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Age-dependent loss of cholinergic neurons in learning and memory-related brain regions and impaired learning in SAMP8 mice with trigeminal nerve damage

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Abstract

The tooth belongs to the trigeminal sensory pathway. Dental damage has been associated with impairments in the central nervous system that may be mediated by injury to the trigeminal nerve. In the present study, we investigated the effects of damage to the inferior alveolar nerve, an important peripheral nerve in the trigeminal sensory pathway, on learning and memory behaviors and structural changes in related brain regions, in a mouse model of Alzheimer's disease. Inferior alveolar nerve transection or sham surgery was performed in middle-aged (4-month-old) or elderly (7-month-old) senescence-accelerated mouse prone 8 (SAMP8) mice. When the middle-aged mice reached 8 months (middle-aged group 1) or 11 months (middle-aged group 2), and the elderly group reached 11 months, step-down passive avoidance and Y-maze tests of learning and memory were performed, and the cholinergic system was examined in the hippocampus (Nissl staining and acetylcholinesterase histochemistry) and basal forebrain (choline acetyltransferase immunohistochemistry). In the elderly group, animals that underwent nerve transection had fewer pyramidal neurons in the hippocampal CA1 and CA3 regions, fewer cholinergic fibers in the CA1 and dentate gyrus, and fewer cholinergic neurons in the medial septal nucleus and vertical limb of the diagonal band, compared with sham-operated animals, as well as showing impairments in learning and memory. Conversely, no significant differences in histology or behavior were observed between middle-aged group 1 or group 2 transected mice and age-matched sham-operated mice. The present findings suggest that trigeminal nerve damage in old age, but not middle age, can induce degeneration of the septal-hippocampal cholinergic system and loss of hippocampal pyramidal neurons, and ultimately impair learning ability. Our results highlight the importance of active treatment of trigeminal nerve damage in elderly patients and those with Alzheimer's disease, and indicate that tooth extraction should be avoided in these populations.

Key Words: *nerve regeneration; Alzheimer's disease; trigeminal nerve; learning; memory; hippocampal CA1; hippocampal CA3; dentate gyrus; basal forebrain; medial septal nucleus; vertical limb of the diagonal band; cholinergic neurons; cholinergic fibers; pyramidal cells; NSFC grants; neural regeneration*

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Introduction

Root surface caries, tooth loss and movement disorders of the mouth and jaw are commonly found in patients with Alzheimer's disease (AD) and are considered risk factors for AD (Olton et al., 1979; Bjertness, 1991; Nagao, 1992; Jones et al., 1993; Sparks Stein et al., 2012; Noble et al., 2013; Teixeira et al., 2014). Kondo et al. (1994) showed that tooth loss is an important risk factor for AD, providing an important clue to the etiology of the disease. Chronic inflammation caused by root surface caries and periodontal disease is also considered a risk factor. The development of periodontal pockets, alveolar bone loss and tooth loss correlate significantly with cognitive impairment in individuals aged over 45 years (Kaye et al., 2010; Noble et al., 2013). Furthermore, serum antibodies to periodontal pathogens are detectable in patients with AD (Sparks Stein et al., 2012; Noble et al., 2013). However, the mechanisms underlying the relationship between AD and root surface caries, periodontitis or tooth loss are poorly understood.

Previous studies have examined the effects of oral dam-

age on learning and memory (Noble et al., 2013; Teixeira et al., 2014). In elderly senescence-accelerated mouse prone 8 (SAMP8) mice, a model for AD, learning and neuronal density in the hippocampal CA1 region are notably diminished after molar extraction; more hippocampal neurons are lost with time since tooth loss, but dental repair improves learning and memory (Onozuka et al., 1999; Watanabe et al., 2002; Noble et al., 2013; Teixeira et al., 2014). In rats, long-term soft-diet feeding reduces afferent impulses from each masticatory muscle, and this leads to impairments in learning and memory, and neuronal loss in the hippocampus (Yamamoto and Hirayama, 2001; Ono et al., 2010). In summary, tooth loss and reduced masticatory function lead to impairments in learning and memory, neuronal damage in learning- and memory-associated brain regions, and acceleration of AD pathogenesis in animal models (Okamoto et al., 2010a; Hiroshi et al., 2013; Hyunyoung et al., 2013; Noble et al., 2013; Teixeira et al., 2014). The mechanisms underlying these observed associations between oral damage, brain damage and AD remain unknown, but may lie in the trigeminal sensory pathway.

The inferior alveolar nerve is an important peripheral nerve in the trigeminal sensory pathway, responsible for proprioception and nociception below the mandibular teeth, mandible and oral fissure. It is a major proprioceptive afferent, stimulated by mastication (Liu et al., 2012; Huang et al., 2013; Ge et al., 2014). After tooth extraction, dental pulp and periodontal sensory nerves can become damaged, and primary sensory neuronal degeneration may occur in the trigeminal system (Gobel, 1984; Kubota et al., 1988). This, in turn, may affect the trigeminal nuclei and ultimately the diencephalon and cerebrum. The decrease in afferent impulses of sensory nerves in the mouth and jaw also leads to the degeneration of target neurons (Cooper and Sofroniew, 1996; Gould and Cameron, 1996). Stimulation by chewing is important in maintaining hippocampal neurons (Onozuka et al., 1999). Eating hard food has been shown to suppress Fos expression in rat cerebral cortex, but this inhibitory effect decreases after bilateral inferior alveolar nerve transection or bilateral somatosensory cortex removal (Ogawa et al., 2003). Therefore, the role of the trigeminal nerve in maintaining learning and memory capabilities, and the effect of damage to the trigeminal nerve, in patients with AD and in animal models deserves further investigation.

In the present study, we explored learning- and memory-related behavior and neuroanatomy in SAMP8 mice during aging, after inferior alveolar nerve transection at 4 months (middle-aged) or 7 months (elderly).

Materials and Methods

Animals and experimental groups

A total of 60 healthy SAMP8 mice were purchased from the Beijing Vital River Laboratory Animal Science and Technology Co., Ltd., China. Of these, 40 mice were aged 4 months, and were equally and randomly divided into four groups: middle-aged experimental groups 1 and 2, and middle-aged control groups 1 and 2 (10 rats per group). The remaining 20 mice were aged 7 months and were equally and randomly divided into an elderly experimental group and an elderly control group (10 rats per group).

Mice in the experimental groups were anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneally) and underwent inferior alveolar nerve transection. A small incision was made in the skin over the masseter, the surface of the inferior alveolar bone was exposed, and the inferior alveolar nerve was isolated. A 1.0 mm length of the nerve was cut and removed, and the wound was sutured. Penicillin (20,000 U) was injected intramuscularly after the surgery. The control groups underwent the same surgery but without ligation of the inferior alveolar nerve; all subsequent experimental procedures were the same as the experimental groups.

Behavioral testing

Learning and memory were assessed using a step-down device (STT-2; Medicine Institute, Chinese Academy of Medical Sciences, Beijing, China) and Y-maze stimulator (MG-3; Huaibei Zhenghua Biological Equipment Co., Ltd., Huaibei, Anhui Province, China) when the mice in middle-aged control group 1 and middle-aged experimental group 1 reached 8 months, and the mice in middle-aged control group 2, middle-aged experimental group 2, elderly control group and elderly experimental group reached 11 months.

Step-down test

This test examined the conditioned passive avoidance reflex. The apparatus comprised a Plexiglas box (60 cm × 12 cm × 18 cm) with an electrified copper grid base, separated into five compartments by opaque walls to allow the testing of five animals at a time. A cylindrical rubber platform, 4.5 cm in height and 4.5 cm in diameter, on which the mice could avoid electric shock, was placed in each compartment. Each mouse was habituated to a compartment for 3 minutes before a 36 V alternating current was applied; the mouse would jump onto the platform to avoid the electric shock. Most mice would then return to the copper grid before rapidly jumping back onto the platform after feeling the electric shock. In a 5 minute acquisition session, the escape latency (time taken for the mouse to jump onto the platform) and the number of errors (number of electric shocks) were recorded, and constituted learning parameters. Twenty-four hours later, the test was repeated for 3 minutes; escape latency and step-down latency (time taken for the mouse to leave the platform) were recorded. If a measure exceeded 3 minutes, a ceiling score of 3 minutes was recorded. In the 3 minute test, escape latency, step-down latency and number of errors constituted memory parameters.

Y-maze test

The Y-maze comprised three similar arms arranged 120° apart around a central joining region, and an electrified grid floor. A signal light was located at the end of each arm. The light was illuminated to indicate that a 50 V electric current would pass through the floor of that arm (*i.e.*, non-safe region), and remained off in the other two arms (*i.e.*, safe re-

gions). The mice were placed in a random arm of the Y-maze and allowed to explore for 3 minutes before the signal light was turned on in a random arm. Once the light was illuminated in the non-safe region, the mice had 10 seconds to escape to a safe region (correct response). If they did not escape within 10 seconds, this constituted an error. The mice were considered to have learned the task when they performed 9 correct responses in 10 tests. The learning rate was measured by the number of sessions required to achieve this criterion. Twenty-four hours later, the test was repeated, and the number of correct responses was a measure of memory.

Tissue collection

After behavioral testing, the mice were anesthetized with 10% chloral hydrate (4 mL/kg, intraperitoneally) and rapidly perfused through the left ventricle of the heart with 50 mL physiological saline at room temperature, followed by 150 mL 4% paraformaldehyde prepared in 0.1 mol/L phosphate buffer, pH 7.2–7.4 (PFA) at 4°C. Brains were removed, post-fixed in 4% PFA for 4–6 hours, and immersed in 20% sucrose overnight. Brain tissue was cut into 20 μm-thick coronal sections with a freezing microtome (Leica, Nussloch, Germany), and every sixth section was collected in serial sets. Three sets of sections were subjected to Nissl staining, acetylcholinesterase (AChE) enzyme histochemistry and choline acetyl transferase (ChAT) immunohistochemistry.

Staining

Nissl staining

Samples were mounted with gelatin and dried at room temperature. After three washes with distilled water, the slides were dipped in 0.25% cresyl violet (Sigma, St. Louis, MO, USA) for 30 seconds, washed again with distilled water, and dehydrated through an alcohol series (dipped in 70%, 80%, 90%, and twice in 100% alcohol, for 30 seconds each). The sections were permeabilized with xylene and mounted with neutral resin. The background was colorless, and Nissl bodies were stained blue-purple.

ChAT immunohistochemistry

The avidin-biotin-peroxidase complex method was used (Cheng et al., 2008; Leng et al., 2008). Sections were washed three times with 0.01 mol/L phosphate buffered saline (PBS) (pH 7.2–7.4) for 5 minutes each time, incubated with 0.3% H_2O_2 at room temperature for 30 minutes, washed three times with PBS as before, treated with 1.5% bovine serum albumin at 37°C for 60 minutes, and then incubated with the primary antibody, goat anti-mouse ChAT polyclonal antibody (1:250; Chemicon, CA, USA) at 37°C for 2 hours and at room temperature overnight. After three further washes with PBS, the sections were incubated with the secondary antibody, rabbit anti-goat horseradish peroxidase (1:1,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China) at 37°C for 2 hours, followed by three PBS washes, and then visualized by reacting with 0.05% 3,3′-diaminobenzidine (DAB) + 0.01% H_2O_2 at room temperature for 15 minutes. After three final washes with PBS, the sections were mounted with gelatin, dried at room temperature, dehydrated with alcohol, permeabilized with xylene, and mounted with neutral resin. The background was light brown, and neurons were stained dark brown. A negative control was not incubated with the primary antibody.

AChE histochemistry

Before staining, incubation solution was prepared with 10 mg acetylthiocholine iodide (Sigma), 24.5 mL reserve liquid I (65 mL acetate buffer, 1 g $Na_3C_6H_5O_7 \cdot 2H_2O$, 75 mg $CuSO_4 \cdot 5\ H_2O$ and double distilled water to 400 mL) and 0.5 mL reserve liquid II (165 mg ferricyanide and double distilled water to 100 mL). Sections were washed twice with 0.1 mol/L acetate buffer (pH 6) for 5 minutes each time (*i.e.*, 2 × 5 minutes), placed in incubation solution for 30–40 minutes at room temperature, and washed with acetate buffer (5 × 3 minutes), 1% ammonium sulfide (1 × 1–2 minutes), 0.1 mol/L sodium nitrate (5 × 3 minutes), 0.1% silver nitrate (1 × 1 minute), and 0.1 mol/L sodium nitrate (5 × 3 minutes). Sections were mounted with gelatin, dried at room temperature, dehydrated with alcohol, permeabilized with xylene, and mounted with neutral resin. The background was colorless, and AChE was visible as brown precipitate. Acetylthiocholine iodide was not added to the incubation solution in the negative control test (Hedreen et al., 1985).

Morphometric analysis

Sections were observed under a light microscope (Zeiss, Axioskop 40, Germany) equipped with a CCD camera (Carl Zeiss, Hallbergmoos, Germany). Images were processed using Axio Vision 4.5 imaging software (Carl Zeiss).

Hippocampal pyramidal neurons

Five Nissl-stained sections of the hippocampus were selected at random from each mouse, and neurons were counted in two randomly selected fields from each section, in the CA1 and CA3 pyramidal layers, under a 40 × objective lens. Counts were taken bilaterally and the mean value was used.

ChAT-immunoreactive neurons in the basal forebrain

Five sections were selected at random from each mouse. The anterior commissure was used to mark the boundary between the medial septal nucleus and the vertical limb of the diagonal band (dorsal and ventral to the anterior commissure, respectively). The number of ChAT-immunoreactive neurons in both regions was quantified in each section using a 10 × 10 grid (1 mm in 1 grid and magnification at 10 × 20 times lens).

AChE-positive fibers in the hippocampal formation

Five stained sections of the hippocampal formation were selected at random from each mouse. The stratum radiatum and stratum lacunosum of the CA1 and dentate gyrus were identified under a 20 × objective lens and cells were counted bilaterally. Image-Pro Plus 6.0 software (Media Cybernetics,

Table 1 Effects of inferior alveolar nerve injury on conditioned step-down passive avoidance in senescence-accelerated mouse prone 8 mice: acquisition session

Group	Escape latency (second)	Number of errors within 5 minutes
Middle-aged control 1	26.20±8.67	1.20±0.44
Middle-aged control 2	44.30±18.40	3.50±2.17
Elderly control	39.70±14.84	3.00±1.49
Middle-aged experimental 1	32.40±9.26	1.30±0.48
Middle-aged experimental 2	46.90±11.14	3.20±1.62
Elderly experimental	63.60±15.31*	5.60±1.84*

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests between corresponding experimental and control groups. *P < 0.05, *vs.* elderly control.

Table 2 Effects of inferior alveolar nerve injury on memory in senescence-accelerated mouse prone 8 mice, 24 hours after step-down training: test session

Group	Escape latency (second)	First step-down latency (second)	Number of errors within 3 minutes
Middle-aged control 1	2.70±0.90	90.20±20.31	1.80±0.72
Middle-aged control 2	5.40±2.32	56.00±19.82	2.80±1.53
Elderly control	4.60±2.31	56.40±17.44	2.40±1.78
Middle-aged experimental 1	2.90±0.86	99.10±22.33	1.70±0.86
Middle-aged experimental 2	4.20±0.79	56.10±21.50	2.50±1.78
Elderly experimental	4.90±1.79	68.10±17.38	2.30±1.16

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests; no significant differences were found between corresponding experimental and control groups.

Table 3 Effects of inferior alveolar nerve injury on learning and memory parameters in the Y-maze in senescence-accelerated mouse prone 8 mice

Group	Trials to criterion	Correct responses after 24 hours
Middle-aged control 1	15.10±6.52	8.20±1.93
Middle-aged control 2	21.30±8.35	5.30±2.63
Elderly control	18.50±5.44	5.80±1.93
Middle-aged experimental 1	16.70±4.67	7.20±2.36
Middle-aged experimental 2	21.90±5.99	4.50±2.22
Elderly experimental	25.90±6.21*	4.50±1.58

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests between corresponding experimental and control groups. *P < 0.05, *vs.* elderly control.

Silver Spring, MD, USA) was used to determine the optical density of AChE-positive fibers per unit area.

Statistical analysis

All data were expressed as the mean ± SD. Means were compared using independent samples t-tests conducted with SigmaStat software (version 3.1, Jandel Corporation, Las Vegas, NV, USA). P < 0.05 was considered statistically significant.

Table 4 Effects of inferior alveolar nerve injury on the number of pyramidal neurons in the hippocampus of senescence-accelerated mouse prone 8 mice

Group	Hippocampal CA1	Hippocampal CA3
Middle-aged control 1	134.41±3.70	173.88±5.26
Middle-aged control 2	114.63±2.80	151.54±4.94
Elderly control	117.56±6.19	154.13±4.25
Middle-aged experimental 1	132.81±3.56	169.13±5.31
Middle-aged experimental 2	112.56±1.94	151.33±4.02
Elderly experimental	75.39±7.43*	115.99±5.62*

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests between corresponding experimental and control groups. *P < 0.01, *vs.* elderly control.

Table 5 Effects of inferior alveolar nerve injury on the number of choline acetyltransferase-immunoreactive neurons in the basal forebrain of senescence-accelerated mouse prone 8 mice

Group	Medial septal nucleus	Vertical limb of the diagonal band
Middle-aged control 1	125.66±12.77	233.21±18.88
Middle-aged control 2	103.12±15.15	194.44±20.55
Elderly control	102.26±8.86	175.46±12.70
Middle-aged experimental 1	127.81±16.21	221.41±21.38
Middle-aged experimental 2	103.70±12.54	185.16±18.49
Elderly experimental	62.76±12.13*	81.62±20.44*

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests between corresponding experimental and control groups. *P < 0.01, *vs.* elderly control.

Table 6 Effects of inferior alveolar nerve injury on the number of acetylcholinesterase-positive nerve fibers in the hippocampus of senescence-accelerated mouse prone 8 mice

Group	Hippocampal CA1	Dentate gyrus
Middle-aged control 1	452,481.4±33,519.88	404,286.1±32,268.21
Middle-aged control 2	420,682.0±38,676.76	372,696.3±35,603.75
Elderly control	414,868.4±20,575.27	388,696.1±50,516.02
Middle-aged experimental 1	446,712.7±35,294.33	398,661.3±40,213.77
Middle-aged experimental 2	414,956.7±34,582.59	378,532.9±38,150.39
Elderly experimental	377,908.0±38,992.66*	327,189.2±45,793.93*

Data are expressed as the mean ± SD (n = 10 rats per group). Means were compared using paired t-tests between corresponding experimental and control groups. *P < 0.01, *vs.* elderly control.

Results

General observations

The inferior alveolar nerve was intact in all sham-operated mice. In the experimental groups, no regeneration of the inferior alveolar nerve was detected in the mandible.

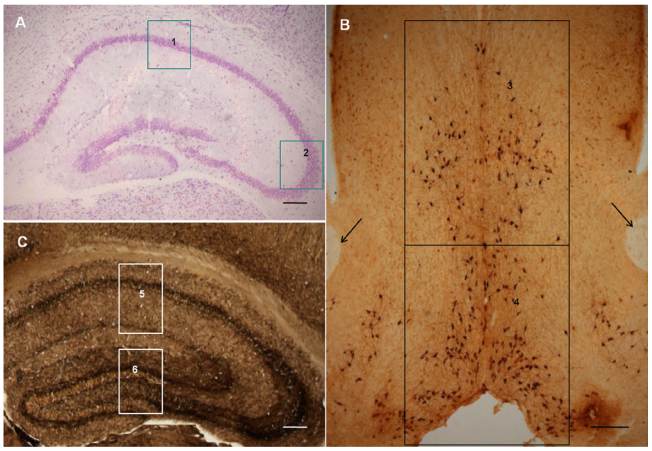

Figure 1 Pyramidal and cholinergic neurons in the hippocampus and basal forebrain in middle-aged control group 1.
(A) Cresyl violet staining: pyramidal layer in hippocampal regions CA1 (box 1) and CA3 (box 2). (B) Choline acetyltransferase (ChAT) immunohistochemical staining: ChAT-immunoreactive neurons in the medial septal nucleus (box 3) and vertical limb of the diagonal band (box 4) in the basal forebrain; arrows point to anterior commissure (boundary marker between medial septal nucleus and vertical limb of the diagonal band). (C) Acetylcholinesterase (AChE) histochemistry staining: AChE-positive fibers in the hippocampal CA1 region (box 5) and dentate gyrus (box 6). Scale bars: 200 μm.

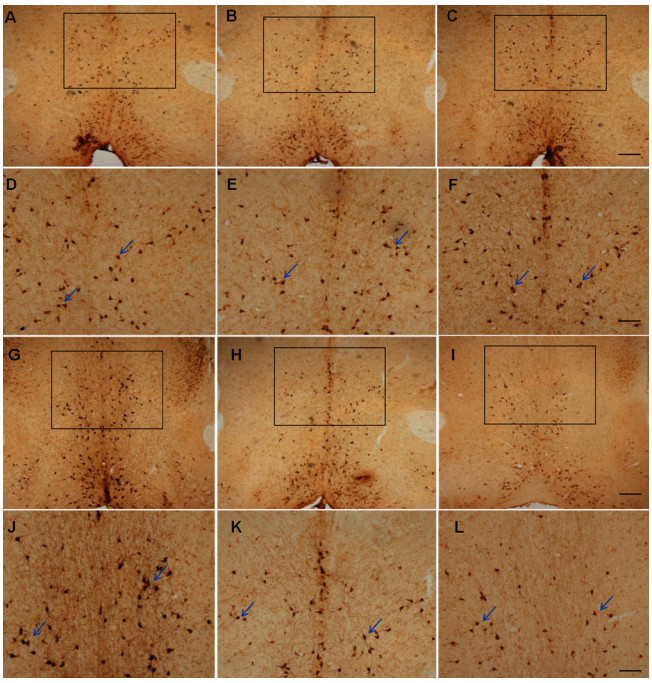

Figure 3 Effects of inferior alveolar nerve injury on choline acetyl transferase (ChAT)-immunoreactive neurons in the basal forebrain of senescence-accelerated mouse prone 8 mice (immunohistochemistry staining, light microscope).
(A–C) Control groups: middle-aged control 1 (A), middle-aged control 2 (B), elderly control (C); (D–F) magnification of panes A–C, respectively. (G–I) Experimental groups: middle-aged experimental 1 (G), middle-aged experimental 2 (H), elderly experimental (I); (J–L) magnification of panes G–I, respectively. Arrows, ChAT-immunoreactive neurons. Scale bars: A–C, G–I, 200 μm; D–F, J–L, 100 μm.

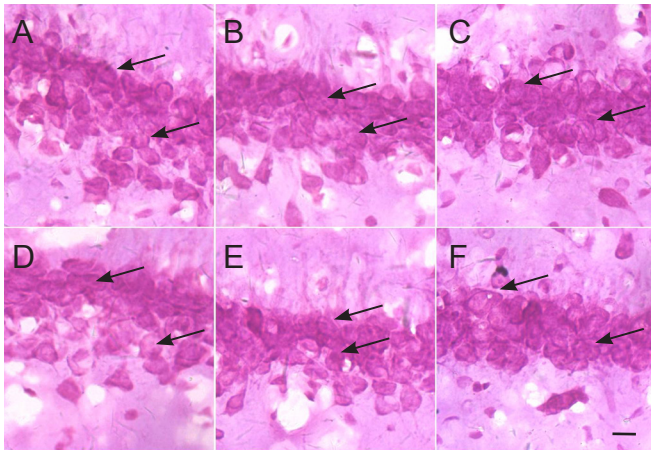

Figure 2 Effects of inferior alveolar nerve injury on pyramidal neurons in the hippocampal CA1 region of senescence-accelerated mouse prone 8 mice (cresyl violet (Nissl) staining, light microscope).
(A) Middle-aged control group 1; (B) middle-aged control group 2; (C) elderly control group; (D) middle-aged experimental group 1; (E) middle-aged experimental group 2; (F) elderly experimental group. Arrows point to pyramidal neurons. Scale bar: 50 μm.

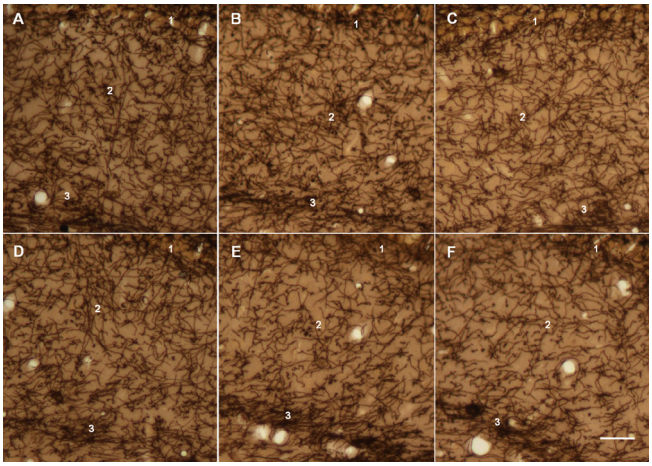

Figure 4 Effects of inferior alveolar nerve injury on acetylcholinesterase (AChE)-positive fibers in the hippocampal CA1 region of senescence-accelerated mouse prone 8 mice (AChE enzyme histochemistry staining, light microscope).
(A) Middle-aged control group 1; (B) middle-aged control group 2; (C) elderly control; (D) middle-aged experimental group 1; (E) middle-aged experimental group 2; (F) elderly experimental group. Scale bar: 100 μm. 1: Pyramidal layer; 2: stratum radiatum; 3: stratum lacunosum.

Effects of inferior alveolar nerve injury on learning and memory in SAMP8 mice

Step-down passive avoidance

Escape latency and number of errors were significantly greater in the elderly experimental group than the elderly control group in the 5 minute acquisition session ($P < 0.05$; **Table 1**). No significant difference in learning was observed between the middle-aged control and experimental groups ($P > 0.05$; **Table 1**).

In the test session, 24 hours after the acquisition session,

no differences were observed between any experimental and control groups in escape latency, step-down latency or number of errors ($P > 0.05$; **Table 2**).

Y-maze test

In the elderly mice, the learning rate (trials to criterion) was significantly poorer in the experimental group than in controls ($P < 0.05$; **Table 3**), but memory (correct responses after 24 hours) was not significantly different from mice in the respective control group ($P > 0.05$). In the middle-aged groups, no significant differences were detected in learning or memory parameters between the control and experimental groups ($P > 0.05$).

Effects of inferior alveolar nerve injury on hippocampal pyramidal neurons in SAMP8 mice

Pyramidal cell layers of different thicknesses were visible in hippocampal regions $CA_{1–4}$ in SAMP8 mice of each group. Four to five layers of neurons were observed in the pyramidal layer of CA1 in middle-aged control group 1 (**Figures 1A, 2A**) and middle-aged experimental group 1 (**Figure 2D**), compared with three to four layers in the remaining two middle-aged groups (**Figure 2B**, **C**) and in the elderly control group (**Figure 2E**). However, there were noticeably fewer CA1 pyramidal neurons (two to three layers) in the elderly experimental group than in the corresponding control group, and the arrangement of cells was disorderly in this group (**Figure 2F**).

Quantitative analysis of Nissl stained neurons revealed significantly fewer pyramidal neurons in the hippocampal CA1 and CA3 regions of the elderly experimental group than in the respective control group ($P < 0.01$); conversely, no significant differences were observed between control and experimental animals in the middle-aged groups ($P > 0.05$; **Table 4**).

Effects of inferior alveolar nerve injury on the number of ChAT-immunoreactive neurons in the basal forebrain of SAMP8 mice

A large number of ChAT-immunoreactive neurons were strongly stained and arranged on both sides of the midline in the medial septal nucleus and vertical limb of the diagonal band in middle-aged control group 1 and middle-aged experimental group 1 (**Figures 1B and 3A, D, G, J**). There were fewer ChAT-immunoreactive neurons in the medial septal nucleus and vertical limb of the diagonal band in the remaining five groups (**Figure 3B, C, E, F, H, I, K, L**). In particular, the number of ChAT-immunoreactive neurons was noticeably lower in the medial septal nucleus in the elderly experimental group (**Figure 3I, L**) than in the other groups.

Quantification revealed that there were significantly fewer ChAT-immunoreactive neurons in the medial septal nucleus and vertical limb of the diagonal band in the elderly experimental group than in the elderly control group ($P < 0.01$; **Table 5**); no significant differences were observed between the middle-aged experimental groups and their respective controls ($P > 0.05$).

Effects of inferior alveolar nerve injury on the number of AChE-positive nerve fibers in the hippocampus of SAMP8 mice

AChE-positive fibers were abundant in the pyramidal layer, stratum radiatum and stratum lacunosum of the CA1 and dentate gyrus in all groups (**Figure 1C** and **Figure 4**). In the CA1 region and dentate gyrus, there were significantly fewer AChE-positive fibers in the elderly experimental group than in the elderly control group ($P < 0.01$); no differences were observed between the other experimental and control groups (**Table 6**).

Discussion

Effects of inferior alveolar nerve injury on learning and memory in SAMP8 mice

SAMP8 mice have many characteristics similar to those seen in AD (Wang et al., 2012; Yuan et al., 2012). Learning and memory capabilities are poorer in SAMP8 mice at the age of 2 months than in age-matched controls, and amyloid deposits can be observed from about 3 months. SAMP8 mice reach maturity at 4–6 months, after which they show several features of rapid aging (Masaomi, 1997; Zhang and Li, 2007; Cheng et al., 2014; Xu et al., 2014). Similarly to patients with AD, the spatial learning and memory capabilities of SAMP8 mice decrease with age, and severe central nervous system degeneration occurs (Masaomi, 1997; Zhang and Li, 2007; Cheng et al., 2014; Xu et al., 2014). In the present study, behavioral analysis did not reveal significant differences in learning or memory between mice that had undergone inferior alveolar nerve transection in middle-aged and sham-operated mice, even when tested in old-aged mice (11 months). However, learning and memory capabilities were notably diminished in elderly mice when transection was performed during the early aging stage (7 months). These data indicate that in SAMP8 mice, the effects of trigeminal nerve damage on learning and memory are limited if the damage occurs before any degeneration of the central nervous system. However, if the injury occurs once degeneration has begun, the central nervous system damage may be potentiated, and learning and memory impairments would be enhanced.

The behavioral results of mice with inferior alveolar nerve injury in our study were consistent with previous reports of the effects of dental damage on cognition (Noble et al., 2009; Okamoto et al., 2010b; Arrivé et al., 2012; Teixeira et al., 2014). Learning and memory were notably impaired in mice exclusively fed a soft diet, suggesting that cognition is affected by mastication (Kawamura, 1989; Yamamoto and Hirayama, 2001). The same study also showed that aging-induced learning impairments were rarely found in the hard feed experimental group, leading the authors to propose that mastication delays brain aging. Kato et al. (1997) removed the molars of young rats, and observed a significant decrease in spatial learning and memory capabilities in the 8-arm maze, 135 weeks later. In several studies by Onozuka and colleagues (Onozuka et al., 1999; Onozuka et al., 2000; Onozuka et al., 2002b) maxillary molars of SAMP8 mice were removed or transected at the gingival margin, leading

to greater impairments in learning and memory as the mice aged. This reduction in learning ability depends on the duration of tooth loss. Long-period molar loss promotes senile impairment of spatial memory (Onozuka et al., 2000). The trigeminal sensory pathway is affected in most cases of dental damage or weakened masticatory afferent stimulation, although there was previously no direct evidence that damage to the trigeminal sensory pathway induced central nervous system degeneration. In the present study, however, we have shown that simple transection of the inferior alveolar nerve, without dental damage, during early aging is sufficient to potentiate later learning impairments in SAMP8 mice, suggesting that trigeminal nerve damage contributes to the mechanism underlying central nervous system impairment after dental injury.

Our behavioral data from the step-down test and Y-maze revealed that after inferior alveolar nerve transection, early aging significantly diminished learning ability. However, measures of memory showed no significant differences between the experimental and control groups. The present findings strongly indicate that simple trigeminal nerve damage only affects learning ability, but does not accelerate memory impairments. This is in contrast to other studies concerning dental damage-induced learning and memory impairments in animal models of AD (Kawamura, 1989; Kato et al., 1997; Onozuka et al., 2000; Yamamoto and Hirayama, 2001; Onozuka et al., 2002b; Noble et al., 2009; Okamoto et al., 2010; Arrivé et al., 2012; Teixeira et al., 2014). Together, these data indicate that dental damage induces central nervous system impairment *via* trigeminal nerve injury, as well as through other factors. After tooth loss, chewing movements are reduced, leading to a reduction in cerebral blood flow and degeneration of mastication-related neocortical areas (Noble et al., 2013; Teixeira et al., 2014). Chewing enhances cerebral functions, such as spatial memory, and increases levels of arousal and alertness, thus improving cognitive ability (Wilkinson et al., 2002; Ono et al., 2009; Hirano et al., 2013; Mariën et al., 2013). Ono et al. (2010) and Teixeira et al. (2014) confirmed that mastication helps maintain cognitive functions in the aging hippocampus. Molar extraction or occlusal imbalance results in abnormal pressure in the stomatognathic system, causing an increase in plasma corticosterone levels, associated with the degeneration of hippocampal neurons and glial cells, and a decline in spatial memory (Onozuka et al., 2000; Watanabe et al., 2001; Yoshihara et al., 2001; Onozuka et al., 2002a; Watanabe et al., 2002). Mitome et al. (2005) found that masticatory activity affects cell proliferation and neurogenesis. Together, this evidence strongly suggests that oral damage leads to central nervous system impairment. Injury of the trigeminal nerve alone may restrict central nervous system damage to sensory afferents, thus inducing impairments in learning related only to these afferents.

Effects of inferior alveolar nerve injury on the hippocampus in SAMP8 mice

The hippocampal formation is a vital component of the limbic system, closely associated anatomically and functionally with the subiculum complex and entorhinal cortex (Simic et al., 1997; Tombaugh et al., 2002; Dillon et al., 2008; Li et al., 2012; Ma et al., 2012; Stevens et al., 2012; Gulbrandsen et al., 2013). The hippocampal formation regulates emotions, learning and memory, behavior, and immunity (Simic et al., 1997; Tombaugh et al., 2002; Dillon et al., 2008; Stevens et al., 2012; Gulbrandsen et al., 2013). The hippocampus is particularly important in spatial learning and memory (West et al., 1994; Simic et al., 1997; Chen et al., 1998; Tombaugh et al., 2002). Aging-related neuropathic damage causes a loss of hippocampal neurons in patients with AD as well as in animal models of cognitive impairment (West et al., 1994; Simic et al., 1997; Chen et al., 1998; Tombaugh et al., 2002).

The hippocampus is very sensitive to aging, being the first structure affected morphologically and physiologically (Bhatnagar et al., 1997; Onozuka et al., 2002a; Watanabe et al., 2002). In the present study, we investigated the survival, distribution and pathological changes in pyramidal cells in the hippocampal CA1 and CA3 regions. After inferior alveolar nerve injury during early aging (7 months), but not middle age (4 months), pyramidal neurons were irregularly distributed, and the number of neurons was notably lower in CA1 and CA3 at the early stage of aging, compared to mice without nerve injury. These results suggest that inferior alveolar nerve injury accelerates pyramidal neuron loss in the hippocampus of SAMP8 mice with degeneration, but does not affect mice without degeneration. The hippocampus is responsible for handling and processing information for short-term memory formation (Simic et al., 1997; Tombaugh et al., 2002; Dillon et al., 2008; Stevens et al., 2012; Gulbrandsen et al., 2013). Our behavioral study showed that inferior alveolar nerve injury was associated with degeneration in the hippocampal formation, leading to impaired short-term memory storage and manifesting as a reduction in learning ability, while long-term (24 hour) memory remained intact.

Our results support those of previous studies showing that molar loss and lack of mastication decrease learning and memory capabilities in elderly SAMP8 mice and reduce neuronal density in the hippocampal CA1 region. Furthermore, hippocampal neuronal degeneration increases with the duration of molar loss (Onozuka et al., 1999). Tooth loss induces changes in hippocampal astrocytes, and neuronal loss in the hippocampus parallels the decrease in learning ability of elderly mice (Onozuka et al., 2000). Hippocampal CA1 neuron loss occurs in the early stages of tooth loss in elderly, but not young, mice (Onozuka et al., 1999; Noble et al., 2013), and in elderly mice without molars, spatial memory impairment correlates with decreases in the number of hippocampal neurons and senile degeneration of astrocytes (Onozuka et al., 1999, 2000). *In vitro* electrophysiological studies (Onozuka et al., 2002a; Watanabe et al., 2002; Noble et al., 2013) demonstrated that the resting membrane potential in hippocampal astrocytes was identical between young and elderly mice without molars. However, when extracellular K^+ concentration was gradually increased, the resultant

increase in membrane potential was lower in elderly mice than in young mice (Onozuka et al., 2002a; Watanabe et al., 2002). Lack of mastication or tooth loss could lead to the degeneration of the hippocampal formation, alterations in related neurons and a decrease in neuroelectrical activity in elderly mice during aging (Onozuka et al., 2002a; Watanabe et al., 2002; Mitome et al., 2005; Noble et al., 2013; Teixeira et al., 2014).

In summary, dental damage affects the structure of the hippocampal formation, probably owing to the sensitivity of the hippocampus to aging, ischemia, free radical damage and inflammation. This may also be an important mechanism underlying the reduction in learning and memory capabilities induced by dental damage. The effects of inferior alveolar nerve injury on learning and memory differ from those of dental damage, but the two types of damage exert similar effects on the hippocampal formation.

Effects of inferior alveolar nerve injury on the cholinergic system of SAMP8 mice

The cholinergic theory of AD was proposed in the 1970s (Davis and Maloney, 1976). The degree of cholinergic dysfunction is positively correlated with cognitive impairment, and improvements in cholinergic function ameliorate learning and memory loss. The cholinergic theory posits that the cholinergic system undergoes severe degeneration, resulting in age-related learning and memory loss, cognitive impairment and dementia. The central cholinergic system, located mainly in the medial septum and diagonal band of the basal forebrain, synthesizes acetylcholine that is transported into the cerebral cortex and hippocampus along projection fibers in multiple pathways, and is involved in new memory formation (Springer et al., 1987). The hippocampal formation contains abundant cholinergic fibers, the majority of which arise from the basal forebrain, notably the medial septal nucleus. AChE-positive fibers in the hippocampus degenerate after injury to the septal-hippocampal cholinergic projection (Springer et al., 1987; Ransome and Hannan, 2012), believed to be an important mechanism by which age-related memory loss occurs (Springer et al., 1987; Ransome and Hannan, 2012).

In the present study, we analyzed cholinergic neurons in the basal forebrain and AChE-positive fibers in the hippocampal CA1 region and dentate gyrus of SAMP8 mice, to explore the effects of trigeminal nerve damage on the septal-hippocampal cholinergic projection pathway. Our results revealed that there were significantly fewer cholinergic neurons in the medial septal nucleus and vertical limb of the diagonal band in the basal forebrain of SAMP8 mice during the early aging stage after inferior alveolar nerve injury. The number of cholinergic nerve fibers in the hippocampal CA1 region and dentate gyrus was notably diminished. Moreover, the loss of cholinergic nerve fibers was significant. These data indicated that inferior alveolar nerve injury led not only to neuronal loss, but also a loss of cholinergic nerve fibers in cholinergic projection areas. Our study supports previous evidence that inferior alveolar nerve injury results in the degeneration of the trigeminal sensory pathway (Gobel, 1984; Kubota et al., 1988). Consistent with previous studies, we found that this degeneration leads to a reduction in afferent impulses from the diencephalon to the cerebral cortex and basal forebrain, a loss of cholinergic neurons in the basal forebrain, and a loss of cholinergic projections from the basal forebrain to the cerebral cortex and hippocampus (Cooper and Sofroniew, 1996; Gould and Cameron, 1996). The reduction in afferent impulses induces a decrease in cholinergic neuronal activity in the basal forebrain, resulting in a further loss of cholinergic nerve fibers, particularly in the hippocampal CA1 and dentate gyrus (Kubota et al., 1988; Cooper and Sofroniew, 1996; Gould and Cameron, 1996; Ogawa et al., 2003).

Dental damage induces cholinergic neuron degeneration in the basal forebrain. Long-term tooth loss causes an age-dependent reduction in potassium chloride-evoked acetylcholine release in the hippocampus of SAMP8 mice (Onozuka et al., 1999). Kato et al. (1997) observed that acetylcholine release was significantly diminished in the parietal cortex of elderly rats after tooth extraction. Other studies have confirmed that the synthesis and release of acetylcholine and dopamine are reduced in the brain after molar extraction (Makiura et al., 2000; Onozuka et al., 2002b; Terasawa et al., 2002). To our knowledge, we are the first to investigate the effects trigeminal nerve damage on cholinergic fibers in the septum and hippocampus, and on learning and memory capabilities. However, dental damage certainly causes trigeminal nerve damage and the degeneration of the cholinergic system in the basal forebrain. The present study has demonstrated that trigeminal nerve damage evokes considerable degeneration of the septal-hippocampal cholinergic system and a loss of hippocampal neurons.

In conclusion, the effects of trigeminal nerve damage on the central nervous system are age-dependent. Trigeminal nerve damage induces cholinergic degeneration in the basal forebrain and a loss of hippocampal pyramidal neurons in elderly SAMP8 mice, ultimately causing impairments in memory and, in particular, learning. This may represent the mechanism underlying the effects of dental damage-induced trigeminal nerve injury on the course of AD. The present results highlight the importance of active dental care in the elderly and patients with AD, and indicate that tooth extraction should be avoided in these populations.

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