

**Memory-related gene expression profile of the male rat hippocampus induced by teeth extraction and occlusal support recovery**

Sachiyo Iida\*, Tetsuya Hara, Daisuke Araki, Chisa Ishimine-Kuroda, Akimasa Kurozumi, Shunichi Sakamoto, Takako Miyazaki, Shogo Minagi

Department of Occlusal and Oral Functional Rehabilitation, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8525, Japan

**\*Corresponding author:**

Sachiyo Iida

Department of Occlusal and Oral Functional Rehabilitation

Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences

2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8525, Japan

Tel: +81-86-235-6687, Fax: +81-86-235-6689

E-mail: sachi@md.okayama-u.ac.jp

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## **ABSTRACT**

**Objectives:** The present study aimed to identify the effect of memory-related genes on male rats tested for spatial memory with either molar teeth extraction or its restoration by occlusal support using experimental dentures.

**Design:** Memory-related genes were detected from hippocampi of Male Wistar rats (exposed to teeth extraction with or without dentures, or no extraction [control]) (7-week old) after behavioral testing (via the radial maze task) using DNA microarray. The time course of the expression of these genes was evaluated by quantitative real-time PCR (on 49-weeks old rats).

**Results:** In preliminary experiments to determine which memory genes are affected by spatial memory training, DNA microarray analysis revealed that thyrotropin-releasing hormone (Trh) and tenascin XA (Tnxa) were up-regulated and Neuronatin (Nnat) and S100a9 were down-regulated after the maze training. The expression of Tnxa, Nnat, and S100a9 of 49-week old rats (during the time-course) via quantitative real-time PCR was consistent with the results of microarrays of the preliminary experiment. Expression of Trh that was evaluated by quantitative real-time PCR did not agree with the results for this gene from the microarray for all groups. Therefore, expression of Trh may have increased in only young trained rats. The expression of S100a9 prior to the maze task was down-regulated in only the extraction group.

**Conclusion:** These results demonstrated that Trh, Tnxa, and Nnat genes were affected according to the degree of memory in male rats. This study also indicated that S100a9 is a memory-related gene, which is affected by the presence of occlusal support.

## 1. Introduction

Neural plasticity is regarded as the fundamental mechanism underlying memory formation, for which the hippocampus plays a vital role given its function in encoding, consolidation and retrieval of memories.<sup>1, 2</sup> Furthermore, the dorsal hippocampus is well recognized to be involved in spatial memory.<sup>3, 4</sup> Hippocampal volumetry is believed to have high sensitivity and specificity in detection of senile dementia.<sup>5</sup>

Various brain sites are affected by tooth loss and mastication. Analysis using positron emission tomography and magnetic resonance imaging has shown that chewing increases neuronal activity in various regions of the cerebral cortex.<sup>6</sup> It has reported that dental extraction may be associated with significant neuroplastic changes within the rat's face primary motor cortex and adjacent face primary somatosensory cortex that may be related to the animal's ability to adapt to the altered oral state<sup>7</sup> and the maturation of network function in the oral somatosensory cortex is impaired by tooth loss during the developmental period.<sup>8</sup>

Epidemiological studies have revealed that tooth loss is a risk factor for senile dementia.<sup>9</sup> For example, in a longitudinal study, a low number of teeth ( $\leq 9$ ) was associated with increased prevalence and incidence of senile dementia.<sup>10</sup> Our group has previously reported of a reduction in the number of pyramidal cells in the hippocampus and decreased spatial memory (using the radial maze) in molar less rats. However, when occlusion was reinstated (by wearing dentures), the number of errors generated in the radial maze was reduced.<sup>11</sup> In order to understand these phenomena, it is necessary to examine the influence of tooth loss and the recovery of the occlusal support on molecular biology, which would be related to memory in the hippocampus.

However, the role of specific memory genes associated with tooth loss remains unresolved. Therefore, in the current study, we investigated the effect of teeth extraction or occlusal support via experimental dentures on hippocampal memory-related genes in Wistar rats tested for spatial learning using the radial maze.

## **2. Material and methods**

### **2.1. Animals**

Male Wistar rats (Japan SLC, Shizuoka, Japan) (n = 52) were housed in groups of 3 or 4 per cage under controlled conditions (22 ± 2 °C, 12:12 light-dark cycle). The animals were given *ad libitum* access to food (MF, Oriental Yeast Co., Tokyo, Japan) and water. All experiments were approved by the Animal Care and Use Committee, Okayama University (protocol no. OKU-2010142).

### **2.2 Experiment design**

Initial experiments focused on the selection of memory-related genes (via microarray analysis) on rats (7 weeks old) tested on the radial maze (n = 5) versus those that were not exposed to the maze (n = 5). To investigate molar extraction (and recovery of occlusion) on memory-related gene expression, rats (6 weeks old) were randomly divided into three groups: (1) no extraction (control) group (n = 14), (2) extraction group (n = 14), and (3) extraction plus denture group (n = 14). Behavioral testing of these groups, via the radial maze, was performed on 49-week old rats, followed by memory-related gene expression analysis, and corticosterone (CORT) level determination. The observation period was 0, 1 and 3 day to evaluate the sequential change of the expression in memory-related gene. Behavioral testing and gene analysis were performed on the same rat on these evaluation days.

### **2.3 Maxillary molar extraction and recovery by occlusion with experimental dentures**

Bilateral maxillary molar teeth were extracted from rats (7 weeks old) under sodium pentobarbital anesthesia (Somnopentyl, 30 mg/kg). The rats in the control group were anesthetized, but no teeth were extracted. To provide a suitable recovery time after teeth extraction, experimental dentures were fitted to rats at 11 weeks of age. Experimental dentures were produced from an impression, made of silicone impression material and resin tray (on 10 weeks old rats). The experimental denture was constructed from the retention part of wire and heat-cured acrylic resin (Acron, GC, Tokyo, Japan) (Fig. 1). Occlusal

adjustments were made until maxillomandibular incisor contacts were obtained. From 47 weeks of age, rats from all groups were kept on a restricted diet, and were pre-trained for behavioral testing.

#### **2.4 Radial maze task**

The 8-arm radial maze (Radial arm maze, Neuroscience, Tokyo, Japan) was used to measure learning and memory performance (Olton and Samuelson<sup>12</sup>). The maze was elevated 50 cm above the floor, with arms (50 cm × 10 cm) projecting at equal angles from a central platform (24 cm diameter). During testing, 45 mg dustless reward pellets (Dustless Precision Pellets, Bioserv, Germany) were placed in small plastic cups affixed 1 cm from the end of each arm. The maze stood in a fixed location at the corner of a room. Prior to maze experiment and in order to enhance motivation for food in the 8-arm radial maze, animals were kept on a restricted diet (water was freely available), and body weight was reduced to 80-85% of normal weight.<sup>13</sup> Preliminary training (10 min, 4 days) for each rat involved handling the rats and then placing them as a group in an open field, followed by individual placement on the central platform of the maze (with food pellets placed throughout the maze). By the 4<sup>th</sup> day, rats were capable of running to the ends of the arms. Rats were allowed to visit the arms until all the food had been collected or until 10 min had elapsed. For the experimental period, rats performed the maze test (max 10 min duration) once per day for 5 consecutive days. An exploration of one arm was recorded if all 4 feet were in this arm, and an error was defined as a re-entry into a previously visited arm.

#### **2.5 Microarray analysis**

The microarray analysis was carried out on rats (7 weeks old) of initial experiments. Ten rats from the initial experiments were anesthetized with sodium pentobarbital (Somnopentyl, 50 mg/kg), and the hippocampi rapidly dissected. The hippocampus was immersed in RNAlater (QIAGEN Inc., Valencia, USA), and the hippocampus was stored at -80 °C until it was processed. Total RNA from hippocampus was extracted by the RNeasy Lipid Tissue Mini Kit (QIAGEN Inc., Valencia, USA), according to the manufacturer's

protocol. The density of total RNA was determined by absorption spectrometry. Total RNA was reverse transcribed into cDNA by the PrimeScript™ RT reagent Kit (TaKaRa, Ohtsu, Japan). RNA hybridizations were carried out using the whole rat gene expression microarray chip (Rat GE 4x44K v3 Microarray Kit G2519F, Agilent Technologies Japan, Tokyo, Japan). The samples were labelled by coupling with Cy3 or Cy5 monoreactive dyes. A flip labeling (dye-swap labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cDNA.<sup>14, 15, 16</sup> Hybridization and wash processes were performed according to the manufacturer's instructions. Data were processed and analyzed with the Agilent DNA microarray scanner (High-Resolution Microarray Scanner G2505, Agilent Technologies, CO, USA). The differentially expressed genes were selected if there was a 2-fold change between the control group and the maze experimental group. The Gene Ontology was used for analysis of gene function. To validate our microarray data and to select the candidate genes in conjunction with memory, Real-Time PCR (qPCR) was conducted, using the SYBR Premix Ex Taq™ II (Takara, Shiga, Japan) on a LightCycler® 1.5(ST300) Instrument (Roche Diagnostics, IN, USA). All reactions were run in triplicate for each gene. Memory-related genes quantified before the radial maze task represented the mean fold difference in expression, which were normalized to gene expression of GAPDH and calibrated against control at day 0 of each gene at baseline level. Memory-related genes quantified after the radial maze task represented the mean fold difference in expression, normalized to GAPDH and calibrated against each group at day 0 baseline level.

## **2.6 Measurement of CORT levels**

Spatial memory depends on proper physiological functioning of the hippocampus, and can be influenced by various factors like stress and elevated CORT levels.<sup>17, 18</sup> Blood samples were taken from the carotid artery, in tubes containing EDTA. The samples were centrifuged (1200 ×g, 10 min at 4 °C), and the serum was collected, and stored at -80 °C

until future use. CORT levels were determined using an Enzyme Immuno Assay kit (Yanaihara Institute Inc, Shizuoka, Japan), according to the manufacturer's instructions.

## **2.7 Statistical analysis**

All data were expressed as the mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used (excluding data from the radial maze task). Two-way ANOVA was performed on the data from the radial maze task, on 49-week old rats. Statistical analysis was performed using the statistical software package, PASW Statistics 18 (SPSS Japan, Tokyo, Japan). Significance was reached at values of  $p < 0.05$ .

## **3. Results**

### **3.1. Initial experiments on rats exposed to the radial maze task for the identification of memory-related genes**

The mean number of errors on the 5<sup>th</sup> trial day of the radial maze task was significantly ( $p < 0.05$ ) decreased in the rats tested on the radial maze compared with the 1<sup>st</sup> trial day (Fig. 2). Microarray analysis of hippocampi from the rats of maze group revealed that of the 304 genes showing greater than 2-fold regulation, 96 and 208 were up-regulated and down-regulated, respectively in rats of the radial maze group. The data from the maze group compared against data from those rats not exposed to the maze. 15 of these genes were selected based on their association with learning and memory, and their expression levels confirmed by qPCR. The qPCR data (Table 1) were similar to the results of the microarray (Fig. 3). The expression of Trh and Tnxa increased approximately 3- and 4-fold, respectively. In addition, the expression of both S100a9 and Nnat decreased by approximately half (Table 1). Genes related to fear memory (Fst and Nts) were regarded as inappropriate for the purposes of our study and showed large individual differences, and were therefore excluded. Other genes showed large individual differences, thus the data for these genes were deemed as inappropriate for future study. Therefore, Trh and Tnxa

(up-regulated genes), and S100a9 and Nnat (down-regulated genes) were selected as memory-related gene for further analysis in this study.

### **3.2. The mean number of errors in the radial maze task**

The mean number of errors on the 3<sup>rd</sup> trial day of the radial maze task was significantly ( $p < 0.05$ ) decreased in the control group and the extraction group compared with the 1<sup>st</sup> trial day (Fig. 4). The number of errors in the denture group on the 2<sup>nd</sup> trial day and the 3<sup>rd</sup> trial day was significantly ( $p < 0.01$ ) lower than those of the 1<sup>st</sup> day. The mean number of errors in the control group and the denture group on the 2<sup>nd</sup> trial day and the 3<sup>rd</sup> trial day was significantly ( $p < 0.05$ ) lower than the extraction group of the each day.

### **3.3. The expression levels of selected memory-related genes before the radial maze task**

No difference was observed for gene expression levels of Tnxa and Nnat before maze training (day 0) between the 3 groups. The expression level of Trh at day 0 was significantly ( $p < 0.001$ ) higher in the denture group compared with the control group or the extraction group. The expression level of S100a9 at day 0 was significantly ( $p < 0.001$ ) lower in the extraction group compared with the control group or denture group (Fig. 5).

### **3.4. The expression levels of the up-regulated genes selected memory-related genes**

On the 1<sup>st</sup> trial day, the expression level of Trh was significantly ( $p < 0.001$ ) reduced (by approximately half) in the control group compared with day 0. However, no significant change was seen in the denture group or extraction group throughout the trial. On the 3<sup>rd</sup> trial day, Tnxa expression levels in the control group or denture group were significantly ( $p < 0.01$  or  $p < 0.05$ , respectively) increased, approximately 2-fold compared with day 0. However, no change was seen in the extraction group throughout the trial (Fig. 6).

### **3.5. The expression levels of the down-regulated genes selected memory-related genes**

The expression level of Nnat in the control group was significantly ( $p < 0.05$ ) reduced (to approximately half) on the 1<sup>st</sup> trial day compared with day 0, and was

significantly ( $p < 0.0001$ ) reduced in the denture group on the 3<sup>rd</sup> trial day compared with day 0. Furthermore, its expression level in the extraction group was significantly ( $p < 0.05$ ) reduced (to approximately half) on the 3<sup>rd</sup> trial day compared with day 0. The expression level of S100a9 in the control group was significantly ( $p < 0.01$ ) reduced on the 3<sup>rd</sup> trial day compared with day 0, and its expression level in the denture group on the 1<sup>st</sup> trial was significantly ( $p < 0.0001$ ) reduced. Furthermore, S100a9 in the extraction group was significantly ( $p < 0.0001$ ) reduced (to approximately half) on the 1<sup>st</sup> trial day compared with day 0 (Fig. 7).

### **3.6. CORT levels**

No significant difference in CORT levels was observed between the three groups (Fig. 8).

## **4. Discussion**

In the present study, it is suggested that Trh, Tnxa, Nnat and S100a9 genes would be affected to memory by the microarray.

Several studies have reported that tooth loss induces neuronal dysfunction in the central nervous system because of the important role that mastication plays in aging brain function.<sup>19</sup> Spatial memory impairment in toothless aged rats may be caused by the deterioration of the cholinergic system.<sup>19</sup> Moreover, aged senescence-accelerated mice (SAM-P8) with fewer molar teeth exhibit hippocampal neuronal loss, and impairment of spatial performance in the Morris water maze test.<sup>20</sup> We have previously reported that the number of pyramidal cells in the hippocampus, and spatial memory were reduced in rats with fewer molar teeth.<sup>11</sup> However, Kurozumi<sup>11</sup> have shown that restoration of occlusal support by dentures decreased the number of the errors in the radial maze.

The hippocampus is not the only brain site affected by tooth loss. This has been confirmed in a number of recent investigations.<sup>8, 9, 10</sup> The candidate genes identified here in hippocampus also play a role at other sites. Trh has multiple actions in mammalian cerebral cortex.<sup>21</sup> S100a9 was found to be increased within neuritic plaques and reactive glia in the

inferior temporal cortex of Alzheimer's disease.<sup>22</sup> Other sites in the brain may contribute to the learning and memory effects of occlusal changes. In this study, we only investigated effects of occlusal changes on hippocampal. Future research is necessary to study sites other than the hippocampus.

The estrus cycle is not relevant in male rats and thus, they were used due to their physiological homogeneity, such as their hormone balance. Therefore, only male rats were used in this study. It has been revealed that male rats tend to perform better than females on the radial maze and circulating levels of estrogen might impair spatial processing in females.<sup>23</sup> Gene expression might be different in the female rat by the hormone influence. Future studies will aim to evaluate gene expression of both sexes.

Young rats were used for selection of memory-related genes because they have better spatial memory learning than do aged rats.<sup>24</sup> The levels of these selected genes were then investigated in aged rats with molar extraction with or without recovery of occlusal support (by experimental dentures).

In rodents, exposure to repeated (or chronic) stressors produces deficits on tasks of learning and memory that rely on the functioning of the hippocampus and medial prefrontal cortex.<sup>25, 26, 27</sup> Although we handled animals in the same way, it is likely that they were fearful because of our handling and the maze task. In fact, genes related to fear memory (Fst and Nts) were altered in some rats, and showed large individual differences. Consequently, these genes were excluded from the following experiment.

Spatial memory depends on the proper physiological functioning of the hippocampus, and can be influenced by various factors like stress and elevated CORT levels.<sup>17, 18</sup> Stress has been evidenced by changes in learning and memory.<sup>28</sup> Higher plasma CORT levels have been shown in animal 10 days after molar extraction.<sup>29</sup> However, our results show no significant differences in CORT levels between the 3 groups, indicating that stress did not influence the affected spatial memory that was observed. Furthermore, because CORT

levels were measured 10 months after tooth extraction, rats may have adapted to the absence of teeth or dentures during the time-period.

The behavioral experiment was carried out to confirm the changes of gene expression in rats with teeth extraction or occlusal support restoration by dentures. Results of the behavioral experiment indicate a significant reduction in the mean number of errors on the 2<sup>nd</sup> or 3<sup>rd</sup> trial days in all groups, furthermore suggesting improved learning and memory.

Trh is a neuropeptide originally discovered for its function as a hypothalamic factor controlling the synthesis and release of thyrotropin from the pituitary.<sup>30</sup> Spatial learning has been shown to increase Trh levels in the septum and hippocampus.<sup>31</sup> Our findings showed that the expression of Trh in the denture group before the maze task was higher than that of the control group or extraction group. The expression of Trh is suggested to be unaffected by occlusal support. Furthermore, our results do not show significant differences in behavior between the control and the denture group. Further studies could focus on clarifying the influence of dentures. The expression of Trh was reduced in the control group on the 1<sup>st</sup> trial day. The expression of Trh in the denture group and the extraction group showed no significant change throughout the observation period. Therefore, these results did not agree with the initial experiment for the selection of memory-related genes in young rats, and also with previously published data showing that Trh concentration is increased in the hippocampus of trained young rats.<sup>31</sup> This discrepancy may be due to age differences as our study used aged rats to investigate the effects of teeth extraction.

Tnxa is a member of the Tenascin family, and is a partially duplicated gene segment that corresponds to intron 32 to exon 45 of Tnxb.<sup>32, 33</sup> Although the function of Tnxa is yet to be determined, structures similar to Tnxa, Tenascin C (Tnc) and Tenascin R (Tnr), have been shown to be related to memory.<sup>34</sup> Results of the present study showed that the expression of Tnxa before maze training was not significantly different between the 3 groups. On the 3<sup>rd</sup> trial day, the expression of Tnxa was increased in control and denture groups, but was unaffected in the extraction group over the trial period. The absence of changes in the

expression of *Tnxa* of the extraction group was not coincident with the behavioral data because the mean number of errors was decreased in this group. Therefore, these results suggest that *Tnxa* may be one of the genes related to learning and memory.

*Nnat* was first identified to be strongly expressed in the rat neonatal brain, and suggested to be involved in neuronal cell differentiation.<sup>35</sup> The neonatal expression of *Nnat* may be involved in olfaction, thus exerting a profound influence over food and water intake.<sup>36</sup> Cognitive impairment in the Phenylketonuria (PKU) mouse has been shown to be due to the high expression of *Nnat*.<sup>37</sup> Our findings showed that the expression of *Nnat* before maze training was not significantly different between the 3 groups. However, the expression of *Nnat* gradually decreased for all groups over the trial days. These results were coincident with the microarray data, thus indicating that *Nnat* may be related to memory function. Therefore, *Nnat* gene may not be influenced by recovery of occlusal support.

*S100a9* is an inflammation-associated calcium binding protein, belonging to the *S100* family.<sup>38</sup> Cerebral ischemia, traumatic brain injury, and Alzheimer's disease (AD) have been reported to be associated with altered expression/function of *S100* family members.<sup>22, 39, 40,</sup><sup>41</sup> Recently, *S100a9* was found to be increased within neuritic plaques and reactive glia, thus suggesting a role in the inflammatory component of AD pathogenesis.<sup>22</sup> Furthermore, inhibition of *S100a9* may be a possible therapeutic target for AD.<sup>38</sup> Our findings showed that the expression of *S100a9* before maze training in the extraction group was lower than that of the control group or denture group. Furthermore, these behavioral experiments showed that the mean number of errors was higher in the extraction group than in the denture group or the control group. Based on these results, the changes in expression of *S100a9* in the extraction or denture group may have thus influenced spatial memory. The expression of *S100a9* was reduced in all groups on the 1<sup>st</sup> or 3<sup>rd</sup> trial day. These results were coincident with the microarray data, and thus indicated that *S100a9* may be related to memory function.

Future studies will aim to evaluate gene expression at specific hippocampal sub-regions by in situ hybridization. In addition, the use of gene-specific knockout animals would provide a greater understanding for the function of each gene.

Overall, the expression of Trh is not related to memory of hippocampus or is changed or increased only in young rats by learning. The quantitative analysis results of Tnxa, Nnat and S100a9 were coincident with those from the microarray data, and results of longitudinal gene expression were consistent with behavioral performance. Furthermore, reduced levels of S100a9 in the extraction group may be a compensatory effect for the decrease in spatial memory. The expression of S100a9 may be affected by occlusal support. These results demonstrate that Trh, Tnxa, Nnat and S100a9 genes may affect memory in male rats.

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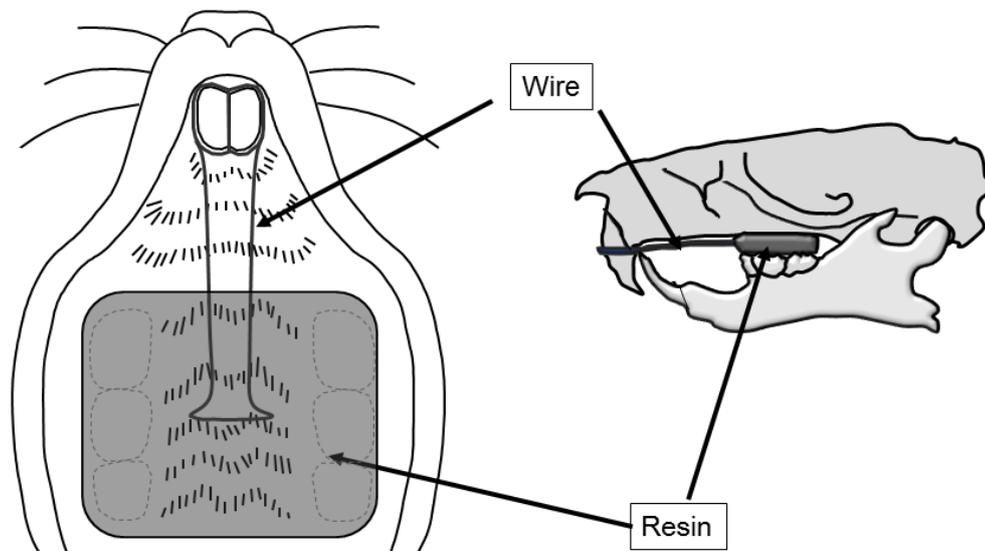
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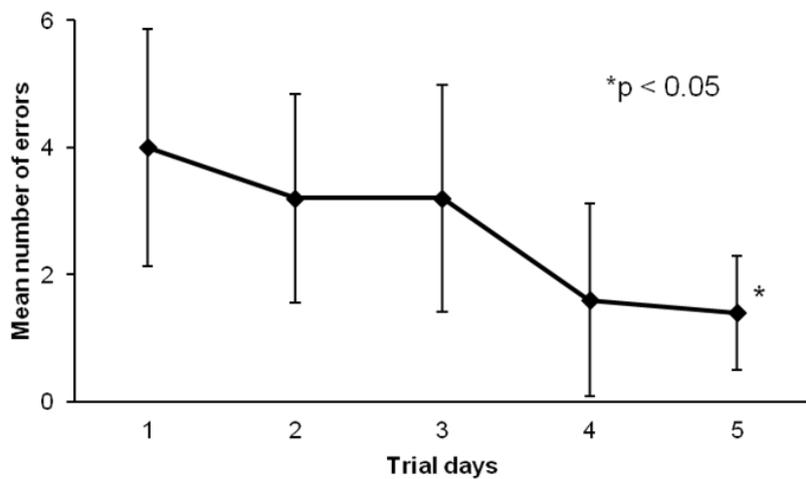
**Table 1. Function of genes and mRNA level**

Gene	Function	mRNA level	
		(control)	(maze task)
Cdh1	Cell-cell adhesion; neuron projection development	1.042 ± 1.034	0.433 ± 0.205
Eif2ak4	RNA-dependent protein kinase activity	0.988 ± 0.037	0.783 ± 0.173
Fst	Actinin binding	1.212 ± 0.506	2.646 ± 0.343
Nnat	Brain development	2.035 ± 0.175	0.930 ± 0.066
Ntrk1	Axon guidance; Learning and memory	0.915 ± 0.689	1.730 ± 0.578
Nts	extracellular region; Working memory	1.633 ± 1.536	5.797 ± 1.118
Otx2	Anatomical structure development	1.730 ± 1.72	0.446 ± 0.251
Pmch	Learning or memory	0.635 ± 0.249	2.042 ± 1.866
Rgs9	Nervous system development	0.978 ± 0.230	0.611 ± 0.196
RGD1564327	Brain development; memory	0.803 ± 1.164	0.160 ± 0.225
Sgk1	Negative regulation of apoptotic process; Long-term memory	1.155 ± 0.671	1.130 ± 0.448
S100a9	Calcium ion binding	0.342 ± 0.081	0.190 ± 0.015
Tac1	Inflammatory response, Long-term memory	0.622 ± 0.342	0.471 ± 0.052
Tnxa	Muscle organ development	0.419 ± 0.054	1.324 ± 0.105
Trh	Hormone-mediated signaling pathway	0.748 ± 0.064	1.848 ± 0.148



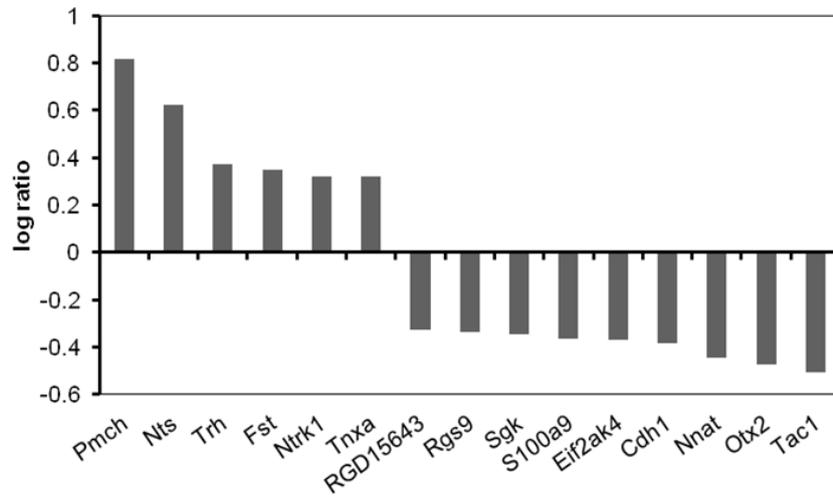
**Figure 1. Schema diagram of the experimental denture**

The experimental denture was constructed from the retention part of wire and the heat-cured acrylic resin. The experimental denture was constructed from heat-cured acrylic resin (Acron, GC, Tokyo, Japan) and the retention part of wire.



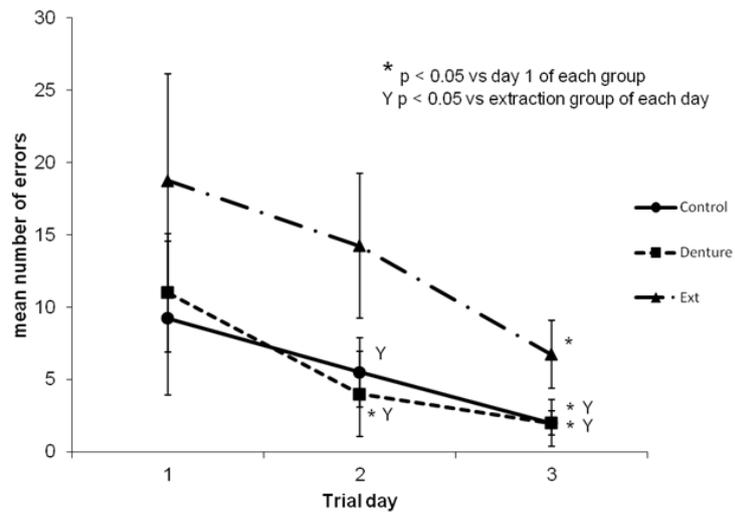
**Figure 2. Mean number of errors from the radial maze task**

It shows the results of maze task. The mean number of errors is plotted versus the day of testing. The mean number of errors on the 5<sup>th</sup> trial day of the radial maze task was significantly ( $p < 0.05$ ) decreased compared with the 1<sup>st</sup> trial day.



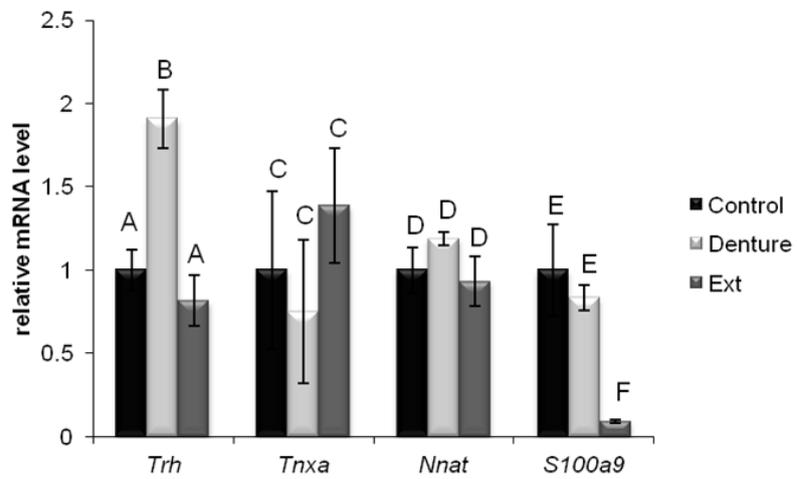
**Figure 3. Micro array results of the 15 candidate genes**

Logarithmic scale ratio of mean fluorescent intensity for all 15 genes. These genes showed greater than 2-fold regulation. Six genes were up-regulated and 9 genes were down-regulated.



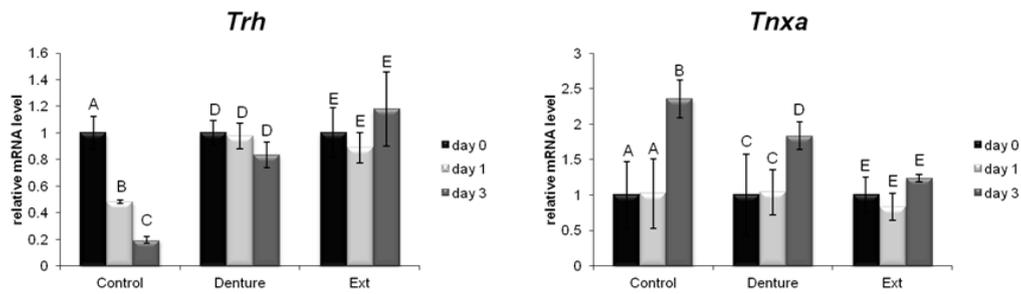
**Figure 4. Mean number of errors from the radial maze task**

Comparison of learning performances in the control group, extraction group, and denture group. The mean number of errors is plotted. The number of errors on the 3<sup>rd</sup> trial day of the radial maze task was significantly decreased in all group compared with the 1<sup>st</sup> trial day.



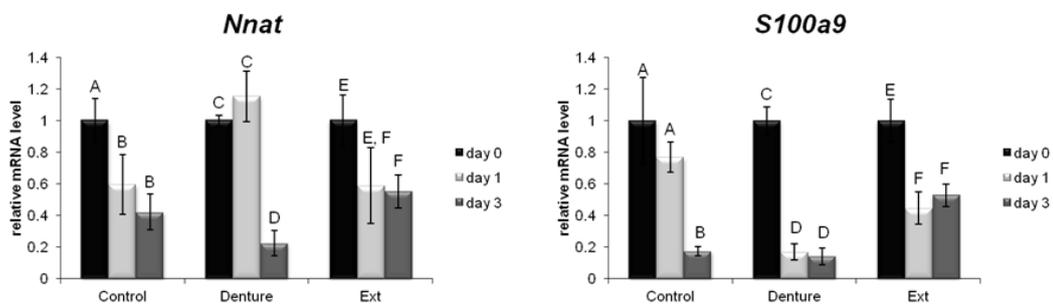
**Figure 5. Profiles of memory-related genes expressed in hippocampi before the maze task.**

Value with same capital letter are not significantly different ( $p < 0.05$ ). The data represent the mean fold difference in expression per time-point, normalized to GAPDH mRNA expression and calibrated to each control 0 day baseline level.



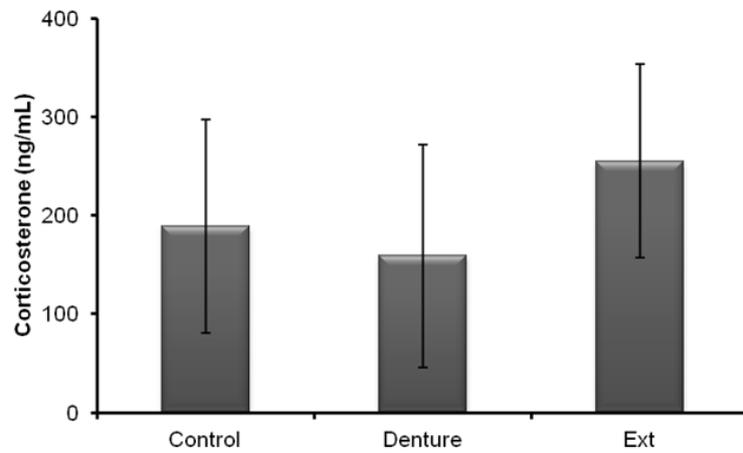
**Figure 6. Profiles of memory-related genes (Up-regulated) expressed in hippocampi over the 3 day trial period of the radial maze task.**

The data represent the mean fold difference in expression per time-point, normalized to GAPDH mRNA expression and calibrated to each 0 day baseline level. Value with same capital letter are not significantly different ( $p < 0.05$ ).



**Figure 7. Profiles of memory-related genes (Down-regulated) expressed in hippocampi over the 3 day trial period of the radial maze task**

The data represent the mean fold difference in expression per time-point, normalized to GAPDH mRNA expression and calibrated to each 0 day baseline level. Value with same capital letter are not significantly different ( $p < 0.05$ ).



**Figure 8. Effect of the stress for loss or recovery of occlusal support and the procedures of experiment on serum levels of CORT.**

Serum level of CORT was measured using an EIA. No significant difference in CORT levels was observed between the three groups ( $p > 0.05$ ).