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# **Increased vertical dimension of occlusion for varying periods differentially impairs learning and memory in guinea pigs**

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## **Abstract**

There is an increasing number of studies showing that occlusal dysfunction impairs learning and memory. We previously demonstrated that the brain has a mechanism to calibrate between the activities of spindle afferents and periodontal-mechanoreceptor afferents for controlling the chewing movement, and the accurate calibration can be done only at the proper vertical dimension of occlusion (VDO). Then, the chewing at an inappropriate VDO may induce a severe mental stress due to a mal-calibration. However, it is not clear how the impairment of learning/memory progresses over the period of stress induced by occlusal dysfunction. We investigated by passive avoidance test how the behavior and learning/memory are altered in guinea pigs in which the VDO was raised by 2-3 mm over the period up to 8 weeks. We found that the guinea pigs reared under the raised occlusal-condition (ROC) for 1 week showed a very high sensitivity to electrical stimulation whereas this did not cause the memory consolidation in the 1st-day retention trial, suggesting that such hypersensitivity rather hampered the fear learning. In the guinea pigs reared under the ROC for 2 and 8 weeks, the learning ability was not largely affected and memory consolidation occurred similarly whereas the memory retention deteriorated more severely in the latter guinea pigs than in the former ones. In the guinea pigs reared under the ROC for 3 and 4 weeks, learning was severely impaired, and memory consolidation did not occur. These results suggest that the occlusal dysfunction for varying periods differentially impairs learning and memory.

**Keywords:** occlusal-bite raising, vertical dimension of occlusion, stress, learning and memory, passive avoidance test, guinea pigs

## 1. Introduction

Based on epidemiological studies, the World Health Organization and the U.S. National Institute on Aging jointly announced that the loss of multiple teeth is one of the risk factors for senile Alzheimer's disease [1]. Subsequently, it was also reported that in aged rats and mice, a decrease in occlusal efficiency caused by crown resection facilitated neuronal cell death in the basal forebrain and hippocampus, which resulted in impairments of learning and memory [2, 3]. These experimental results may suggest that the learning-memory impairment may be caused not only by the reduction of the trigeminal sensory information due to the removal or resection of the crown but also by the failure of effective occlusion or its control.

We previously proposed that the brain has a mechanism to calibrate the information on the intended muscle tension against that on the resulted periodontal ligament pressure during the power-closure phase of mastication in order to control the jaw movement near the mandibular resting position [4]. In clinical dentistry, it is known that when the vertical dimension of occlusion (VDO) or occlusal height is excessively high or low, patients show various symptoms such as headache, tenderness of masticatory muscles, postural control disorder and sleep disorder, probably because masticatory motor control does not work properly at such a VDO [5, 6]. In view of these reports, the occlusal height of the natural dentition in humans is considered to be maintained within the optimal range for executing normal chewing [7-11]. On the other hand, in guinea pigs, teeth eruptions are continuously occurring whereas VDO is maintained at the optimal height by occlusal abrasion [12]. It has been demonstrated that when a bite-raising device is applied to the incisors, the molars erupt about one week later, and the molars occlude with a high VDO [13-15]. However, when the occlusal-bite raising device is removed, the VDO returns to its original occlusal height in a few days by occlusal abrasion through the activity of primary sensory neurons in the mesencephalic trigeminal nucleus [16, 17]. In our previous studies, it was also demonstrated that the accurate

calibration between the two sensations arising from muscle spindles and periodontal mechanoreceptors [4] or mucous membrane mechanoreceptors can be done only at the original or optimal VDO, suggesting that the original or optimal VDO is the best calibration point for performing accurate isometric contraction during power-closure phase [18]. Therefore, it is conceivable that the chewing at a raised VDO may induce a stress due to a mal-calibration between the two sensations, which may further cause temporomandibular joint disorders, and consequently may facilitate senile dementia or induce a transient impairment of memory and learning [19-26]. On the other hand, it has recently been reported that left-right asymmetries of occlusion can also cause cognitive impairment [27, 28] although such occlusion may also cause stress as a result of mal-calibration or mal-occlusion.

We here investigated how the behavior and learning/memory are altered in those guinea pigs in which the original VDO was raised by 2-3 mm by attaching a bite raising device to their incisors, and also investigated whether such alteration is modified depending on the period of the raised occlusal condition.

## **2. Materials and methods**

The study was conducted following the Helsinki Declaration and carried out according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines version 2.0. All experimental procedures were approved and performed in accordance with the relevant guidelines of the Ethical Guidelines Committee for Animal Care of Osaka University Graduate School of Dentistry (DENT: H26-013-0).

### **2.1. The bite-raising device**

The bite-raising device was made as previously reported [16] (Fig. 1A). Under the anesthetized condition with intra-peritoneal injection of sodium pentobarbiturate (30 mg/Kg) and atropine sulfate (5 mg/Kg), the orthodontic band was attached to the mandibular incisors

with dental bonding agent. Thereafter, the resin bite-raising appliance was made by attaching a flat stainless steel metal plate ( $4 \times 4 \text{ mm}^2$ ) on top of the lower incisors with an immediate-curing resin, which subsequently created a gap of approximately 2-3 mm between the upper and lower molar teeth and prevented the reduction of the raised VDO due to occlusal abrasion (Fig. 1A). It is known that it takes approximately less than a week for those guinea pigs to establish a new VDO raised by 2-3 mm at which occlusal contacts between erupted upper and lower molar teeth are made [16]. To confirm that the occlusion of the molars was sufficiently elevated, we used a 3D micro-CT for laboratory animals (3D x-ray CT Rm\_CT2, Rigaku Corporation, Tokyo, Japan) before, immediately after, and 7 days after the attachment of the bite-raising device (Fig. 1B, C, D, respectively). The control animals underwent a sham operation, where they were handled and anesthetized like the bite-raised animals but did not receive the bite-raising procedure. Pentobarbiturate was purchased from Dainippon Pharmaceutical Co., Ltd. (Tokyo, Japan), atropine sulfate from Tanabe Seiyaku Co. (Tokyo, Japan), dental bonding agent (Megabond® FA) from Kuraray Noritake Dental Inc. (Tokyo, Japan), orthodontic band (Unitech™ Band) from 3M Japan Co., Ltd. (Tokyo, Japan), immediate-curing dental resin (Clearfil® ES2) from Kuraray Noritake Dental Inc.

## **2.2. Passive avoidance experimental apparatus**

A passive avoidance experimental apparatus (MPB-R020, MELQUEST, Toyama, Japan) was used to study the behavior and learning/memory of guinea pigs. The apparatus consists of two rooms, a light room ( $200 \times 200 \times 200 \text{ mm}$ ) and a dark room ( $200 \times 200 \times 200 \text{ mm}$ ), which were separated with a guillotine door ( $80 \times 80 \text{ mm}$ ). Metal poles with a diameter of 5 mm are placed at 14 mm intervals on the entire floor of the dark room, through which electric stimuli can be delivered by a shock generator (SG-1000E, MELQUEST). Once the guinea pigs moved from the light room to the dark room, the guillotine door was manually closed for 20 s to give an electrical stimulation at an intensity of 2.0 mA with the duration of 0.5 s with a shock

generator.

### **2.3. Experimental animals and breeding conditions**

A total of 40 male Hartley guinea pigs at 4 weeks of age (Japan SLC, Hamamatsu, Japan) were purchased, and 2-3 animals were housed together in a cage under the condition at a room temperature of  $23 \pm 1^{\circ}\text{C}$  and with a humidity of  $65 \pm 5\%$ . Guinea pigs were maintained in a 12-hour light/12-hour dark cycle starting from 8 am at the animal experiment facility attached to our institution. After acclimating to the rearing environment for 1 week, the guinea pigs at 5 weeks of age were divided into two groups: the control group without occlusal elevation ( $n = 5$ ) and the occlusal-bite raised group. The latter was further divided into 5 groups according to the date on which the series of 6 trial sections for the passive avoidance test was initiated after rearing under the raised occlusal condition. The five initiated dates are as follows; 7 days ( $n = 5$ ), 15 days ( $n = 7$ ), 22 days ( $n = 7$ ), 27 days ( $n = 8$ ) and 57 days ( $n = 8$ ), respectively, after the occlusal contact was confirmed to be recovered 7 days after the bite-raising device was attached [12, 16]. Thus, the 5 experimental groups were defined as follows; 1-week, 2-week, 3-week, 4-week and 8-week occlusal-bite raised groups. The control and the bite-raised animals were fed ad libitum with pellets [29] (Oriental yeast Co., Ltd., Tokyo, Japan) and water.

### **2.4. Weight measurement**

Guinea pigs were weighed on one day before and every day for one week and every week for 14th day to 35th day after the bite-raising device was attached to the guinea pigs, in order to obtain a measure of their health condition.

### **2.5. Behavioral study**

We performed the behavioral study to clarify whether the varying period of the raised occlusal-bite condition affects the basal behavior and the abilities of learning and memory

differentially. This behavioral experiment for the passive avoidance test consisted of six serial sections of trials: a control trial, an acquisition (learning) trial on the next day, and memory retention trials examined at 1, 2, 7, and 14 days after the acquisition trial. The guinea pigs were placed in a light room, and the latency was defined as the time taken for the animal to move into the dark room after the opening of the guillotine door. In the control trials, when the guinea pigs entered the dark room from the light room, the guillotine door was closed for 20 s without electrical stimulus, and then the trials were repeated 10 times. In the acquisition trials, when the guinea pigs entered the dark room from the light room, an electrical stimulation was manually applied 5-10 s after the guillotine door was closed. The guillotine door was closed for 20 s to prevent the guinea pigs from returning to the light room in response to an electrical stimulus. This was repeated 10 times. In the retention trials, even when the guinea pigs entered into the dark room, electrical stimulation was not applied during the 20 s period of guillotine closure. The guinea pigs that did not enter the darkroom within 300 s after the guillotine door was open were removed from the experimental apparatus [30, 31] and kept in the cage for 180 s before resuming the respective trials.

## **2.6. Statistical analyses**

Two-tailed paired or unpaired Student's *t*-test, two-way repeated measures (RM) ANOVA followed by post hoc Fisher's protected least significant difference (PLSD) test, and Wilk's lambda were used to examine the statistical differences in body weight, the latency to enter into the dark room, and the relationship between the latency and the number of times the animals avoided to enter the darkroom within 300 s, respectively. Two-way RM ANOVA was performed with the two factors. The different groups (control, 1-week, 2-week, 3-week, 4-week and 8-week occlusal-bite raised group; 6 levels) can be represented as a between-subjects factor. The conditions (trials) examined in the subjects within each group (trials; control, acquisition and retention at 1, 2, 7 and 14 days after acquisition trial; 6 levels) can be

represented as a within-subjects factor as each subject receives the same series of trials (repeated measures). The significance level was set at 5% or less ( $p < 0.05$ ). Statistical results are given as a  $p$  value, unless  $p < 0.001$ .

### **3. Results**

#### **3.1. Weight change**

Body weights were measured on immediately before and 1-7, 14, 21, 28, and 35 days after the attachment of the bite-raising device. Compared to the control group, the body weights of the occlusal-bite raised groups were significantly lower on all measurement days from the 1st day after the installation of the bite-raising device, while showed similar weight gain trends as the control group 3-4 days after the installation (Fig. 2A and B). To examine the time course of the body weight decrease caused by the persistent bite-raising, the mean body weights of the control group were subtracted from those of the bite-raised groups. There were two phases of the body weight decrease; the fast phase peaked at 5th day and was followed by the slow phase that peaked at 14th day and showed a tendency of recovery (Fig. 2C), thus showing a biphasic decrease with an increase in the period of the raised occlusal condition, as decomposed into the fast (blue dotted curve) and slow (red dotted curve) components by a mathematical fitting to the body weight change.

A total of 6 guinea pigs died, one each on the 7th and 8th day after the device was attached, in the 2-week, 3-week and 8-week occlusal-bite raised groups, respectively. These guinea pigs showed a continuous decrease in body weight from the time after they were attached with the bite-raising device until death (Fig. 2D). One guinea pig in the 4-week occlusal-bite raised group was excluded from the experiment because it died on the 51st day after attaching the bite-raising device, although it did not show continuous weight loss.

### **3.2. Behavior and ability of learning/memory changed depending on the period of the raised occlusal condition**

In order to evaluate the changes in behavior and learning/memory ability following bite-raising for 5 different periods, the mean latency to enter the dark room was plotted against the respective 6 different trials (Figs. 3 and 4). Two-way RM ANOVA was performed to evaluate the differences among the control and occlusal-bite raised groups (Group effect,  $F(5, 27) = 3.79$ ,  $p = 0.010$ ; Trial effect,  $F(5, 135) = 28.67$ ,  $p < 0.001$ ; Group  $\times$  Trial effect,  $F(25, 135) = 2.50$ ,  $p < 0.001$ ) (Fig. 3) while  $t$ -test was performed to evaluate the differences between the trials in the respective groups (Fig. 4).

#### **3.2.1. Control group**

In the control guinea pigs in which VDO was not increased, the guinea pigs showed quite normal behavior in response to the passive avoidance test (Fig. 3A and B, black circles; Fig. 4A). In the control trial examined at 5th weeks of age, the animals quickly entered the dark room as soon as the guillotine door was open, as revealed by a mean latency of  $31 \pm 17$  s, and in the acquisition trial, the mean latency sharply increased to  $188 \pm 25$  s due to the electrical stimulation in the dark room. In the retention trial one day after the acquisition trial, the mean latency was usually slightly but significantly increased to  $274 \pm 15$  s from  $188 \pm 25$  s in the acquisition trial (Fig. 3A,  $p = 0.026$ , two-way RM ANOVA followed by post hoc Fisher's PLSD; Fig. 4A,  $p = 0.009$ ,  $t(4) = -4.642$ , paired  $t$ -test), suggesting that the post-learning memory consolidation might have been caused [32]. After that, there were no significant changes in the mean latencies among the retention trials examined at 2, 7, and 14 days after the acquisition trials, showing that memory was well maintained over 14 days (Fig. 3A and B, black circles; Fig. 4A).

### 3.2.2. One-week occlusal-bite raised group

However, in the 1-week occlusal-bite raised group (Fig. 3B, orange circles; Fig. 4D), the animals appeared to hesitate to enter the dark room and the mean latency was slightly but insignificantly longer than that of the control group in the control trial. In the acquisition trial, the mean latency sharply increased to  $194 \pm 31$  s similar to that of the control group, whereas in the retention trial one day after the acquisition trial a sharp and significant decrease in the mean latency was observed (Fig. 4D,  $p = 0.021$ ,  $t(4) = 3.715$ , paired  $t$ -test), indicating that the post-learning memory consolidation did not occur. Then, the increase in the mean latency in the acquisition trial did not necessarily mean the learning, but may simply reflect the transient hypersensitivity reaction to the electrical stimulation. Indeed, the mean latencies in the retention trials examined 1, 2 and 7 days after the acquisition trial were significantly shorter than those in the control group (Fig. 3B,  $p = 0.004$ ,  $p = 0.007$ ,  $p = 0.006$ , respectively, post hoc Fisher's PLSD), indicating that the learning/memory was suppressed or hampered presumably due to the rearing under the raised occlusal condition for one week. There was no significant difference in the mean latency in the retention trials at 14 days between the control group and the 1-week occlusal-bite raised group due to the large variability of the mean latency in the 1-week occlusal-bite raised group (Fig. 3B).

### 3.2.3. Two-week occlusal-bite raised group

The animals of the 2-week occlusal-bite raised group (Fig. 3A, red triangles; Fig. 4B) quickly entered the dark room as soon as the guillotine door was open in the control trial, as revealed by a mean latency of  $19 \pm 9$  s. In the acquisition trial, the mean latency sharply and significantly increased to  $132 \pm 49$  s due to the electrical stimulation in the dark room. In the retention trial one day after the acquisition trial, the mean latency was also significantly increased (Fig. 3A,  $p = 0.037$ , post hoc Fisher's PLSD; Fig. 4B,  $p = 0.046$ ,  $t(4) = -2.851$ , paired  $t$ -test), suggesting that the post-learning memory consolidation might have been caused. This

consolidated memory was maintained only in the retention trial examined 2 days after the acquisition trial, and declined slowly at 7 and 14 days as revealed by gradual but significant decreases in the mean latency with increases in the date of retention trial to 14 days (Fig. 3A, red triangles,  $p < 0.001$  and  $p < 0.001$ , post hoc Fisher's PLSD; Fig. 4B). These observations may indicate that the memory retention was suppressed or hampered by rearing under the raised occlusal condition for 2 weeks.

### **3.2.4. Three-week and four-week occlusal-bite raised group**

In contrast to the control and the 2-week occlusal-bite raised group (Fig. 3A, black circles and red triangles; Fig. 4A and B), in the 3-week and 4-week occlusal-bite raised groups, the mean latency in the 1st-day retention trial did not significantly increase from that in the acquisition trial (Fig. 3B, green squares and pink diamonds; Fig. 4E and F), indicating the impairment of memory consolidation. Especially, in the 3-week occlusal-bite raised group (Fig. 3B, green squares; Fig. 4E), the latency to enter the dark room in the control trial largely varied and the mean latency was longer than any other groups, and there was no significant difference in the mean latency between the control and acquisition trials. This observation indicates that the control condition was unstable and restless among individual guinea pigs and among trials, and the learning was hampered, due to the rearing under the raised occlusal condition for 3 weeks. On the other hand, in the 4-week occlusal-bite raised group, the control condition was not so unstable (Fig. 4F) compared to the 3-week one as revealed by the mean latency and SD value (Fig. 4E). However, the mean latency in the 1st-day retention trial was significantly shorter than that in the control group (Fig. 3B,  $p = 0.023$ , post hoc Fisher's PLSD), indicating that the memory consolidation did not occur as strongly as in the control group. Consistent with this observation, there was no significant increase in the mean latency between the acquisition and the retention trial one day after that (Fig. 4F,  $p = 0.246$ ,  $t(6) = -1.285$ , paired  $t$ -test), indicating the impairment of memory consolidation.

### 3.2.5. Eight-week occlusal-bite raised group

In contrast, in the 8-week occlusal-bite raised group (Fig. 3A, blue inverted triangles; Fig. 4C), the mean latency in the control trial was as short as that in the control group. In the acquisition trial, the mean latency sharply increased to  $147 \pm 57$  s (Fig. 3A, blue inverted triangles; Fig. 4C). In the retention trial one day after the acquisition trial, the mean latency was significantly longer than that in the acquisition trial (Fig. 3A,  $p = 0.017$ , post hoc Fisher's PLSD; Fig. 4C,  $p = 0.043$ ,  $t(5) = -2.698$ , paired  $t$ -test), indicating that the memory consolidation occurred. However, this consolidated memory was not maintained but sharply decreased even in the retention trial examined at two days after the acquisition trial, and further deteriorated slowly at 7 and 14 days, as revealed in the significantly smaller mean latency than those in the control (Fig. 3A,  $p = 0.004$ ,  $p < 0.001$ ,  $p < 0.001$ , post hoc Fisher's PLSD). These observations may indicate that the memory retention was more severely suppressed or hampered in the 8-week occlusal-bite raised group, compared to that in the 2-week one.

### 3.3. Relationship between the mean latency and the number of avoidance responses

The maximum latency was limited to 300 s, as in the trial in which the guinea pigs did not enter the darkroom within 300 s after the guillotine door was open. If the maximum latency was not limited to 300 s, the mean latency in any trials would be a larger value than the present one because there were many trials in which the guinea pigs avoided entering the dark room within 300 s. To circumvent such an underestimation and to more accurately assess the effects of bite-raising on the behavior, the number of avoidance responses was evaluated separately from the mean latency, and the mean latency was plotted against the number of avoidance responses (Fig. 5). Using Wilk's lambda test, statistical significance in the avoidance-latency relationship was examined between the control and occlusal-bite raised groups.

There was a significant difference in the avoidance-latency relationship for the acquisition trial only between the control and the 4-week occlusal-bite raised group (Fig. 5F)

while no significant difference in the control conditions between the two groups (Fig. 5E), indicating that sufficient learning was not acquired in the 4-week occlusal-bite raised group compared to the control. This was not detected in two-way RM ANOVA analysis of the latency change shown in Fig. 3B, possibly due to the underestimation of the avoidance.

The guinea pigs in the 2-week, 3-week, 4-week, and 8-week occlusal-bite raised groups except the 1-week one received electrical stimulation  $6.8 \pm 1.5$ ,  $6.2 \pm 1.5$ ,  $6.7 \pm 1.5$ , and  $6.7 \pm 2.1$  times, respectively, during acquisition trials, which are not necessarily significantly but relatively larger than the number of received electrical stimulation in the control group ( $4.4 \pm 2.8$  times) (unpaired *t*-test:  $p = 0.028$ ,  $t(8) = -2.683$  vs 2-week;  $p = 0.180$ ,  $t(8) = -1.470$  vs 3-week;  $p = 0.020$ ,  $t(10) = -2.752$  vs 4-week;  $p = 0.080$ ,  $t(9) = -1.969$  vs 8-week). This indicates that the guinea pigs showed a tendency not to avoid pain stimuli, suggesting that the threshold for pain stimuli was raised by stress, which may have resulted in an apparent decrease in learning ability.

## **4. Discussion**

### **4.1. Changes in body weight induced by bite-raising may reflect changes in cortisol**

In the present study, the body weight showed a biphasic decrease with an increase in the period of the raised occlusal condition; the first fast phase apparently peaked at 5th day and was followed by the second slow phase that apparently peaked at 14th day and then showed a tendency of recovery (Fig. 2C). However, given that the body weight decrease is composed of the fast and slow components and the rising and decay phases of the fast component are overlapped by the rising phase of the slow component, the real peak of the fast component (blue upward arrow) is earlier than the apparent first peak (black arrow) and that of the slow component (red arrowhead) is slower than the apparent second peak observed at 14th-15th day (black arrowhead) (Fig. 2C).

Such a decrease in the body weight may be caused by two factors. Firstly, as a new occlusal contact between upper and lower molar teeth can be achieved within a week after attaching the bite-raising device, the fast phase of the body weight decrease observed until 5th day of the bite-raising procedure may be largely due to the lack of effective mastication of food. Secondly, a part of the fast phase and the slow phase of the body weight decrease may be due to the activation of hypothalamic-pituitary-adrenal axis in response to stress induced by raising the occlusal-bite. Indeed, when VDO in macaque monkeys was raised by 2-3 mm in the molar teeth, urinary free cortisol ( $\mu\text{g}/24\text{ h}$ ) showed a biphasic increase; the first fast phase peaked in a few days and the second slow phase peaked 21 days after the onset of the VDO elevation, and the body weight showed a less clear biphasic decrease [19]. In the present study, the peak time point of the slow phase of the body weight decrease may be between 14 and 21 days (Fig. 2). Thus, the body weight change does not completely coincide with but largely reflects the biphasic increase in urinary free cortisol. However, we have not measured corticosterone concentration in the present study. In addition, macaques and guinea pigs may be different in the pattern of mandibular movement or mastication and hence the effects of occlusal-bite raising on the mastication may also be different, causing stress differentially. Therefore, hypothalamic-pituitary-adrenal axis evaluation should be done also in guinea pigs in a future study.

#### **4.2. Early phase of stress responses that affect learning and memory**

The locus-coeruleus (LC)-norepinephrine (NE) system is a major stress response system that initiates arousal and modulates cognitive function in response to stressors through its regulation of the prefrontal cortex (PFC). It has been hypothesized that stress-induced impairments of *executive function* [33, 34] arise from excessive LC-NE drive to the medial PFC (mPFC) [35], resulting in the impairment of inhibitory circuit in mPFC [36]. Stress hormones such as corticotropin-releasing factor that would also increase following stress can

enhance the activity of LC neurons. Indeed, in the 1-week occlusal-bite raised group, the animals were very sensitive to electrical stimulation applied after they entered the dark room in the acquisition trial whereas this did not lead to the memory consolidation in the 1st-day retention trial (Figs. 3B and 4D). This suggests that the short-term stress may have caused the hypersensitivity to the pain stimulation presumably by hyperactivation of LC-PFC circuits [35] or by increases in excitatory synaptic transmission in PFC [37]. Therefore, the enhanced avoidance during the acquisition trial in the 1-week occlusal-bite raised group may have been caused by the hypersensitivity but not by the learning during the acquisition trial, thereby not leading to the memory-consolidation. However, in the 2-week occlusal-bite raised group, an adaptation of the hypersensitivity may have occurred in accordance with the decline of the first fast phase of free cortisol increase [19], and therefore the learning was not disturbed and the subsequent memory consolidation was clearly observed (Figs. 3A and 4B). In contrast to the learning, the memory retention was deteriorated in the 2-week occlusal-bite raised group. As the downregulation of neuroprotective adiponectin receptor 2 (AdipoR2) occurs in hippocampus as early as 3 days after repeated restraint stress (RS) while it occurs in PFC at 7 days after repeated RS [38] and as hippocampus is more susceptible to cortisol than PFC is [39, 40], memory deterioration preceded learning impairment as observed in the 2-week occlusal-bite raised group.

#### **4.3. Mid-term and late phases of stress responses that affect learning and memory**

The learning appeared to be largely disturbed in the 3-week and 4-week occlusal-bite raised groups in which the examination was initiated 3-4 weeks after rearing under the raised occlusal condition. This may be not only the direct effect of stress hormones but also the secondary effects of stress hormones, such as the glucocorticoid-mediated upregulation of polysialylated neural cell adhesion molecule (PSA-NCAM) expression [41] that participates in the processes of synapse formation and remodeling [36] between GABAergic cells and

pyramidal cells in PFC [42] and also between granule neurons in dentate gyrus (DG) and CA3 pyramidal neurons [43]. Such a secondary effect of stress hormones may have affected the activity of PFC, leading to further impairments of *executive function* as reflected in confused behaviors especially in the control trial in the 3-week occlusal-bite raised group (Fig. 4E) and in the acquisition trial in the 4-week occlusal-bite raised group (Fig. 4F), together with the partial inhibition of memory consolidation in both the 3-week and 4-week occlusal-bite raised groups (Fig. 4E and F).

In these groups, it was not possible to properly evaluate the memory retention because of impairments of *executive function*. Behaviors in the memory retention trial reflect neither memory retention nor memory deterioration due to the impairment of executive function. It has been reported that following RS of 6 h/day repeated for 3 weeks, the generation of new neurons and their survival in the DG of adult male rats is progressively impaired while PSA-NCAM expression is upregulated to induce adaptive structural plasticity [44]. However, this upregulation of PSA-NCAM expression vanished and the generation of new neurons and their survival in the DG were further impaired after 6 weeks RS, suggesting that irreversible impairment of hippocampus function occurs after 6 weeks RS [44]. It has also been reported that after 21 days RS a significant downregulation of neuroprotective AdipoR1 was induced in the hippocampus without downregulation in PFC [38]. These studies suggest that RS for 3 to 6 weeks impairs the functions of PFC and hippocampus, causing the reversible second phase of learning and memory impairments as observed in the 3-week and 4-week occlusal-bite raised groups, and the latest irreversible phase of memory impairment after 6 weeks as observed in the 8-week occlusal-bite raised group. Thus, in the 8-week occlusal-bite raised group, the avoidance learning may still be possible without confusion although the strength of learning is relatively weak, and the memory consolidation occurred whereas the memory retention was severely impaired. Thus, only in the 8-week occlusal-bite raised group, there was a clear

unilateral decline in memory retention comparable to a forgetting curve.

#### **4.4. Removal of the bite-raising device may affect the memory retention**

In the present study, the guinea pigs in which VDO was raised for varying periods were subjected to a series of examination trials over the period of 2 weeks without removing the bite-raising device. It may not be unreasonable to regard that the control, acquisition and 1st-day retention trials were examined in the respective occlusal-bite raised group. However, the retention trials examined at 7 and 14 days after the acquisition trials do not necessarily reflect the original condition of the respective occlusal-bite raised group because the bite-raising device was not removed over the period of the retention trials. The retention trials examined at 7 and 14 days after the acquisition trial in the respective occlusal-bite raised group would rather reflect the behaviors contaminated with those that were observed in the group reared under the raised occlusal condition for one or two weeks longer periods than the original group. However, if the bite-raising device is removed after the acquisition trial, the guinea pig would exert a rigorous occlusal abrasion to reduce VDO [16] which itself would reduce stress sharply [45], consequently affecting the behavior during the memory retention trials. Thus, it can be concluded that the control, acquisition and 1st-day retention trials are reliable and differentially reflect the preceding reared period under the raised occlusal condition while the retention trials at 7 and 14 days after the acquisition trial may not reflect the nature of the defined group. Nevertheless, the 8-week occlusal-bite raised group showed a clear decline in the memory retention at 7 and 14 days after the acquisition trial, suggesting that there may be no more alterations in stress-related signaling between 8 and 10 weeks of the occlusal-bite raising. It is known that the impairment of learning system which was revealed as the degeneration of basal forebrain cholinergic neurons was induced 15 weeks after the masticatory dysfunction caused by horizontal resection of molar tooth crowns in aged rats [29].

#### **4.5. Limitation of the present study**

In this study, although we have examined the control animals which underwent a sham operation for handling and anesthesia like the occlusal-bite raised animals, we have not examined another control group which received the sham treatment of a non-bite-raising device only with a metal plate. Given the rationale for the present experiment proposed based on our previous studies [4, 18], such sham treatment with the non-bite-raising device that would cause a transient difficulty in food intake due to the attachment of a metal plate to incisor teeth would not cause cognitive impairment. It is also unlikely that guinea pigs in such a sham group die at the same rate as that of the bite-raised group. This is because the death in the bite raised group was chiefly caused by the lack of effective mastication of food due to the gap space between upper and lower molar teeth for 5-7 days until the gap space disappears, as represented by the weight loss pattern in the dead bite-raised guinea pigs, which is almost similar to that of the survived bite-raised guinea pigs at least for the initial five days. Therefore, as long as the bite is not raised, the sham device would cause neither cognitive impairment nor death although it may cause a transient difficulty in food intake. However, this kind of sham operation would be helpful to more firmly establish the present conclusion.

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## **CRediT authorship contribution statement**

**H. Toyoda:** Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. Funding acquisition. **Y. Fujinami:** Investigation, Formal analysis, Data curation, Writing – review & editing. **M. Saito:** Formal analysis, Data curation, Visualization, Writing – review & editing. **Y. Maeda:** Investigation, Writing – review & editing. **Y. Kang:** Conceptualization, Supervision, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

## **Declarations of interest**

The authors have no conflicts of interest.

## **Data availability**

Data will be made available on request.

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## Figure legends

### Fig. 1. The bite-raising procedure in guinea pigs.

(A) The bite-raising device. The immediate-curing dental resin was bonded to the top of the mandibular incisors to fabricate a resin bite-raising appliance, to the upper surface of which a flat stainless steel metal was attached. (B-D) The 3D micro-CT images obtained before, immediately after, and 7 days after the attachment of the bite-raising device. The red interrupted lines represent the occlusal plane (B and D). The upper and lower red interrupted lines represent the occlusal planes of the upper and lower jaws, respectively (C).

### Fig. 2. Body weight changes in control and occlusal-bite raised guinea pigs.

(A) Body weights measured immediately before and 1-7, 14, 21, 28 and 35 days after just anesthetizing the no bite-raised control group ( $n = 5$ ) and after attaching the bite-raising device under anesthesia in the occlusal-bite raised groups (1-week,  $n = 5$ ; 2-week,  $n = 5$ ; 3-week,  $n = 5$ ; 4-week,  $n = 7$ ; 8-week,  $n = 6$ ). (B) The time course of the body weight changes same as in (A), but the mean values of the respective occlusal-bite raised groups were averaged to perform the subtraction between the control and the occlusal-bite raised groups. (C) Body weight decrease obtained by subtracting the body weights of the control group from those of the occlusal-bite raised group. The body weight decreased in a biphasic manner with an increase in the period of rearing under the raised occlusal condition (black line). The fast and slow components (blue and red dotted lines, respectively) were obtained by a mathematical fitting of the body weight change with the following function:  $-53.36 \times x \times \exp(-0.41 \times x) + 15.16 \times x \times \exp(-0.06 \times (x - 5.18))$ . Black arrow and arrowhead indicate the first and second peaks of the body weight change at 5th and 14th-15th day, respectively. Blue arrow and red arrowhead indicate the peaks of the fast and slow components, respectively. (D) Body weight changes in 6 guinea pigs, 2 each in the 2-week, 3-week and 8-week occlusal-bite raised groups, which

died either 7 or 8 days after the device was attached.

**Fig. 3. Differential changes in the mean latency in respective trials during passive avoidance test between the control and occlusal-bite raised groups.**

(A) The mean latency in the control, acquisition, and retention trials at 1, 2, 7 and 14 days after the acquisition trial in the control group ( $n = 5$ ) and the 2-week and 8-week occlusal-bite raised groups ( $n = 5$  and 6, respectively). (B) The mean latency in control, acquisition, and retention trials at 1, 2, 7 and 14 days after the acquisition trial in the control group ( $n = 5$ ) and 1-week, 3-week and 4-week occlusal-bite raised groups ( $n = 5, 5$ , and 7, respectively). There were significant differences between the control and any of the occlusal-bite raised groups. Two-way RM ANOVA: Group effect,  $F(5, 27) = 3.79, p = 0.010$ ; Trial effect,  $F(5, 135) = 28.67, p < 0.001$ ; Group  $\times$  Trial effect,  $F(25, 135) = 2.50, p < 0.001$ , followed by Fisher's PLSD,  $\#p < 0.05$  (vs control group).

**Fig. 4. Differential changes in the mean latency among respective trials during passive avoidance test in the control and occlusal-bite raised groups.**

(A-F) The mean latency in control, acquisition, and retention trials at 1, 2, 7 and 14 days after the acquisition trial in control group ( $n = 5$ ) and 2-week, 8-week, 1-week, 3-week, and 4-week occlusal-bite raised groups ( $n = 5, 6, 5, 5$  and 7, respectively).  $*p < 0.05$ , paired  $t$ -test. The bar graphs with error bars represent the mean latency in respective groups (mean  $\pm$  SD). The plots with error bars represent the mean latency in each guinea pig (mean  $\pm$  SE). Significant learning and significant memory consolidation were observed only in the control, 2-week and 8-week-occlusal-bite raised groups.

**Fig. 5. Differential avoidance-latency relationships among the control and respective**

**occlusal-bite raised groups in control, acquisition and retention trials at 1 and 14 days.**

(A-D) Relationship between the mean latency and the number of avoidance responses was compared among the control group ( $n = 5$ ) and 2-week or 8-week occlusal-bite raised groups ( $n = 5$  and 6, respectively) in control, acquisition, and retention trials at 1 and 14 days.  $p$  values were obtained by Wilk's lambda. (E-H) Relationship between the mean latency and the number of avoidance responses was compared among the control group ( $n = 5$ ) and 1-week, 2-week, or 4-week occlusal-bite raised groups ( $n = 5, 5$ , and 7, respectively) in control, acquisition, and retention trials at 1 and 14 days.  $p$  values were obtained by Wilk's lambda. Note a significant difference in the avoidance-latency relationship for the acquisition trial only between the control and the 4-week occlusal-bite raised group (F), which was not detected by ANOVA analysis in Fig. 3B. The oblique dotted line represents the avoidance-latency relationship in which a number of avoidance responses in the ten trials gives a mean latency when the latency in the un-avoided trials was zero. Therefore, all the data points should appear above the oblique dotted line.









