Extracellular Glutamate Release in the Edentulous Rat Hippocampus following Tetanic Stimulation: in vivo Study by Microdialysis

Keiji Okuda, DDS, Hiroshi Inoue, DDS, PhD, and Yasushi Sakuma, DDS

a Department of Removable Prosthodontics and Occlusion, Osaka Dental University, Osaka, Japan

Clinical significance

The amount of glutamate released extracellularly in a functional hippocampus was measured by microdialysis in rats following extraction of the maxillary molar teeth. As compared to normal rats, the rats with extracted maxillary molars showed significantly lower extracellular levels of glutamate in the hippocampus, suggesting that tooth loss may also impair learning and memory, which represents significant information in the field of dental prosthetics.

Abstract

Purpose: Loss of molar teeth in rats has been reported to be associated with impairment of spatial memory, which may be related to hypo function of the hippocampus. However, the effects of tooth loss on the higher brain functions still remain unclear. In the present study, the glutamate levels in the hippocampus were measured as an index of the functions of the hippocampus, in order to elucidate the neurochemical changes in the brain in edentulous rats.

Methods: At the start of this study, 4-week-old male Sprague-Dawley rats were divided into 3 groups: EXT, in which all the maxillary molar teeth were extracted; ANE, in which anesthesia was administered, but no tooth extraction was performed; UNT, in which neither anesthesia was administered nor tooth extraction was performed. A guide cannula and recording electrode were then fixed in the right hippocampus of the rats at 7 weeks of age. A dialysis probe with a stimulation electrode was introduced into the guide cannula after allowing a 1-week recovery period, and the glutamate levels in the hippocampus were measured in the rats at 8 weeks of age. Tetanic stimulation (100 pulses of 0.2 ms duration at 100 Hz) was applied to the hippocampus through the stimulation electrode under monitoring by extracellular recording via the recording electrode when the glutamate levels had stabilized. The fluctuations in the glutamate levels following the tetanic stimulation were compared among the three experimental groups using a brain microdialysis system.

Results: The extracellular glutamate concentrations within the hippocampus increased in all 3 experimental groups following tetanic stimulation. However, the edentulous rats exhibited lower increases in the glutamate levels as compared with the sham and untreated groups.

Conclusion: Decreased afferent information from periodontal sensory receptors resulting from molar tooth loss in rats appears to cause hippocampal hypo function. Tooth loss may thus cause impairment of memory and learning in humans.

Key words: glutamate, hippocampus, electrical stimulation, in vivo microdialysis, rat, tooth loss

Introduction

The effects of mastication on the central nervous system have begun to be examined, and numerous studies are being conducted to investigate the effects of reduced masticatory function due to tooth loss on the brain.1-6 Using aged mice, Onozuka et al.1 reported that poor mastication by removal of the molar teeth crowns reduced the number of cells in the CA1 region of the hippocampus and caused impaired spatial learning and memory as assessed by the water-maze test. Yamamoto et al.7 noted that the number of synapses formed in the hippocampus and cerebral cortex was lower in mice given soft foods than in mice given hard foods. In rats, molar tooth loss has been shown to cause impairment of spatial learning and memory and reduce the levels of acetylcholine in the hippocampus.2 This impairment of spatial learning and memory is believed to be caused by...
hypofunction of the hippocampus and cerebral cortex, which in turn may be due to hindered hippocampal communication systems or reduction in the number of pyramidal cells in the hippocampus. An epidemiological study reported that tooth loss represented a risk factor for Alzheimer’s dementia. However, few studies have experimentally clarified whether tooth loss may also cause impairment of learning and memory.

Glutamate (Glu) is a major excitatory neurotransmitter in the central nervous system, playing an important role in learning, memory, neurological differentiation and generation, as well as in neuronal death. The present study investigated the effects of tooth loss on the neurological and biochemical functions of the hippocampus, to clarify whether tooth loss may also be associated with impaired learning and memory.

Changes in the Glu levels in the hippocampus following tetanic stimulation, which elicits long-term potentiation (LTP), were ascertained by microdialysis in rats.

**Materials and methods**

**Experimental animals**

This study was conducted using 18 male Sprague-Dawley rats (4-weeks-old; Japan SLC, Hamamatsu, Japan) with a mean body weight of 91 g (range: 78-98 g). The rats were kept under the usual experimental conditions (23±1°C:55±5% humidity) and provided access to solid food (MF: Oriental Yeast, Tokyo, Japan) and tap water ad libitum.

Light was provided for 12 h/day (08:00–20:00 hours). The study was conducted at the Osaka Dental University Central Dental Research Animal Laboratory in accordance with animal study guidelines established at the Osaka Dental University, with the approval of the animal study committee (approval number: 04–03013).

**Experimental procedures**

Figure 1 shows the experimental procedures. Between 4 and 7 weeks of age, the rats were weighed every week. The animals were then divided into 3 groups (Fig. 2): the extraction group (EXT), the anesthesia group (ANE), and the untreated group (UNT). In the EXT group, mice were administered 50 mg/kg of pentobarbital sodium (Tokyo Kasei, Tokyo, Japan) intraperitoneally at 4 weeks of age, and the maxillary molar teeth were extracted with Delicate C osteoclastic forceps (GC, Tokyo, Japan). In the ANE group, the mice were administered pentobarbital sodium intraperitoneally in the same manner as above, however, the maxillary molars were not extracted. In the UNT group, neither pentobarbital sodium was administered nor was any dental extraction performed. The rats were then kept under the same environment for 3 weeks. At 7 weeks of age, a guide cannula and recording electrode were placed and fixed at the dental resin in all the rats.

After allowing a 1-week recovery period, the changes in the hippocampal Glu levels were measured in the 8-week-old rats by microdialysis.
Cannula and recording electrode placement and fixation
In all the three animal groups, the guide cannula and recording electrode were placed 1 week before the microdialysis measurement, to avoid the effects of surgical invasion on the functions of the hippocampus. Following the administration of 50 mg/kg of pentobarbital sodium intraperitoneally at 7 weeks of age, each rat was fixed in an SR-5S brain immobilizer (Narishige, Tokyo, Japan). An MI-AG-4 guide cannula (Eicom, Kyoto, Japan) was then placed 3.8 mm posterior and 2 mm lateral to the bregma, so that its lower tip was 2.5 mm from the dura mater. An OM204-25 concentric recording electrode (Unique Medical, Tokyo) was placed 5.8 mm posterior and 5 mm lateral to the bregma so that its lower tip was 4 mm from the dura mater. According to the map of the rat brain published by Paxinos et al.,\textsuperscript{16} the cannula and electrode were placed in the right hippocampal CA1 region. To prevent the cannula and electrode from rotating or falling out, 2 stainless steel screws M1.4×2 (Naniwaneji, Osaka, Japan) were placed in the cranium and Unifast II fast-curing dental resin (GC, Tokyo) was used for fixation. Until the day of the microdialysis measurement, the guide cannula contained an MI-AD-4 dummy cannula (Eicom) and was capped using an AC-1 cap nut (Eicom).

Confirmation of the cannula and electrode position
At the end of the study, all the rats were euthanized by administration of a lethal dose of pentobarbital (Tokyo Kasei), the cerebrum was excised, and brain tissue sections were prepared. The location of the dialysis probe and the recording electrode were confirmed by gross observation of India ink perfusing through the dialysis membrane and the electrode (Fig. 3).

Measurement of Glu in the hippocampus
Figure 4 shows a block diagram depicting the schema of the Glu measurement. On the day of the microdialysis measurement, inhalational anesthesia was induced by the administration of 4% isoflurane and 96% oxygen at a flow rate of 3 L/min. After removing the dummy cannula, a dialysis probe with two stimulating electrodes with a membrane length of 1 mm (WE-A-I-4-01, Eicom) was introduced through the guide cannula. The stimulating electrode was positioned at the center of the membrane length. For measurement of the hippocampal Glu levels, artificial cerebrospinal perfusion fluid (Harvard, Massachusetts, USA) was used to perfuse the dialysis probe at a flow rate of 1 µL/min using an ESP-64 microsyringe pump (Eicom). The perfusion fluid was passed into the first automatic injector. To quantify Glu, a 10^{-6} mol of standard Glu solution was passed into the second automatic injector at a flow rate of 1 µL/min using a microsyringe pump. The mobile phase for Glu was prepared by adding 250 mg/L of hexadecyltrimethylammonium bromide (Nacalaitesque, Kyoto, Japan) to 15 mM phosphate buffer (pH 7.5). The mobile phase was degassed using a DG-300 degasser (Eicom),...
and delivered into the machine and the column at a flow rate of 0.5 mL/min using an EP-300 pump (Eicom). The artificial cerebrospinal perfusion fluid and standard Glu solution recovered every 10 min using the 2 automatic injectors were separated on a GU-GEL analysis column (Eicom) with a delay of 90 s. The eluate was then passed through an E-ENZYMPAK oxygen column (Eicom) to generate hydrogen peroxide, which was then captured by an ECD-300 electrochemical detector (Eicom) to specifically quantify the levels of Glu. The temperature of the analysis and oxygen columns was maintained at 33°C using a constant-temperature ATC-300 bath (Eicom). The resultant data were analyzed using a POWERCHROM chromatogram analyzer (Eicom), and recorded on to a computer to calculate the hippocampal Glu levels.

**Experiment**

The glutamate measurement was conducted between 10:00 and 18:00 in all the rats, considering the effect of the circadian rhythm. When the Glu levels had stabilized by ≥2 h after the introduction of the dialysis probe, 6 baseline measurements were obtained. Next, to experimentally stimulate the hippocampus, an ETC-UNIT 7801 electric stimulator (UGO BASILE, Comerico-Varese, Italy) was used to apply 3 tetanic stimulations (pulse width, 0.2 ms; 100-Hz square wave; stimulation intensity, 5 mA; duration, 1 s) during the seventh sampling under monitoring by extracellular recording. Voltage signals obtained by the recording electrode were amplified using an EMG-100 bioelectric amplifier (Biopack Systems, California, USA), digitized using an MP-100 A/D converter (Biopack Systems) and recorded on to a computer, to ensure that the hippocampus was stimulated. The Glu levels were measured in a total of 25 samples (250 min), and the hippocampal Glu levels before and after the stimulation were compared between the groups. For statistical analysis, repeated-measures analysis of variance (ANOVA) was conducted using time and group as the main variables. Tukey's test for HSD (honestly significant difference) was conducted for multiple comparisons. The level of significance was set at \( P < 0.05 \) for all the tests, and all the tests were performed using the SPSS software (SPSS Japan, Tokyo, Japan).

**Results**

**Changes in body weight**

Figure 5 shows the serial changes of the mean body weight in the 3 experimental groups. Repeated-measures ANOVA revealed significant correlations between the time and the body weight \( (P < 0.001) \) in all the groups, with all the rats exhibiting increase of body weight over time. However, the changes in the body weight exhibited no significant correlations with the group \( (P=0.804) \) or any time/group interaction \( (P=0.973) \). Thus, the changes in the body weight were similar among the three groups.

**Changes in hippocampal Glu release**

Figure 6 shows the changes in the amount of Glu released in the hippocampus following tetanic stimulation. Repeated-measures ANOVA was used to ascertain the chronological changes in the 25 samples, which confirmed significant correlations to the group \( (P=0.039) \) time \( (P<0.001) \), as well as to the interaction between group and time \( (P<0.001) \). In regard to the 6 baseline samples, a significant correlation was demonstrated with time \( (P<0.001) \), and the amount of Glu released decreased gradually with time in all the groups. However, the amount of Glu released in the hippocampus was not significantly correlated to the group \( (P=0.263) \) or the interaction between group and time \( (P=0.064) \). In sample 7 (immediately after the hippocampal stimulation), the amount of Glu released exhibited a significant correlation to the group \( (P=0.002) \). Multiple comparisons by
Tukey’s test revealed that the degree of increase in Glu release with sample 7 was significantly lower in the EXT and ANE groups than in the UNT group; no significant difference was noted, however, between the EXT and ANE groups. In sample 8 (10 min after the stimulation) again, the amount of Glu released was significantly correlated to the group \((P=0.002)\), and multiple comparisons by Tukey’s test revealed that the degree of increase in Glu release was significantly lower in the EXT group than in the UNT and ANE groups; no significant difference was, however, noted between the UNT and ANE groups. No significant correlations between the group and the amount of Glu released were noted in the remaining samples.

Discussion

Body weight changes

Studies have shown that tooth extraction decreases the weight of the masseter muscle, but does not affect the body weight in mice, and that extraction of the maxillary molars does not affect the body weight in rats. In the present study, the body weight increased by about 50 g/week in all the rats (Fig. 5), and since no significant differences in body weight were noted among the groups, tooth extraction and anesthesia did not appear to affect feeding or nutrition. The rats were able to eat normally because the incisors were not extracted, and because the food was made by packed powder, no problems with digestion or absorption were encountered.

Changes in hippocampal Glu due to tooth loss

Mastication is the part of the digestive process in which the food is crushed, and it is controlled by reflex movements. However, efficient masticatory movements require afferent information from the peripheral receptors and regulatory mechanisms from the higher centers of the central nervous system. Periodontal tissues are innervated by sensory nerve endings and contain the peripheral receptors that activate the masticatory mechanisms. When teeth are lost, the periodontal membrane disappears, with loss also of the periodontal mechanoreceptors. Kimoto et al conducted a morphological study in rats and proved that tooth loss caused not only the loss of receptors, but also the death of neurons in the mesencephalic nucleus of the trigeminal nerve. In the present study, the molars were extracted from relatively young rats (4 week of age) in the EXT group; the rats in this group must have received reduced sensory input from the periodontal membrane to the central nervous system.

In recent years, several studies have investigated the effects of loss of the molar teeth on the central nervous system. It was reported that when the maxillary molars were extracted to accelerate aging, the number of pyramidal cells in the CA1 region of the hippocampus decreased, and spatial learning, as assessed by the water maze test, became impaired. Yet another study revealed that when the molars were extracted, the number of pyramidal cells in the CA1, CA3 and CA4 regions of the hippocampus decreased and spatial learning and memory, as assessed using an 8-directional maze, became impaired, with the most marked impairments being observed after extraction of the maxillary molars. Studies have reported that tooth loss reduces the hippocampal levels of acetylcholine. In addition, reduced information input has been shown to induce neuronal death, suggesting a possible reduction in the number of pyramidal cells in the hippocampus following tooth loss. Tooth loss may thus affect the functions of not only the mesencephalic nucleus of the trigeminal nerve, but also of the cerebral cortex and the hippocampus.

However, to the best of our knowledge, no previous studies have investigated the effects of tooth loss on the functions of the hippocampus in animals with unrestricted movements. The present study used the microdialysis method to measure the levels of Glu in the hippocampus, which...
is closely involved in learning and memory formation. Glu has been known to function as an excitatory neurotransmitter and to act as an endogenous excitotoxin. These two special functions are mediated through glutamate receptors at central synapses. The glutamate receptors belong to three families of receptors, namely, the NMDA receptors, AMPA/kainate receptors and the metabotropic receptors. 

Electrophysiologically, activation of the NMDA receptor is an absolute prerequisite for induction of the LTP in the hippocampus, which models synaptic plasticity and is hypothesized to be the neurobiological mechanism underlying memory processes. Therefore, to experimentally place the hippocampus in a functional state, 3 tetanic stimuli were delivered at 100 Hz, and changes in the amounts of Glu released in the hippocampus following the stimulation were compared among the groups. Such tetanic stimulation is known to elicit LTP, which is considered to be a neurological mechanism involved in learning and memory.

In this study, no significant differences in the hippocampal levels of Glu were noted at rest among the three experimental groups. Thus, the experimental procedures did not affect the hippocampal levels of Glu. Next, after the tetanic stimulation, the amount of extracellular Glu released in the hippocampus increased in all the three groups. However, the amount of Glu released immediately after the stimulation was lower in the EXT and ANE groups than in the UNT group. Kirino et al. clarified that pentobarbital protects the neurons in the CA1 region of the hippocampus from ischemic injury, but Hanning et al. found that repeated pentobarbital administration decreased α-bungarotoxin binding in the cerebral cortex, suggesting changes in the cortical nicotinic receptors. Furthermore, a mouse study documented marked suppression of respiration following pento-barbital administration. These findings suggest that anesthesia at a younger age affects the function of the hippocampus.

In sample 8, the level of Glu was lower in the EXT group than in the other two groups. This suggests that masticatory disturbance caused by tooth extraction may reduce the amount of Glu in the synapses of the hippocampus, hindering the neurological and biochemical functions of the hippocampus. These findings indicate that the amount of Glu released in the hippocampus decreased as a result of a decrease in the number of neurons in the CA1 region of the hippocampus.

Furthermore, reduced numbers synapses and networks of hippocampal neurons and reduced Glu release at the synapses of stimulated pyramidal cells might also be involved. This decrease in the number of neurons could be caused by tooth extraction, which produces painful stimuli and peripheral nerve injury and abnormal excitation of the central nervous system, and may result in a number of possible outcomes: excessive Glu release, which acts as an excitotoxin; loss of sensory organ function resulting in decreased afferent information; decreased cerebral blood flow causing cerebral ischemia and consequently, excessive Glu release. However, these are merely conjectural, as the level of Glu in the hippocampus was not measured during the tooth extraction in the present study. One study reported a 26% decrease in the number of cells in the CA-1 region of the hippocampus following mastication failure for one week. Because the measurements were made one month after tooth extraction in the present study, the effects of a reduced neuron count may have been present. At the cellular level, direct interactions between glutamatergic and cholinergic neurons occur continuously, and glutamatergic neurons suppress the functional changes in the activity of the cholinergic neurons. Subsequently, one study reported that a combination of memantine hydrochloride, an NMDA antagonist, and an acetylcholine esterase inhibitor served as the optimal treatment for Alzheimer's dementia, and that this combination therapy may be used routinely in clinical settings in the future. Therefore, tooth loss may exacerbate the symptoms associated with Alzheimer's dementia or become a risk factor for this disease.

The present results indicate that decreased afferent information from periodontal sensory receptors as a result of molar tooth loss in rats may cause hippocampal hypofunction. Thus, tooth loss may cause impaired memory and learning in humans.

Acknowledgments: The authors would like to thank the members of the Department of Removable Prosthodontics and Occlusion, and Associate Professor Tera Maeda and members of the Department of Anesthesiology for their kind advice and help. This study was performed in the Laboratory Animal Facilities of the Institute of Dental Research, Osaka Dental University, in accordance with the guidelines for animal research at Osaka Dental University.
References


15. The animal experiment committee, Osaka Dental University. Guidelines for animal research at Osaka Dental University. 1-3, 2001.


