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ORIGINAL ARTICLE

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Effects of a liquid diet on the response properties of temporomandibular joint nociceptive neurons in the trigeminal subnucleus caudalis of growing rats

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Structured Abstract

Objective – To investigate whether low mechanical loading on the temporomandibular joint (TMJ) when ingesting a liquid diet affects the response properties of neurons in the trigeminal spinal tract subnucleus caudalis (Sp5C) in growing rats.

Materials and Methods – Shortly after weaning, 2-week-old male rats were fed chow pellets (control) or a liquid diet (experimental). Firing activities of single sensory units were recorded from the Sp5C at 4, 5, 7, and 9 weeks. Neurons were functionally classified by their responsiveness to TMJ stimuli. The responses of Class II and III neurons to TMJ stimuli were investigated.

Results – In both neuron classes, the firing threshold in the experimental group was significantly lower than in the control group at all time points, but remained static in the control group throughout the experimental period, whereas it peaked in the experimental group at 4 weeks, decreased at 5 weeks, and remained stable thereafter until 9 weeks. Similarly, the initial firing frequency was significantly higher in the experimental group than in the control group, but remained static in the control group throughout the experimental period, whereas in the experimental group, it was at its lowest at 4 weeks, increased at 5 weeks, and stayed stable thereafter until 9 weeks.

Conclusion – Differences in TMJ loading arising from variable diet consistency during growth may affect the functional characteristics of Sp5C neurons.

Key words: liquid diet; nociceptive neuron; rat; Sp5C; Temporomandibular joint



Introduction

The mammalian facial skeleton is optimized for countering or dissipating masticatory stress (1). The teeth, periodontal tissue and temporomandibular joint (TMJ) are subjected to loading during mastication, which in mammals is a complex, coordinated, and acquired behavior that is learned after birth (2, 3). Masticatory ontogeny has been divided into three stages: the fetal period, during which the masticatory organs move, but do not function; the infancy period, in which the animal feeds by sucking, while the structures and movements of relevant organs develop gradually; and the juvenile period, which occurs following weaning and is where adult feeding patterns are increasingly refined (3). The most important period in relation to an increase in loading borne by the TMJ is the juvenile period, because this period is associated with extensive changes in morphology. For example, the deciduous teeth exfoliate, the permanent teeth erupt, the jaws greatly increase in mass and muscle develops (3).

Many studies have sought to alter experimentally the force delivered to the TMJ. As forces exerted on the teeth are partially dissipated at the TMJ, models in which dietary consistency is modified have been frequently employed to achieve a decrease in joint loading in vivo. These studies have established that animals fed a liquid diet are characterized by significantly thinner condylar cartilage (4), a pronounced decrease in the overall width and length of the condylar surface (4), a decrease in the growth rate of the mandibular angle (5) and significant differences in the type and size of fibers in the masseter muscle (5). These results showed that physiological loading of the TMJ during development is important for promoting the growth of the mandibular condyle and for maintaining the normal function and structure of the TMJ and suggest indirectly that loads transmitted to the condyle may be reduced in rats fed with a liquid diet.

Ingestive behavior that results from continuous changes in the physical consistency of diet is related to the development and maturation of the peripheral nervous system. Sensory neurons that supply the TMJ are classified as either mechanoreceptive or nociceptive neurons. It has been shown that the response properties of TMJ-mechanoreceptive neurons change after feeding with a liquid diet (6). The nociceptive system provides warning signals to identify internal and external noxious stimuli and is a basic physiological defense mechanism. However, the functional characteristics of TMJ-nociceptive neurons under subphysiological loading are still unknown. The trigeminal spinal tract subnucleus caudalis (Sp5C) contains the cell bodies of secondary trigeminal neurons that transmit nociception. It was hypothesized that the response properties of Sp5C neurons in rats fed a liquid diet were different from those in their counterparts fed chow pellets. This study therefore investigated whether low mechanical loading on the TMJ when ingesting a liquid diet affects the response properties of TMJ-nociceptive neurons in Sp5C in growing rats.

Materials and methods

The experimental procedures described here were approved by the Animal Welfare Committee and performed in compliance with the Animal Care Standards of Tokyo Medical and Dental University (#0100204C, #0110197A, #0110337A).

Animal preparation

Fifty-six 2-week-old male Wistar albino rats were used. All infants were fed by their mother and were examined every 12 h to confirm weaning to prevent an experimental group from having any experience of chewing a solid diet. Soon after weaning, the rats were randomly divided into control and experimental groups (n = 28 in each group). The control group was fed chow pellets (CE-2, CLEA Inc, Tokyo, Japan), while the experimental group was fed a liquid diet consisting of CE-2 powder mixed with water in a blender at a ratio of 1:4 (w/v), dispensed through a graduated feeding tube (Dyets[®], Inc., Bethlehem, PA, USA). Food and water were freely accessible at all times. The body weights of rats in both groups were monitored throughout the entire experimental period to assess their general health status.

For electrophysiological recordings, all rats were lightly anesthetized with thiamylal sodium (Isozol[®], Yoshitomi Pharmaceutical, Osaka, Japan; 60 mg/kg) administered intraperitoneally (i.p.). The depth of anesthesia was monitored by checking pupil size, flexor and corneal reflexes, and heart rate. When a firm pinch applied to the tail resulted in increased respiratory and heart rate, a supplemental injection of 5 mg/kg i.p. of thiamylal sodium was administered.

After incision of the facial skin overlying the left TMJ, the posterior parts of the temporal and masseter muscles were detached to expose the left TMJ region. The left mandibular condyle and left TMJ capsule were thus made visible and accessible for both electrical and mechanical stimulation. A short-acting local anesthetic (10 μ l of 2% lidocaine; AstraZeneca Canada Inc., Mississauga, ON, Canada) was infiltrated subcutaneously prior to incision of the facial skin overlying the left TMJ (7).

The animals were then transferred in the prone position to a stereotaxic frame (models SN-2 and SM-15M, Narishige Scientific Instruments, Tokyo, Japan). The head was secured rigidly by a metal plate attached to the skull. To prevent movement of the mandibular condyle, the maxillary and mandibular incisors were fixed in the intercuspal position using dental resin (Fig. 1A).

Stimulation and recording

When the rats were 4, 5, 7, and 9 weeks old (n = 7 in each group), the firing activities of single sensory units were recorded from the left Sp5C, which contains the cell bodies of secondary trigeminal neurons that transmit nociception (8–11). To allow for placement of the recording electrode, a midline incision was first made in the scalp, and a small aperture, about 3.0 mm wide, was then made in the skull by means of a stereotaxic microengine. The brain surface was bathed in warm liquid paraffin. Through this aperture, monopolar tungsten microelectrodes (250- μ mdiameter shaft with an 8.0 degree tapered tip, AC impedance of 9.0 M Ω , A-M Systems Inc, Carlsborg, WA, USA) were inserted into the left Sp5C, 14–14.5 mm caudal to the bregma, 2–3 mm lateral to the midline, and 7.5–8.5 mm below the cortical surface, following the stereotaxic coordinates previously reported for the recording of activities of Sp5C neurons (7, 9, 12, 13).

First, electrical stimuli with constant-current single pulses (3 mA, 0.1 ms) were applied to the left TMJ capsule to determine whether the Sp5C neurons receive afferent inputs from the TMJ. If these units received afferent inputs from the TMJ, unitary responses to the electrical stimuli were recorded. Electrical stimuli of the left TMJ were delivered by a pair of stainless steel needles (1-mm interpolar distance) placed on the capsule of the left TMJ.

Second, mechanical stimuli were applied to the left TMJ capsule to examine the responsiveness of Sp5C neurons to various stimuli and therein to classify them according to their responses into Class I (excited only by innocuous stimuli), Class II (excited only by noxious stimuli), or Class III (excited by both types of stimuli) responders (14). It has been reported previously that a force level of <1 g is considered to be a tactile stimulus, 20-50 g is defined as a pressure stimulus and 100–500 g is a pinch stimulus (15). In our study, weak mechanical stimuli using a von Frey device (evaluator size: 4.31, target force: 2.0 g) were applied at two strokes per second with a reciprocating motion, and noxious mechanical stimuli using another von Frey device (evaluator size: 6.45, target force: 180 g). Class I neurons responded to 2.0 g but not to 180 g, whereas Class II neurons showed the inverse response. Class III neurons responded to both stimuli. Class II and III neurons are considered to receive nociceptive information from the TMJ, while Class I neurons function as mechanoreceptive neurons (14). Thus, this experiment focused on the functional



Fig. 1. Experimental design. (A) Schematic drawing of the experimental setting. The head of the rat was stabilized in a stereotaxic apparatus. A small aperture, *ca.* 3.0 mm wide, was prepared in the skull, and monopolar tungsten microelectrodes were inserted into the trigeminal spinal tract subnucleus caudalis (Sp5C). Mechanical stimulation was applied to the left temporomandibular joint (TMJ) with a von Frey device attached to a force transducer. (B) Histological identification of the electrode position based on electrolytic markings and signs of electrode penetration. Asterisks indicate a representation of the Sp5C drawn from a frontal section of the brain 14.04 mm below the bregma. (C) Data analysis. The firing threshold (a) was defined as the magnitude (g) of mechanical stimulation when the first spike response occurred. The initial firing frequency (b) was defined as the firing frequency (Hz) when the mechanical stimulation reached 100 g.

characteristics of Class II and III neurons to investigate the response properties of TMJ-nociceptive neurons in the Sp5C.

Finally, mechanical stimuli were applied to the left TMJ to investigate the response properties of Sp5C neurons to noxious stimuli. Mechanical stimuli were applied to the left TMJ with a von Frey device (evaluator size: 6.10, target force: 100 g) attached to a force transducer and an amplifier (TBM 4 and FORT 250, World Precision Instruments, Sarasota, FL, USA). Pressure stimuli (*ca.* 100 g) were applied manually in a ramp-and-hold fashion by pushing with a force transducer. The stimuli in this study were always applied in a direction perpendicular to the sagittal plane (16).

Spike signals were recorded and amplified by a differential amplifier (DAM-80, WPI, Sarasota, FL; 1000 \times gain, 300 Hz and 3.0 KHz for low and high filters, respectively). All data were cap-

tured by means of a CED 1401 interface and stored on a computer hard disk. The data were later analyzed offline with Spike2 software for Windows, Version 4.02a (Cambridge Electronic Design, Cambridge, UK).

Histological identification of the electrode position

The electrode position was marked using 10 μ A negative current for 10 s at the end of each recording track (17). At the end of each experiment, rats were euthanized by an overdose of thiamylal sodium (120 mg/kg i.p), and their brains removed. Brains were embedded in paraffin, cut into 5 μ m sections, and stained with crystal violet, so that we could histologically identify the tip of the electrode position based on the electrolytic markings and signs of electrode penetration (Fig. 1B).

Data analysis

The effects of low masticatory loading under feeding with a liquid diet on Sp5C neurons were assessed using two response properties (Fig. 1C): the firing threshold and the initial firing frequency. The firing threshold was calculated as the magnitude of mechanical stimulation required to evoke the first spike response, or a firing rate of greater than two standard deviations above baseline neuronal activity. The initial firing frequency was calculated as the number of spikes in the previous period set by bins (1 bin = 0.1 s) when the pressure reached 100 g.

Statistical analysis

All data are expressed as the mean \pm SD. All data were compatible with a normal distribution, confirmed using an *F*-test (F < F_{.05} = 4.28). All data were compared using generalized linear models with running multiple comparisons using the Sidak correction test. All statistical analysis was performed at a 5% significance level (*p* < 0.05) using SPSS for Windows (version 16.0J) software (SPSS Inc., Chicago, IL, USA).

Results

All infants were weaned about at 3 weeks and were randomly divided into two groups. The mean body weights (g) of the rats in the control and experimental groups at 4, 5, 7, and 9 weeks are shown in Fig. 2. Mean body weight in the control and experimental groups increased continuously throughout the experimental period, and there were no significant differences between rats of the same age in the two groups.

From the 56 rats (n = 7 in each group) used in this experiment, 344 neurons were recorded from the left Sp5C. When stimuli were applied to the TMJ, the 344 neurons could be assigned into one of the three classes according to their responsiveness. The numbers of Sp5C neurons in each group are summarized in Table 1. In Class II, a single neuron in each rat was recorded in the control and experimental groups



Fig. 2. Mean body weights in the control and experimental groups. There were no significant differences between groups at each age. Error bars indicate standard deviation (SD).

Table 1. Number of classified neurons in the trigeminal spinal tract subnucleus caudalis (Sp5C) in the control and experimental groups at each age according to their responses to stimuli applied to the temporomandibular joint (TMJ)

	Control group			Experimental group		
	Class I	Class II	Class III	Class I	Class II	Class III
4 weeks	13	7	22	15	7	20
5 weeks	12	7	22	14	7	25
7 weeks	14	7	23	14	7	24
9 weeks	14	7	21	15	7	20

at each age, thus all neurons in this class were analyzed. In Class III, 3–4 neurons per rat were recorded in the control and experimental groups at each age; therefore, a neuron that was first recorded from each rat was investigated.

Most single sensory units were subjected to three trials of ramp-and-hold stimulation applied to the left TMJ. Typical examples of responses from the Class II neurons recorded from the left Sp5C at 9 weeks in both groups are shown in Fig. 3. In the experimental group at 9 weeks, the firing threshold was significantly lower than that in the control group, whereas the initial firing frequency was significantly higher than in the control group.

In both the control and experimental groups, the firing threshold in the Class II neurons was significantly higher than in the Class III neurons at each recording age. In both the Class II and III neurons, the firing threshold in the experimental group was significantly lower than that in the control group at each recording age



Fig. 3. Typical examples of responses from Class II neurons recorded from 9-week-old animals in the control (A) and experimental (B) groups. Note the lower firing threshold and higher initial firing frequency in the experimental group at 9 weeks compared with the control group.

(Fig. 4Aa, Ab). Also, this parameter in the control group remained static throughout the study period, whereas that in the experimental group peaked at 4 weeks, decreased at 5 weeks, and remained relatively stable thereafter until 9 weeks (Fig. 4Aa, Ab).

In both the control and experimental groups, there were no significant differences between the initial firing frequency in the Class II and III neurons at each recording age. In both the Class II and III neurons, the initial firing frequency was significantly higher in the experimental group than in the control group at each recording age (Fig. 4Ba, Bb). Also, this parameter in the control group remained static throughout the experimental period, whereas that in the experimental group was at its lowest at 4 weeks, increased until 5 weeks, and stayed relatively stable thereafter until 9 weeks (Fig. 4Ba, Bb).The percentage decrease in the average number of spikes during a 1 s period immediately before (N_{before}), and after (N_{after}) the cessation of pressure application was calculated in the control and experimental groups. This rate of decline in the firing frequency was calculated as (N_{before}- N_{after} / N_{before} × 100 (%). In both the Class II and III neurons, the rate of decline in the firing frequency was significantly lower in the experimental group than in the control group at each recording age (Fig. 4Ca, Cb). Also, this parameter remained static in the control group throughout the study period but, in the experimental group,

peaked at 4 weeks before declining and then stabilizing at later time points (Fig. 4Ca, Cb).

Discussion

With regard to trigeminal nerves, it has been shown that both TMJ (6) and periodontal-mechanoreceptive neurons (18) mature by 5 weeks. The nociceptive system provides warning signals to identify internal and external potentially noxious stimuli and is a basic physiological defense mechanism. However, the mechanisms by which TMJ-nociceptive neurons develop and mature are still unclear. In rats, A-fiber afferents penetrate the spinal dorsal horn at embryonic day 17 and are followed by C-fiber afferents at embryonic day 20 (19). In humans, sensory fibers are present by 9 weeks of gestation, spinal dorsal horn neurons are present by 13 weeks and synaptic connections between these fibers can be observed by 19 weeks (20). During development, both A- and C-fiber afferents form synaptic connections at the spinal dorsal horn, and neuronal networks mature (19). Even though the trigeminal sensory systems are known to be functionally complex compared with the spinal system, description of the central mechanisms of trigeminal pain has often relied on analogies to condiwithin the spinal cord. tions Structural homology between the Sp5C and the dorsal horn of the spinal cord has been reported (21, 22). In



Fig. 4. The firing threshold (A), the initial firing frequency (B) and the rate of decline in the firing frequency (C) of temporomandibular joint (TMJ)-nociceptive neurons (a, Class II; b, Class III) in the control and experimental groups (7 units per group). Error bars indicate SD. * denotes significant (p < 0.05) differences between the experimental and control groups within the same classification of neurons at each age. [#]indicates significant (p < 0.05) differences between the Class II and Class III neurons of the same age.

this study, both the firing threshold and the initial firing frequency in the control group remained static throughout the experimental period, as they did also in the experimental group after 5 weeks. These findings indicate that the functional properties of Sp5C neurons mature by 5 weeks, when functional molar occlusion and a change in the dietary contents from liquid to hard diet can be achieved. In addition, the firing threshold in the experimental group was significantly lower, and the initial firing frequency significantly higher, than in the control group at each age. In this study, all of the significant changes were found at the earliest time point (4 weeks), and all values stayed relatively stable after 5 weeks. Rats are generally weaned at 3 weeks, and thus the observed changes may initialize within a week and be complete in 2 weeks. This interesting finding suggests that this 2-week period could be critical for the maturation of the functional characteristics of Sp5C neurons.

Previously, it has been reported that a prolonged period of feeding with a liquid diet after weaning impedes functional development of mastication and leads to immature mastication in growing rats (23). In a previous report describing, the effects of a liquid diet on TMJ- mechanoreceptive neurons, the firing threshold and the maximum instantaneous frequency were significantly lower and higher, respectively, in the experimental group than in the control group at each recording age (6). These results are similar to the changes in response properties seen in TMJ-nociceptive neurons under low mechanical loading of the TMJ by the liquid diet. This suggests that changing the consistency of food to a liquid diet impedes the masticatory environment during the critical period of masticatory learning and impedes the development of mature mastication. As a result, the Sp5C neurons in the experimental group have become hypersensitive due to under-stimulation.

According to the neural basis of the Weber– Fechner law, the processes between a stimulus and the subjective response to it consist of both logarithmic and linear steps. In this sense, there are two factors: a phasic factor that decreases with time and a tonic factor that persists during stimuli (24). The changes in the firing threshold and the firing frequency may reflect the tonic factor, while the rate of decline in the firing frequency may reflect the phasic factor. Therefore, low mechanical loading during development due to the administration of a liquid diet affects both the tonic and phasic factors (i.e., affects both the logarithmic and linear steps) and may affect adaptation by the peripheral stimulation of Sp5C neurons.

Conclusions

Differences in mechanical loading of the TMJ through differences in diet consistency during

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growth may affect the functional characteristics of the neurons in the Sp5C. Therefore, ingestive behavior that results from continuous changes in the physical consistency of the diet appears to be related to the functional maturation of Sp5C neurons.

Clinical relevance

Mastication is controlled by central pattern generators whose activities are modified by sensory feedback. Changes in dietary consistency lead to incomplete development of peripheral nervous system and thus alter masticatory pattern. In this experiment, all significant changes occurred at 4 weeks and stayed stable after 5 weeks, highlighting a critical period for the functional maturation of TMJ-nociceptive neurons in rats. This period is equivalent to the juvenile period in humans, when molar occlusion and a change in dietary contents from liquid to hard can be achieved. Therefore, the dietary environment during growth may be important in acquisition of masticatory function.

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