, and synaptophysin expression in the hippocampus later in life of SAMP8 mice.
*Design:* We examined the effects of tooth loss soon after tooth eruption (1 month of age) on plasma corticosterone levels, learning ability in the Morris water maze, newborn cell proliferation, survival and differentiation in the hippocampal dentate gyrus, and synaptophysin expression in the hippocampus of aged (8 months of age) SAMP8 mice.
*Results:* Aged mice with early tooth loss exhibited increased plasma corticosterone levels, hippocampus-dependent learning deficits in the Morris water maze, decreased cell proliferation, and cell survival in the dentate gyrus, and suppressed synaptophysin expression in the hippocampus. Newborn cell differentiation in the hippocampal dentate gyrus, however, was not affected by early tooth loss.
*Conclusion:* These findings suggest that learning deficits in aged SAMP8 mice with tooth loss soon after tooth eruption are associated with suppressed neurogenesis and decreased synaptophysin expression resulting from increased plasma corticosterone levels, and that long-term tooth loss leads to impaired cognitive function in older age.



## 1. Introduction

Learning and memory critically depend on the hippocampus, a bilateral brain structure located in the temporal lobe. New cells are generated throughout life in the subgranular cell layer of the hippocampal dentate gyrus (DG) (Gage, 2002), where they mature into functional neurons with axons extending into the hippocampal CA3 region (Hastings, 1999; Stanfield & Trice, 1988). Neuronal cell proliferation, differentiation, and survival are regulated at several levels (Lee et al., 2006), and are likely related to hippocampus-mediated learning ability (Gould, Beylin, Tanapet, Reeves, & Shors, 1999). Neurogenesis in the DG decreases with aging (Gould, Reeves et al., 1999), and the age-dependent cognitive impairment is likely related to the age-related decline in neurogenesis (Bondolfi, Ermini, Long, Ingram, & Jucker, 2004). Newborn cell generation is affected by various factors, including psychologic stress and environmental complexity (Czéh et al., 2002; Torner et al., 2009; Kempermann, Brandon, & Gage, 1998; van Praag, Kempermann, & Gage, 1999). Synaptophysin is a major integral protein of the synaptic vesicle membrane that is involved in the regulation of neurotransmitter release (Bähler, Benfenati, Valtorta, & Greengard, 1990). Synaptophysin-positive synaptic boutons are a sensitive correlate of cognitive deficits (Calhoun et al., 1998).

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Exposure to stress leads to reduced expression of synaptophysin in the hippocampus (Thome et al., 2001).

Impaired mastication is an epidemiologic risk factor for learning and memory dysfunction, such as dementia and Alzheimer's disease, as well as for mortality (Shimazaki et al., 2001; Shigetomi, 1998; Kondo, Niino, & Shido, 1994). Recent studies using senescence-accelerated mouse prone 8 (SAMP8) reported that tooth loss soon after tooth eruption accelerated age-related increases in plasma corticosterone levels, spatial learning deficits, neuronal loss, and increase in astroglial responsiveness in the hippocampus (Kondo et al., 2016; Kubo et al., 2010). These findings suggest that long-term tooth loss and reduced mastication impair hippocampus-related cognitive function later in adulthood. The mechanisms underlying the effect of early tooth loss on neurogenesis in the hippocampal DG, however, are not well understood.

In the present study, we examined whether tooth removal soon after tooth eruption affects cell proliferation, cell survival, and newborn cell differentiation in the DG; synaptophysin expression in the hippocampus; and learning ability in aged SAMP8 mice.

## 2. Materials and methods

### 2.1. Animals and experimental protocol

We used male 1-month-old SAMP8 mice (n = 66) in the present study. Mice of this strain mature normally up to 6 months of age and then exhibit features of accelerated aging with a median lifespan of only 12 months compared with the 2–3 year median lifespan of the parent strains. The characteristics of the SAMP8 strain are reported elsewhere (Flood & Morley, 1998). The mice were bred under conventional conditions and then housed in plastic cages in groups of five under controlled temperature ($23 \pm 1\,^{\circ}C$), humidity ($55 \pm 2\%$), and light (12 h; light period, 0600–1800; dark period, 1800–0600). Food and water were available *ad libitum*. The experiment was undertaken in accordance with the guidelines for laboratory animal care and use of Asahi University. The ethics committee of Asahi University School of Dentistry approved the study.

Removal of the upper molar teeth was performed as described previously (Kondo et al., 2016). Briefly, mice at 1 month of age were anesthetized with sodium pentobarbital (35 mg/kg) and the upper molar teeth were extracted using tweezers. As tooth development in mice continues for 25 days after birth (Hamashima, 1963), the molars were extracted at one month of age to ensure complete tooth removal. Control animals underwent the same anesthetic procedures, but their molars were not removed. The mice were maintained under the same controlled conditions after surgery for 8 months. Body weight was measured immediate before surgery, and at the end of 1st, 4th, and 8th months after surgery.

### 2.2. Plasma corticosterone levels

To examine the effects of early tooth loss on plasma corticosterone levels in aged mice, plasma corticosterone levels were measured in experimental and control mice 8 months after molar removal (n = 5/group). Blood was sampled at the beginning of the dark period (2000 h), when corticosterone levels are typically highest (Olariu, Cleaver, & Cameron, 2007). Mice were then decapitated and blood was collected in 2.0-ml micro-centrifuge tubes without anticoagulant and centrifuged at $3500 \times g$ for 10 min at $4\,^{\circ}C$. The serum was stored at $-80\,^{\circ}C$ until measurement of the corticosterone levels by radioimmunoassay at the SRL Laboratories in Tokyo, Japan.

### 2.3. Morris water maze test

The Morris water maze test is commonly used for examining hippocampus-related learning and memory (Morris, 2007). The Morris water maze test was performed for both experimental and control mice (n = 8/group) as described previously (Kondo et al., 2016). Briefly, a stainless steel tank (90 cm in diameter and 30 cm in depth) was filled with water ($\sim 25\,^{\circ}C$) to a height of 22 cm and the water surface was covered with floating polystyrene foam granules with a ~2-mm diameter. A platform was submerged 1 cm under the water surface at a constant position near the center of one of the four quadrants of the pool. The mice were placed into the water from 1 of 4 points around the perimeter of the tank and given 4 learning trials daily for 7 consecutive days (a total of 28 trials). The sequence of the starting positions for each of the four trials was randomly changed every day. The latency to swim to the platform was monitored using a CCD video camera linked to a computer system (Move-tr/2D, Library Co., Ltd, Tokyo, Japan). On the last day of training, all animals underwent a visible probe test 2 h after the last training trial.

### 2.4. Immunohistochemistry

We examined the cell proliferation and survival following intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg; Sigma-Aldrich, St, Louis, MO; 10 mg/ml dissolved in 0.9% NaCl solution) into the mice (n = 10/group) 5 times with 3-h intervals at 8 months after the molar removal procedure, as described previously (Takagi et al., 1999). Either the next day (n = 5/group) or 22 d (n = 5/group) after the BrdU injection, the mice were anesthetized with pentobarbital sodium (40 mg/kg) and trans-cardially perfused with 30 ml of saline at $37\,^{\circ}C$, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at $4\,^{\circ}C$. The brains were removed and fixed in 2% paraformaldehyde fixative overnight at $4\,^{\circ}C$.

Brain sections with a thickness of 25 μm were prepared using a cryostat (CM1850, LEICA, Wetzlar, Germany). The DNA was denatured by incubating the sections for 2 h in 50% formamide/2 x SSC (0.3 M sodium chloride and 0.03 M sodium citrate) at $65\,^{\circ}C$, for 30 min in 2N HCl at $37\,^{\circ}C$, and then neutralizing them for 10 min in 0.05 M Tris-buffered (TBS, pH 8.5). A standard immunohistochemical procedure was used to process floating sections with the ABC method. First, brain slices were rinsed with phosphate-buffered saline (PBS), incubated with 1% $H_2O_2$ for 10 min at room temperature, rinsed again with PBS, and incubated for 60 min at room temperature with 5% normal goat serum. After the third rinse with PBS, the sections were incubated with rabbit polyclonal anti-BrdU antiserum (Abcam PLC, Cambridge, UK) diluted 1:200 in PBS containing 0.3% Triton X-100 for 48 h at $4\,^{\circ}C$, 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 again with PBS and 0.05 M Tris-HCl buffer (pH7.6), 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.

To investigate the effects of early tooth loss on newborn cell differentiation in the hippocampal DG, double-immunofluorescence with markers for mature granule neurons (NeuN) and astroglial glial fibrillary acidic protein (GFAP) was used to detect colocalization with BrdU. BrdU (50 mg/kg) was injected intraperitoneally into mice (n = 5/group) 5 times with 3-h intervals and, the brains of the mice were collected 22 days after BrdU injection, as described above.