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Influence of tooth-loss and concomitant masticatory alterations on cholinergic neurons in rats: immunohistochemical and biochemical studies

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Abstract

The influence of tooth loss on the viability of cholinergic neurons was examined in rats. At 25th postnatal week, rats were divided into the three groups; a control group fed a solid diet, a soft diet group fed a powder diet and a molar crown-less group in which all molar crowns were removed and the powder diet was given. At 15 and 35 weeks post-treatment, the number of choline acetyltransferase (ChAT)-positive neurons in the nucleus of the diagonal band/medial septal nucleus (NDB/MS) was significantly smaller in the molar crown-less group than in the control group ($P < 0.0l$). This was not the case in the pedunculopontine tegmental nucleus or (PPT) or in the trigeminal motor nucleus. Biochemical assay showed no statistically significant differences in choline concentrations in the hippocampus between the control and the molar crown-less group both at 15 and at 35 weeks post-treatment. Nevertheless, acetylcholine (ACh) concentration in the hippocampus of the molar crown-less group was significantly lower than that of the control group at 15 weeks post-treatment ($P < 0.05$). Taken together, a decrease of oral sensory information may have caused a reduction in the number of ChAT-positive neurons selectively in NDB/MS, which in turn caused a decline of ACh concentrations in the hippocampus.

Keywords: Mastication; Cholinergic neurons; Nucleus of the diagonal band; Pedunculopontine tegmental nucleus; Acetylcholine

1. Introduction

As a loss of tooth was reported to be a risk factor for Alzheimer-type dementia (Isse et al., 1991), occlusal-masticatory function or malfunction may affect on the higher brain function. In fact, an impairment of spatial memory due to a decrease in acetylcholine (ACh) level in the cerebral cortex was caused in 135 weeks old rats by tooth extraction (Kato et al., 1997). A similar impairment of spatial memory has been reported to occur in aged mice by extracting or cutting the molar teeth at young ages (Onozuka et al., 1999), and also in adult rats by feeding soft-diet after the weaning period (Yamamoto and Hirayama, 2001). In these studies, both the neuronal density in the CA1 hippocampus (Onozuka et al., 1999) and the synaptic formation in the hippocampus and the parietal cortex (Yamamoto and Hirayama, 2001) have been reported to decrease.

The nucleus of the diagonal band and the medial septal nucleus (NDB/MS), together with the nucleus

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basalis of Meynert, are known to be involved in leaning and/or memory. Cholinergic neurons in NDB/MS project to both the hippocampus and the cerebral cortex (Johnston et al., 1981; Davies and Feisullin, 1982; Sofroniew et al., 1987; Houser et al., 1983; McKinney et al., 1983; Mesulamm et al., 1983a,b; Pearson et al., 1983), whereas those in the nucleus basalis of Meynert send less axons to the hippocampus in comparison with the cerebral cortex (Lehmann et al., 1980; Rye et al., 1984).

In the present study, therefore, we aimed to investigate the influence of dietary—and occlusal loss—related changes on cholinergic neurons in NDB/MS and the possible subsequent changes in ACh concentration in the hippocampus in male rats using immunohistochemical and biochemical methods, respectively. The immunohistochemical results were compared with those obtained from cholinergic neurons in the pedunculopontine tegmental nucleus (PPT) and the trigeminal motor nucleus (Vmo). The number of ChAT-positive neurons in NDB/MS and the concentrations of ACh in the hippocampus changed differentially following dietary changes and deprivation of occlusion.

2. Materials and methods

Experiments were carried out on 84 male Wistar rats (25 weeks old, 400–450 g). They were divided into three groups: (1) a control group (fed a solid diet), (2) a soft diet group (fed a powder diet containing the same components as the solid one) and (3) a molar crown-less group (fed the powder diet) (Fig. 1). In the molar crown-less group, at 25th postnatal week, rats were anesthetized with sodium pentobarbital (35 mg/kg), and all maxillary and mandibular molar crowns were cut off at the gingival margin with a dental turbine. In the control and the soft diet group, animals were given anesthesia alone, without undergoing removal of the molar crown. The experimental timetable and the numbers of animals used in each experiment were given in Table 1. The rats were kept on a light-dark cycle (light up through 08:00–20:00) in an air-conditioned room (22±2 °C, 55–65% humidity). This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals by the Animal Research Committee in Health Sciences University of Hokkaido.

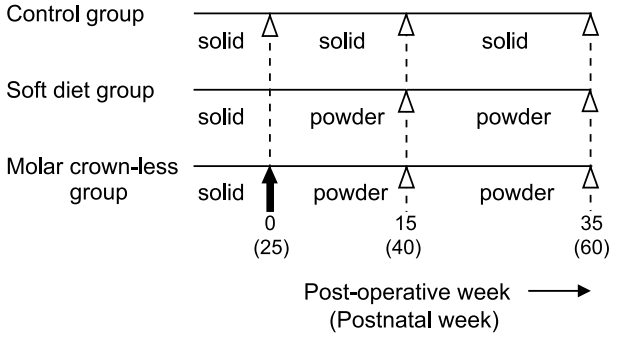

Fig. 1. Time schedule of the experiment. Rats were divided into three groups and treated as follows; (l) a control group in which the diet was consistently given in a form of solid pellet, (2) a soft diet group in which the form of diet was changed from solid to powder at 25th postnatal week, (3) a molar crown-less group in which all molar crowns were removed and the form of diet was changed from solid to powder at 25th postnatal week (arrow). Open arrowhead shows the time of observation.

2.1. Immunohistochemistry

At 15 weeks post treatment (40 weeks old) and at 35 weeks post treatment (60 weeks old), the rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg) and perfused transcardially with 0.1 M phosphate buffered saline (pH 7.2–7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4). The brain was removed and serial transverse sections were made with Microslicer (Dosaka EM, Kyoto Japan) at 50 μm thickness. The sections were processed with 3% H_2O_2 in 0.1 M phosphate buffered saline (PBS) for 30 min to block endogenous peroxidase activity. The sections were rinsed three times (10 min each) with 0.1 M PBS and then incubated in 5% normal goat serum (NGS) for 1 h to reduce background staining. Following this incubation, the sections were incubated rabbit anti-sera against choline acetyltransferase (CHEMICON, Temecula, CA, USA; diluted at 1:1000) in 0.1 M PBS containing 1% NGS and 0.5% Triton X-100 at 4 °C for 2 days. The sections were washed with 0.1 M PBS three times (10 min each), and then incubated overnight at 4 °C in biotinylated goat anti-rabbit IgG (1:200, Nichirei, Tokyo, Japan) in 1% NGS containing 0.5% Triton X-100. The sections were washed with 0.1 M PBS two times and with 0.05 M Tris buffered saline (TBS, pH 7.6) two times (10 min each), and then incubated for 2 h in streptavidin conjugated with horseradish peroxidase for 2 h (1:400, Nichirei, Tokyo, Japan) in 0.05 M TBS containing 1% Triton X-100. After several rinses with TBS, sections were reacted 0.05% 3,3′-diaminobenzidine tetrahydrochloride containing 0.003% H_2O_2. After rinsing with TBS and PBS, these sections were mounted on the slide grass, counterstained with 1% Neutral Red, dehydrated with graded alcohol, and coverslipped.

The locus and the number of labeled neurons were examined by light microscopy. In every third section, the count was made on cells whose nucleus was seen under 10 × objective (Fig. 2D). No corrections were made for double count because the maximal diameter of labeled somata was less than 50 mm.

Table 1
Number of animals used in each experiment

Group	Post-operative week (postnatal week)	ChAT immunohistochemistry	Ch and ACh concentration
Control	0 (25)	6	6
	15 (40)	6	7
	35 (60)	6	6
Soft diet	15 (40)	6	8
	35 (60)	6	6
Molar crown-less	15 (40)	6	7
	35 (60)	6	5

2.2. Acetylcholine and choline concentration

At 15 and 35 weeks post-treatment, the right hemisphere of rat brain was removed after microwave irradiation for l.5 s (5 kW), and was dissected into seven regions according to the method of Glowinski and Iversen (1966) to allow measurement of ACh and choline (Ch) concentration. The tissues were stored at −80 °C until assay. Extraction of tissue ACh and Ch was performed with aliquots of 0.1 N perchloric acid including ethylhomocholine as an internal standard. The homogenates were centrifuged for 15 min (10 000 rpm, 2 °C). The supernatant was neutralized with 0.2 N $KHCO_3$ and was filtrated (0.22 μm Millipore filter) and injected into an high performance liquid chromatography (HPLC) system (EP-10, EiCOM, Kyoto, Japan) connected to an immobilized enzyme reactor and an electrochemical detector (ECD, ECD-100, EiCOM, Kyoto, Japan), as reported previously (Matsumoto et al., 1990). Protein levels were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

2.3. Statistical analysis

Statistical analysis of the number of ChAT-positive neurons and ACh and Ch concentration were done by the one-way analysis of variance (ANOVA) and Scheffe multiple range test using SPSS.

3. Results

3.1. The number of ChAT-positive neurons in NDB/MS

ChAT immunohistochemistry showed that the numbers of ChAT-positive neurons in NDB/MS of the soft diet group and the molar crown-less group were smaller than those of the control group, at 15 and 35 weeks post-treatment as shown in Fig. 3 and in Table 2. There was no significant age-related change in the number of ChAT-positive neurons in NDB/MS of the control group when compared between 15 and 35weeks post-treatment. However, the numbers of ChAT-positive neurons in the molar crown-less groups at 15 and 35 weeks post-treatment were significantly smaller than those of the control group ($P < 0.0l$). At 15 and 35 weeks post-treatment, there was no significant difference ($P > 0.05$) in the number of ChAT-positive neurons either between the control and soft diet groups or between the soft diet and molar crown-less groups. The remaining ChAT-positive neurons in NDB/MS of the molar crown-less group, showed prominent shrinkage of dendrites. In the following sets of experiments, it was addressed whether these changes in cholinergic neurons were selective for NDB/MS or not.

The PPT is another source of major cholinergic projection neurons in the central nervous system. The influence of dietary-changes and occlusal loss was examined on cholinergic neurons in the PPT as well as those in the trigeminal motor nucleus (Vmo). At 15 and 35 weeks post-treatment, there were no significant differences in the number of ChAT-positive neurons in the Vmo and in the PPT any two groups (e.g. Figs. 4 and 5), although there appeared to be a slight shrinkage in dendrites of the molar crown-less group of the Vmo, but not in PPT, in comparison with the control group.

3.2. The concentration of ACh and Ch in the hippocampus

The concentration of ACh and Ch in the hippocampus of each group is shown in Table 3. There was no significant difference in the concentration of Ch between any two groups. In contrast, the concentration of ACh in molar crown-less groups was significantly lower than that of control group ($P < 0.05$), at 15 weeks post-treatment. At 35 weeks post-treatment, however, there was no significant difference in the concentration of ACh between the control and molar crown-less groups, although the mean ACh levels in the molar crown-less groups was lower than that of the control. These observations may indicate that the ACh concentration was transiently decreased following removal of molar teeth.

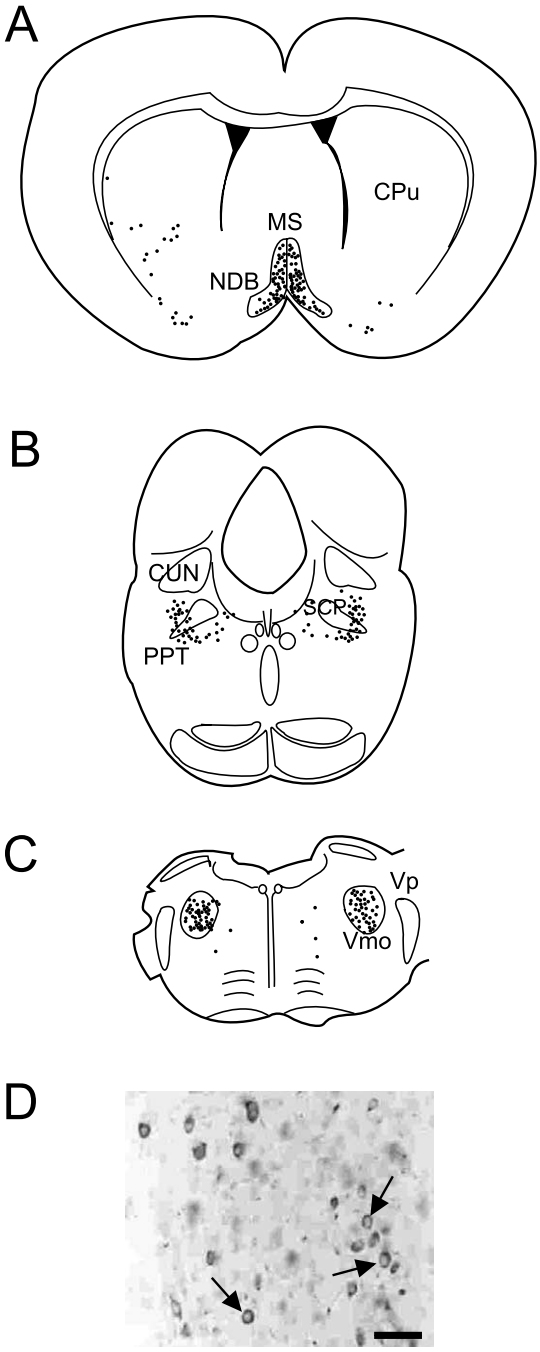

Fig. 2. Illustrations summarizing the distribution of ChAT immunoreactive neurons in forebrain (A), midbrain (B) and pons (C). Abbreviations are as follows: CPu, caudate/putamen; CUN, cuneiform nucleus; NDB, nucleus of the diagonal band; MS, medial septum; PPT, pedunculopontine tegmental nucleus; SCP, superior cerebellar peduncle; Vmo, trigeminal motor nucleus; Vp, trigeminal main sensory nucleus. D, ChAT immunoreactive neurons in NDB/ MS. The neurons whose nucleus could be seen (arrows) were counted. Scale bar = 50 μm.

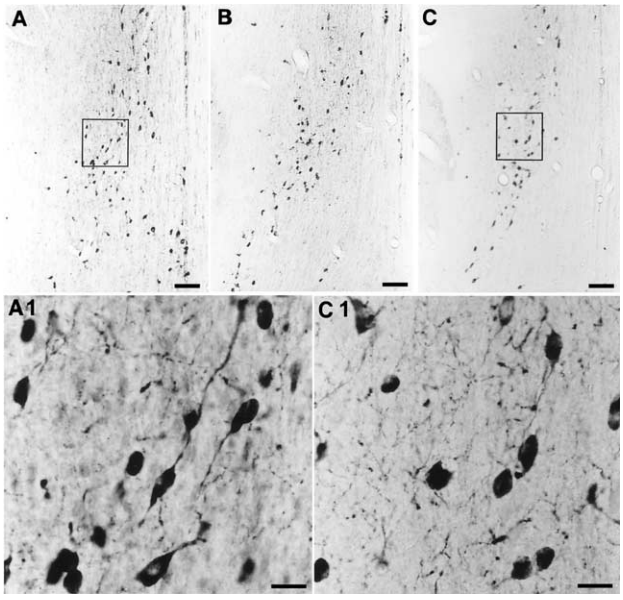

Fig. 3. Photomicrographs of ChAT-positive neuron in the nucleus of the diagonal band/medial septal nucleus (NDB/MS) at 15 weeks post-treatment. A, control group. B, soft diet group. C, molar crown-less group. Scale bar = 100 μm. A1, a higher magnification view of the boxed area in A. C1, a higher magnification view of the boxed area in C. Scale bar = 30 μm. Note that the number of ChAT-positive neurons in B and C is smaller than that in A.

Table 2
The number of choline acetyltransferase immunoreactive neurons

Post-operative week (postnatal week)	0 (25th)	15th (40th)	35th (60th)
NDB/MS			
Control	1496 ± 176.1	1427 ± 212.9	1694 ± 305.1
Soft diet		1207 ± 97.1	1319 ± 139.5
Molar crown-less		1040 ± 91.1[a]	964 ± 226.3[a]
PPT			
Control		564 ± 33.8	
Soft diet		475 ± 18.9	
Molar crown-less		503 ± 77.5	
Vmo			
Control		1215 ± 263.4	
Soft diet		1095 ± 83.4	
Molar crown-less		1209 ± 68.1	

Values are mean ± S.D. NDB/MS, nucleus of the diagonal band and the medial septal nucleus. PPT, pedunculopontine tegmental nucleus. Vmo, trigeminal motor nucleus.

[a] $P < 0.01$ compared with the control group.

4. Discussion

Interference with cholinergic function in the hippocampus and cerebral cortex produce impairments of memory and cognitive performance (Drachman, 1977; Olton et al., 1982; Bartus et al., 1983), as seen in Alzheimer's disease (Smith and Swash, 1978). A neurochemical investigation of patients with Alzheimer's disease and senile dementia has demonstrated a reduction in the density of presynaptic nerve terminals of cholinergic neurons in the hippocampus and cerebral cortex (Whitehouse et al., 1982). For such Alzheimer-type dementia, a loss of teeth is reported to be a risk factor (Isse et al., 1991). In agreement with this

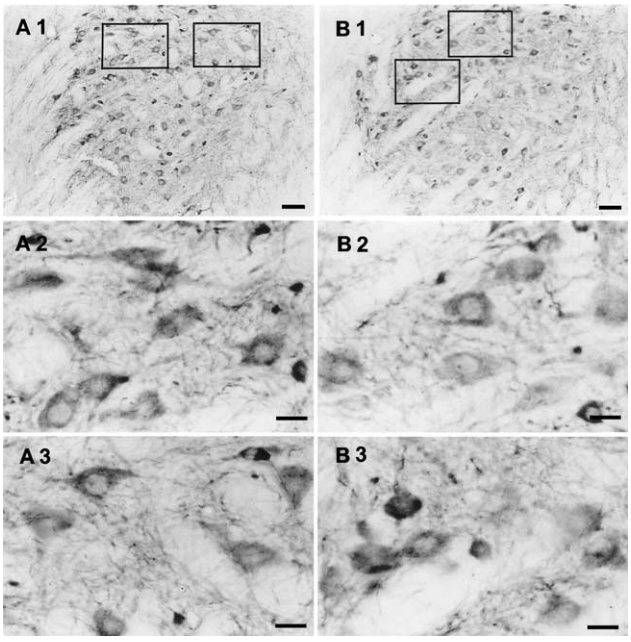

Fig. 4. Photomicrographs of ChAT-positive neurons in the trigeminal motor nucleus (Vmo) at 15 weeks post-treatment. A1, the control group. B1, the molar crown-less group. Scale bar = 100 μm. A2 and 3, higher magnification views of the boxed areas in A1. B2 and 3, higher magnification views of the boxed areas in B1. Scale bar = 30 μm. Note that no apparent difference is observed in the number of ChAT-positive neurons between A and B.

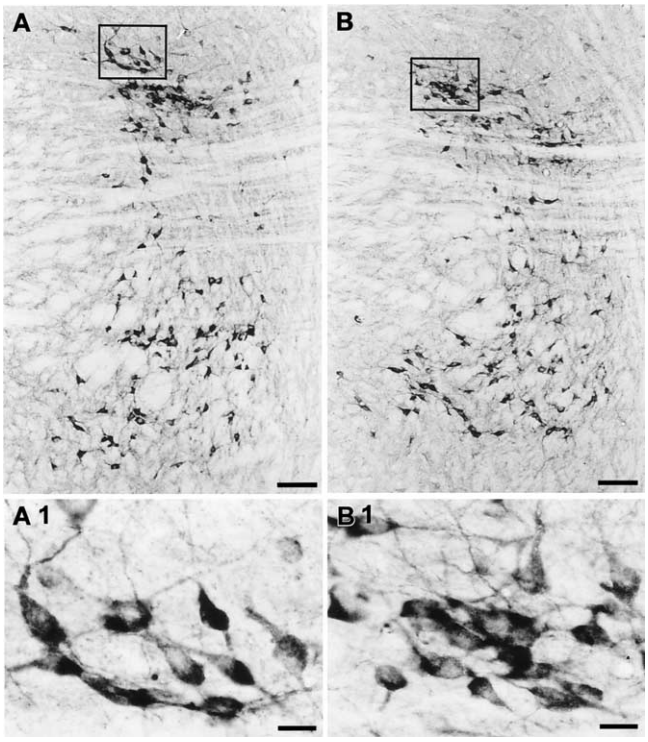

Fig. 5. A and B show low power photomicrographs of ChAT-positive neurons in the pedunclopontine tegmental nucleus (PPT) at 15 weeks post-treatment of the control and molar crown-less groups, respectively. Scale bar = 100 μm. A1, a higher magnification view of the boxed area in A. B1, a higher magnification view of the boxed area in B. Scale bar = 30 μm. Note that no apparent difference is observed in the number of ChAT-positive neurons between A and B.

epidemiological study, the distinct decrease in the number of ChAT-positive neurons in NDB/MS was observed following a decrease in sensory information from intact proprioceptors in the periodontal ligament in the present study. Sensory information arising from periodontal ligament, muscle spindle and temporomandibular joint play important roles for modulating masticatory and/or occlusal functions (Mizuno et al., 1983; Shigenaga et al., 1988a,b; Li et al., 1995). Loss of posterior occlusal stop may affect the musculature, temporomandibular joints, teeth, and periodontium (Osborn and Lammie, 1974). Therefore, wide variety of sensory information would have been altered by the removal of molar crowns, which might have consequently resulted in an unusual neuronal cell death in NDB/MS, although the causal neuronal connections or pathways are not clear.

Teeth extraction can induce cell death in the trigeminal mesencephalic neurons (Kimoto, 1993), and tooth pulp extraction induces degenerative changes in primary trigeminal axons and in their target neurons of the nucleus caudalis (Gobel and Binck, 1977). The causal relationship is clear in these studies. By contrast, the causal neuronal relationship to the cell death of cholinergic neurons in NDB/MS is not clear. Cell death can be caused either by excitotoxicity through activation of glutamate receptors and/or free radical nitric oxide (NO). NO is produced through the activity of nitric oxide synthetase (NOS) (Vincent, 1994). Especially, cholinergic neurons in NDB/MS are reported to be 300 times more susceptible to NO than those in PPT (Fass et al., 2000). This is presumably due to the sparse presence of superoxide dismutase, preventing the formation of peroxynitrite, in neurons of NDB/MS in comparison with those of PPT (Kent et al., 1999).

Table 3
The concentration of acetylcholine (ACh) and choline (Ch) in the hippocampus

Post-operative week (postnatal week)	Ach (nmol/mg protein)	Ch (nmol/mg protein)
0 (25*th*)		
Control	0.293 ± 0.038	0.191 ± 0.069
15*th* (40*th*)		
Control	0.222 ± 0.020	0.166 ± 0.027
Soft diet	0.210 ± 0.024	0.206 ± 0.071
Molar crown-less	0.183 ± 0.035[a]	0.166 ± 0.038
35*th* (60*th*)		
Control	0.226 ± 0.025	0.196 ± 0.079
Soft diet	0.196 ± 0.024	0.180 ± 0.040
Molar crown-less	0.209 ± 0.029	0.187 ± 0.069

Values are mean ± S.D.
[a] $P < 0.05$ compared with the control group.

Cholinergic neurons in NDB/MS receive cholinergic inputs from PPT (Vertes, 1988; Semba et al., 1988; Sarter and Bruno, 2000), and cholinergic neurons in PPT are considered to exert presynaptic inhibition onto glutamatergic inputs to NDB/MS (Rasmusson et al., 1994). On the other hand, trigeminal sensory neurons are reciprocally connected with parabrachial regions, including parabrachial nucleus, cuneiform nucleus and PPT (Hayashi and Tabata, 1989, 1990, 1991; Yoshida et al., 1997). Taken together with their vulnerability to NO, it may not be unreasonable to assume that decreases in the excitability of PPT cholinergic neurons possibly induced by decreases in trigeminal sensory inputs would allow excessive glutamatergic activation to induce a production of NO in cholinergic neurons in NDB/MS, consequently causing cell death of these cholinergic neurons.

It was reported that the activity of ChAT was reduced remarkably in the cerebral cortex and the hippocampus of patients with senile dementia (Iizuka, 1986). Therefore, the loss of ChAT-positive neurons in this study might be related with senile dementia. In this study, however, any significant decrease in the number of ChAT-positive neurons in relation with aging was not found. This is in accordance with the report by Sofroniew et al. (1987). The aging process of the central cholinergic neuron is known to involve major changes in ACh synthesis, storage and the release mechanism (Perry, 1980). In the present study, biochemical assay of ACh and Ch were performed in the hippocampus, which receives projections from neurons in NDB/MS, to investigate the influence of possible neuronal loss in NDB/MS. The present study did not show any differences in Ch concentration among all the groups, whereas a significant decrease in ACh concentration in the hippocampus was seen only in the molar crown-less group. Thus, the decrease in ACh levels in the hippocampus is likely to result from the decline of ChAT activity. Therefore, it is strongly suggested that the decrease of the number of ChAT-positive neurons in NDB/MS resulted in the decrease of ACh synthetic ability in the hippocampus. However, this decrease in ACh level in the molar crown-less group was transient whereas the number of ChAT-positive neurons remained smaller than that of the control group. This discrepancy can be explained by the following possibilities. First, ChAT activity remained at a low level due to a permanent loss of cholinergic neurons in NDB/MS while the activity of acetylcholine esterase (AChE) in the postsynaptic membrane of the hippocampus neurons might also be decreased in compensation of decreases in ACh synthetic ability. Second, the remaining or survived cholinergic neurons in NDB/MS might have increased their ability to synthesize ACh in their presynaptic terminals.

Based on the present study, it is speculated that the deprivation of the oral sensory input caused by the decreased mechanical stress of teeth causes a decrease in the number of ChAT-positive neurons in NDB/MS and a decline in ACh concentrations in the hippocampus. It was suggested that there is a close relationship between the masticatory function and the learning and/or memory ability.

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