(Form 14)

Summary of the Final Examination Results

Report No.	Diploma No. 601		Applicant	Ashis Dhar
Examination Committee Members	Chief Examiner	Dr. TAMATSU Yuichi	Degree	Doctor of Philosophy in Dental Science
	Examiner	Dr. MATSUGUCHI Tetsuya	Examiner	Dr. SAITO Mitsuru
	Examiner	Dr. NAKAMURA Norifumi	Examiner	Dr. SHIMA Kaori

The chief and four examiners interviewed applicant **Ashis Dhar** on 02/15/2021. The applicant was asked to explain the thesis and answer related questions. We asked the following questions and received satisfactory answers.

Question 1: Did you fill the drug in the sockets?
Answer: After the extraction, the sockets were cleaned up with normal saline and clean cotton. We used gum closer glue after the application of Fluoro-Gold (FG) into the sockets.

Question 2: You described the maxillary molars on both sides were extracted. Why did you extract the maxillary teeth? How much did the masticatory function and the amount of food decrease when the molars on both sides are removed?
Answer: For the research convenience and to keep the mice physically fit, I had to do that. Because in the mandibular region there is vital issue of tongue. On the other hand, it is hard to extract teeth in the lower region in mice. After the extraction we provided powder food to the experimental mice. So, there was no evidence observed due to extraction, especially body weight.

Question 3: At what rostrocaudal level was the block #11 located?
Answer: The block #11 was located approximately at bregma −5.51 mm. In this point the neuronal damages of the trigeminal mesencephalic nucleus (Vmes) happened more than the other part.

Question 4: Anti-IBA1 antibody can be seen in the Table 1, but I cannot find any descriptions related to IBA1 in the main text and the figures. Did you try to evaluate the activities of macrophage and/or microglia?
Answer: When we had started to plausible our hypothesis, we needed to see the microglial expression around the respected area. To observe the most impacted area we needed to see that data. We did that to set up our baseline study.

Question 5: Explain the Figure 1 procedure according to your research? Which part was the Vmes part and which part shows Ruffini nerve ending?
Answer: Figure 1(B) showed a retrograde projection from PDL to a Vmes neuron using FG. FG was not visible in the nucleus and it was observed in the cytoplasm. Figure 1 (C, D, E) showed an anterograde (AAV-GFP) injection result from Vmes to Ruffini nerve endings. In that figure we explained, from Vmes to PDL projection existences in the wild type mice case; which was unrevealed before our study.

Question 6: Is it difficult to do retrograde and anterograde staining in the same mouse?
Answer: It is difficult to keep mice alive after two surgeries at a time. We did not do it take the consideration of the mice health issues.

Question 7: How to find size of the Vmes? What's the difference between a neuron with and without the black hole?
Answer: After the FG staining in the Vmes, we measured the size of the Vmes. The procedure was used as follows, continuous Z slices were taken for a single cell and the one with the largest cross-sectional area (including nucleus) was taken. FG retrograde tracer stained in the cytoplasm region and black hole actually represent the nucleus of the mesencephalic nucleus (Vmes).

Question 8: In Figure 2, what does the difference in the size of Vmes between periodontal ligament (PDL) and masseter muscle (Mm) mean?
Answer: We observed 2 different size of Vmes neuron in our experiments (large size and small size). In the figure 2 we wanted to reveal which size of Vmes was projected to PDL. We divided the Vmes according to projection from Vmes to PDL and Vmes to Mm. We found Vmes projected to PDL was smaller than the Vmes to Mm. This is because group sensory nerve fibers (Aα nerve fibers) are the thickest type of nerve fibers, while nerve fibers derived from root membrane mechanoreceptors are thinner Aβ nerve fibers.

Question 9: Do you think that the difference in number of Piezo2-positive neurons between control and tooth-extracted animals shown in Figure 3D and E exclusively reflected death of neurons innervating the upper molars? How about other effects, for example, decreased activity of jaw-closing muscles due to the intake of powder diet?
Answer: Yes, as we clearly show the projection from Vmes to PDL in our research and tooth extraction induces the neuronal degradation. Tooth extraction is the one of vital cause of Vmes neuronal death within one-month after extraction in C57BL/6J mice case. There may be other reasons of this factor, that need to further research.

Question 10: What was the reason why you used only one section of the block #11 for ATF3 immunostaining? I guess that you stained all sections but showed the result obtained from the block #11 only in Figure 4. Why?
Answer: In our study, we founded ATF3 IR Vmes was very few in number. That's why among 11blocks we checked all section from # 9 block. The article showed similar parts of all case (5th, 10th, 15th). Anti-ATF3 IR Vmes was found in the section containing Vmes. We will try to show it in our next study.

Question 11: Could you detect immunoreactivities for ATF3 and/or those for cleaved caspase-3 in neurons of the trigeminal motor nucleus (Vmo)?
Answer: We also think the same, this part needs further investigation too, this the first step. In future we will try to do it. Thanks for the reminder.

Question 12: You used cleaved-caspase 3 as a marker of apoptosis. Generally, doesn't necrosis happen after neuronal degeneration?

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Does apoptosis happen in Vmes after the extraction?
Answer: I used cleaved caspase-3 as marker of cell death for Vmes neuron. There is some apoptotic effect of Vmes after the extractions. It is very obvious.

Question 13: You showed two patterns of TDP-43 distribution: cytoplasmic and neuronal cytoplasmic inclusions (NCIs) types of TDP-43. What is the functional difference between these two patterns? Did you find both types in the same mouse? Are there any difference of protein conformation or modification such as phosphorylation of TDP-43 in the two types?
Answer: In the retina, muscle and glial cells, the accumulation of cytoplasmic species of TDP-43 were observed in a few studies. In case of NCIs type we observe pathological type crescentic TDP43 around the nucleus. The functional difference between these 2 types still not clear. In our study after extraction the number of cytoplasmic types increased but decreased in 2month experimental cases. NCIs type increased in one month and 2 months both. After the tooth extraction, we observed cytoplasmic and NCIs type within one mouse.

Question 14: Did you try to stain TDP-43 in Vmes?
Answer: No, in our study we observed Vmo changes by the Anti-TDP-43 primary anybody. Based on previous study, we wanted to focus on revealing the motor degeneration after extractions.

Question 15: As you show in Figure 6, NCI of TDP-43 was observed even in control group. How do you think the reason of the phenomenon? Is it related to aging or something?
Answer: The main reasons of control group NCIs was suspicious too. Aging is the one of the candidates of the appearance of cytoplasmic type or NCIs type.

Question 16: I think that dual or triple immunostaining experiments using the combination among anti-Piezo2, anti-ATF3, and anti-cleaved caspase-3 antibodies could more clearly elucidate the correlation between the neurodegeneration of Vmes neurons and these molecules related to neuronal regeneration and apoptosis. Did you try to perform multiple immunostaining methods?
Answer: Yes, we performed multiple staining, but not with the above combination. We performed Piezo2-ATF3 and TDP43-ChAT staining. We make it as a precious note for future study. Thanks for your suggestion.

Question 17: Did you do any kind of research about masseter muscle after extraction? Like bite power test.
Answer: We did not conduct any test for masseter muscle, because our point of interest was to show the effect of extraction induced neuro-degenerated Vmes causes degeneration also in Vmo. We wanted to see the immediate effect after that.

Question 18: Why didn't you experiment with unilateral tooth extraction instead of bilateral tooth extraction?" Isn't it better to compare the healthy side and the affected side with unilateral tooth extraction? Or you did in your research?
Answer: Yes, we tried bilateral and unilateral extraction. In case of ipsilateral case there was no effect of non-extracted side, so we used bilateral extracted mice. To observe the size of the masseter muscle, FG was injected into the non-extracted side of the masseter muscle while to observe the Vmes-PDL projection we injected FG into the extracted socket of the other side of the same mouse.

Question 19: When you compared tooth-extracted mice with non-extracted mice, did you consider the possibility that the results might have been affected by malnutrition and disturbed chewing behaviors caused by tooth loss?
Answer: We also agreed this point. Actually, at 4–5days after the extraction, mice started to regain the body weight. However, in our study, we measured the weight and physical conditions of the extracted mouse at days after tooth extraction, we did not find significant differences. Therefore, in our study, there was no incidence of appetite loss.

Question 20: You showed Vmes projected to Vmo, also higher neuron projected to Vmo? How do you define that?
Answer: Vmo has the connection in the other part as well. In our study we did not observe that part or the follow up of the Vmo degeneration. Surely it has some impact on the other part, because neuron has the close connectivity.

Question 21: In the Discussion, you mean that Vmes neurons located at the block #12, may showed a functional shift. What kind of functional shift may occur in Vmes neurons? Do you assume the postsynaptic change at glutamatergic synapses as reported by the cited literatures?
Answer: Vmes neuronal death happen, maybe it is the primary neuron directly connected with PDL. Accordingly, Vmes functionally cannot be productive due the neuronal apoptosis. Due to reasons, we mention it there.

Question 22: Trigeminal ganglion (TG) neurons also innervate periodontal mechanoreceptors. Please briefly explain the difference in fate of neurons and/or glial cells after tooth extraction between the TG and the Vmes.
Answer: For the clarification of the tooth extraction procedure, we also stained trigeminal ganglion with ATF3 damaged marker. We found the damaged positive cell into TG as well. So, our study does support the TG and Vmes study. As well as we want to mention that due the total connection loss from PDL caused Vmes neuronal death.

Question 23: Explain the edentulous person Vmes situation, according to your research? Does gum part have Vmes projection?
Answer: Yes, according to the literature review of the previous research, we know that Vmes has the projection in gum part too. For the edentulous person that part still active, though the extraction causes Vmes neuronal death, but others cause may have accelerated this effect. Masticatory function is more important issue to provide good nutrition in the Vmes part.

Question 24: How did the dentist make a suggestion to the patient? Please explain based on your research. What dentist can do to prevent this degeneration after extraction?
Answer: Firstly, my suggestion to the patient is, maintain the number of teeth. It's very important for brain. After extraction I think it better to make the extracted part active. So, any kind of prosthesis like, implant of the tooth or crown bridge is more effective after the tooth extraction. Need to make masticatory function more active to keep the neuron healthy.

From the above results, the five reviewers confirm that the applicant possesses the academic skills and knowledge required to complete the doctoral course and is qualified to receive the degree of Doctor of Philosophy in Dental Science.