An Operant Conditioning Model to Assess Changes in Feeding Behavior Associated with Temporomandibular Joint Inflammation in the Rat

Paul D. Thut, PhD
Professor Emeritus
Department of Biomedical Sciences
Dental School

Tracey O. Hermanstyne, BS
Graduate Student
Neuroscience Program
Department of Biomedical Sciences
Dental School

Natasha M. Flake, DDS, PhD
Department of Biomedical Sciences
Dental School
University of Maryland, Baltimore
Baltimore, Maryland

Michael S. Gold, PhD
Associate Professor
Department of Medicine
University of Pittsburgh
Pittsburgh, Pennsylvania

Correspondence to:
Dr Michael Gold
University of Pittsburgh
Department of Medicine
3500 Terrace Street
Room E1440 BST
Pittsburgh, PA 15261
Fax: + 412 383 8663
E-mail: goldm@dom.pitt.edu

Aims: *To develop and validate a model in which to assess a loss of function associated with temporomandibular joint (TMJ) inflammation in awake, freely moving rats.* ***Methods:*** *The dependent variable in the model was the time between food rewards (pellets), or interfeeding interval (IFI). IFI was quantified after rats were trained to "bar-press" for food. To validate use of the IFI as a surrogate for temporomandibular disorder (TMD) pain, we determined the impact of several manipulations, including changes in pellet size, the presence and severity of inflammation of the TMJ, masseter muscle, or skin (induced with complete Freund's adjuvant [CFA]), and the influence of preadministration of the nonsteroidal anti-inflammatory drug indomethacin (4 mg/kg). Furthermore, in order to determine whether a change in IFI reflected an increase in the time rats spent eating, rats were videotaped, and the amount of time spent eating, grooming, and exploring was analyzed.* ***Results:*** *Inflammation of the TMJ or masseter muscle resulted in significant dose- and pellet size–dependent increases in the IFI. Inflammation of the skin overlying the TMJ had no effect on IFI. Pre-administration of indomethacin reversed the inflammation-induced shift in the IFI. An inflammation-induced increase in IFI was associated with an increase in feeding time.* ***Conclusions:*** *Our model constitutes a relatively fast and sensitive method with which to assess changes in feeding behavior associated with TMJ inflammation. Only 2 days of training are required to obtain a stable baseline IFI. It is possible to detect changes in IFI as small as 40% with 12 rats per group.* J OROFAC PAIN 2007;21:7–18

Key words: inflammation, nociception, pain, temporomandibular joint disorders

Temporomandibular disorders (TMD) are associated with chronic and debilitating pain.[1,2] Clinical evidence suggests that TMD pain may reflect a number of underlying problems, including trauma to the temporomandibular joint (TMJ) and/or related structures,[2,3] parafunctional habits such as clenching or bruxism,[4] psychosocial factors such as stress,[5] systemic disorders such as lupus,[2] and occlusal disharmony[2] (but see Gesch et al[6]). However, while it is appreciated that a number of factors may contribute to TMD pain, our mechanistic understanding of this syndrome, and consequently, the ability to effectively treat the syndrome, remains extremely limited.[2,3]

A number of animal models of TMD pain have been developed. However, each is associated with significant limitations. Ren and colleagues described 2 behavioral assays to assess the impact of

TMJ inflammation in rats.[7,8] Changes in mechanical sensitivity were assessed by measuring withdrawal thresholds in response to von Frey filaments applied to the skin overlying the TMJ in unrestrained rats.[7] Changes in thermal sensitivity were assessed by measuring withdrawal latencies in response to a noxious thermal stimulus applied to the skin overlying the TMJ in lightly anesthetized rats.[8] Both assays were relatively sensitive, revealing large drops in mechanical and thermal thresholds in the presence of inflammation. However, both assays involve stimulation of tissue remote from that directly influenced by injury or inflammation (ie, the TMJ or masseter) and therefore are likely to reflect changes in central nervous system processing (ie, allodynia and secondary hyperalgesia) rather than changes in the affected tissue. Another indirect assay of TMJ sensitivity was developed by Schutz and colleagues, who assessed alterations in the sleep patterns in rats with inflammation of the TMJ induced by complete Freund's adjuvant (CFA).[9,10]

Roveroni and colleagues employed a model in which oral motor activity and/or facially directed motor activity were assessed in response to formalin injection into the TMJ.[11,12] Similar models utilizing mustard oil[13,14] or hypertonic saline[14] as noxious stimuli have also been employed. Although these assays should enable assessment of ongoing nociceptor activity, alone, they do not yield information about the changes in sensitivity associated with oral motor behaviors that can be a debilitating component of TMD pain observed in humans.

Ro recently developed a use-dependent assay of oral-motor function, in which rats are trained to bite a force transducer for a reward.[15] Inflammation of the masseter muscle resulted in a significant reduction in bite force and successful attempts to generate a threshold force. While this model enables an assessment of the impact of inflammation on oral motor function, training rats to successfully perform in this assay takes considerable time and effort.

Meal pattern analysis is another use-dependent assay of TMJ function developed by Bellinger and colleagues.[16–19] With this approach, feeding behavior was analyzed over a 24-hour period. Results from this analysis indicated that TMJ inflammation was associated with dramatic alterations in feeding behavior,[19] the most specific of which was an increase in meal duration.[16] While this is clearly a powerful approach for assessing the impact of inflammation on a behaviorally relevant endpoint, the 24-hour observation period may preclude the assessment of short-acting therapeutic interventions that may ultimately be useful in humans for the treatment of TMD pain.

The present study was performed in order to determine whether it would be possible to adapt the Bellinger model in an operant conditioning paradigm to both increase assay sensitivity and decrease the amount of time feeding behavior must be monitored. The specific aim was to develop and validate a model in which to assess a loss of function associated with TMJ inflammation in awake, freely moving rats. Preliminary results of this study were presented in abstract form.[20]

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 150 to 175 g at the start of the experiments were used in all experiments. Rats were housed in groups of 2 in the University of Maryland–Baltimore Dental School animal facility on a 12:12 reverse light cycle. All experiments were approved by the University of Maryland Dental School Institutional Animal Care and Use Committee and performed in accordance with NIH guidelines as well as guidelines established by the International Association for the Study of Pain for the use of laboratory animals in research.

Behavioral Methods

Shaping and Training. A typical experiment was run as follows. Rats were received from the supplier Tuesday morning of week 1, and were placed in the vivarium with only water available ad libitum. On Wednesday through Friday of week 1, in 1-hour sessions, rats were easily shaped and trained to press a bar for 45-mg food-pellet rewards on a fixed ratio schedule of 4 lever presses being required before a food reward was delivered (FR4). After the training session on Friday, rats were placed in their home cage with food available ad libitum; food was available ad libitum until Sunday morning. Rats received another training session on Monday of week 2; this final 1-hour FR4 training session was administered using 45-, 97-, or 190-mg pellets, depending on the size of the pellets to be used in the test sessions. Baseline data were acquired on Tuesday, and experimental data were collected on Wednesday. Rats were then deeply anesthetized prior to performing the Evans blue plasma extravasation (PE) assay (see the fol-

lowing section) in order to obtain an independent measure of the extent of tissue inflammation. Forty-five mg pellets were used during training and shaping sessions to reduce the possibility that the animals would become satiated and stop responding. Data collection occurred at the same time of day for each group of animals; 2 groups of animals were run between 10 am and 12:30 pm. Times of individual rewards were digitally recorded, and the times between each feeding event were calculated and recorded as the interfeeding interval (IFI). The modular test chambers (ENV-008), sound attenuating environmental chambers (ENV-022M), pellet dispensers (ENV-203-97IR), response levers (ENV-110M), and computer interface hardware (SG-6080 and DIG-716P1) and software (MEDState Notation) were purchased from MED Associates. Pilot studies revealed that the FR4 schedule ensured that animals responded purposefully for rewards and consumed them immediately with little or no spillage. Lower fixed ratios resulted in very irregular IFIs and as many as 50 pellets uneaten at the end of a 1-hour session. FR4 is not a stressfully high rate requiring stamina nor is any timing or cue recognition required by the animal which might be influenced by analgesic drugs. The feeding behavior could be maintained on this moderate response rate over a 1-hour experimental session, which was long enough to study the effects of both short- and long-duration drugs on the inflammation-induced behavioral decrements observed.

Because animals had access to food ad libitum between Friday afternoon and Sunday morning, it was not necessary to supplement food intake to maintain body weight within 10% of that expected of age-matched controls. Water was always available, ad libitum, in the home cages.

Inflammation-induced Changes in Feeding Behavior. The session on Tuesday of week 2 was used as the baseline session. Immediately following this session, tissue was inflamed, sham-injected with saline, or left untreated. The 1-hour session on Wednesday of week 2 was the test session. It was expected that inflammation of the TMJ and/or muscles of mastication would cause rats to feed more slowly than saline-treated or untreated control rats, thus increasing the IFIs. Postinflammation IFIs were also obtained for comparison of pre- and postinflammation data.

Tissue Inflammation. Inflammation of the TMJ was performed as previously described.[21] Rats were anesthetized with 55 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine. The skin overlying the TMJ was shaved, and the injection target was identified by palpating the zygomatic arch and mandible. A 25-gauge needle was inserted just inferior to the zygoma until it hit the ramus of the mandible; the needle was then turned superiorly until it was stopped by the glenoid fossa of the squamosal bone. Inflammation was induced by injecting CFA (50 µL; 1:1 in physiologic saline; Sigma). Noninflamed rats received vehicle injections (50-µL physiologic saline). Inflammation of the masseter was induced in a similar fashion, except that CFA was injected over 6 sites along the length of the muscle and over the TMJ. Inflammation of the skin overlying the TMJ also was induced in a similar fashion, except that the needle was directed into the skin overlaying the joint. In some experiments, both higher and lower concentrations of CFA were used. Bilateral injections were employed where indicated. Following injection, animals were monitored while recovering from anesthesia and kept warm with a heating pad set to 37°C.

Indomethacin (Sigma), a nonsteroidal anti-inflammatory drug (NSAID), was used in a group of rats to assess the impact an NSAID had on inflammation-induced changes in feeding behavior. Indomethacin was dissolved in physiologic saline containing 1% sodium carbonate ($NaCO_3$) at a concentration of 4 mg/mL. Rats received an injection of either 1 mL/kg of indomethacin (ie, 4 mg/kg intraperitoneally) or vehicle intraperitoneally. This concentration of indomethacin was chosen based on results of previously published studies (eg, Zhang et al[22]).

Evans Blue PE Assay. Immediately following the test session, rats were anesthetized using the aforementioned anesthetic combination. Evans blue (50 mg/mL in saline) was injected into the femoral vein. Ten minutes later, rats were perfused transcardially with ~200 mL of phosphate buffered saline, and TMJ, muscle, and skin were collected. Skin overlying the TMJ was collected first (~8 mm^2), then muscle overlying the TMJ, leaving primarily the articulation (including portions of the squamosal bone, zygomatic process, and condyle) and immediately surrounding tissues. Finally, the TMJ was dissected to a standardized size, such that the dissected joint measured 8 mm^2 when positioned with the zygomatic process facing upward. Left and right TMJs were placed in a 15 mL tube containing 2 mL of dimethylsulfoxide. Evans blue was extracted from tissue over 3 to 4 days at room temperature on a rocking platform. Aliquots of the solution (1 mL) were then spun at 10,000 rpm for 20 minutes. Evans blue concentration was assessed by spectrophotometry at 620

Table 1 Experimental Groups

Experiment/brief description	Group 1	2	3	4	5	6
1 Assessment of effect of pellet size on IFI	45-mg pellet TMJ saline (n = 12)	45-mg pellet TMJ CFA (n = 12)	97-mg pellet TMJ saline (n = 6)	97-mg pellet TMJ CFA (n = 6)	190-mg pellet TMJ CFA (n = 6)	
2 Real-time analysis of rat behavior in the feeding chambers	Unilateral TMJ injection of CFA (50 µg/50 µL, n = 4)	Unilateral TMJ injection of saline (50 µg/50 µL, n = 4)				
3 Assessment of the magnitude of inflammation on changes in feeding behavior	Intact (n = 5)	TMJ saline (n = 6)	TMJ CFA (50 µg; n = 5)	TMJ CFA (25 µg; n = 6)	TMJ CFA (12.5 µg; n = 3)	TMJ CFA (6.3 µg; n = 3)
4 Assessment of the impact of the site of CFA injection (50 µg/ 50 µL) on changes in feeding behavior	Unilateral skin (n = 6)	Bilateral skin (n = 6)	Unilateral muscle (n = 6)	Bilateral muscle (n = 8)	Bilateral TMJ (n = 6)	Unilateral TMJ (n=6)
5 Assessment of the impact of indomethacin on changes in feeding behavior associated with a bilateral TMJ injection of CFA (50 µg/ 50 µL)	Injection of indomethacin (4 mg/kg intraperitoneally; n = 8) 30 minutes prior to start of feeding session	Injection of indomethacin vehicle (1% $NaCO_3$, n = 8) 30 minutes prior to start of feeding session				

nm, a wavelength at which absorbance is linearly related to dye and albumin concentration.[23] The amount of Evans blue dye extracted per joint was calculated based on a standard curve. Tissue was dried at 67°C. The magnitude of PE was quantified as µg Evans blue/g dry weight of tissue.

Data Analysis. In preliminary studies, a number of different parameters were assessed in order to identify the most sensitive measure with which to assess changes in feeding behavior. These included *(1)* the amount of food consumed, *(2)* meal duration, *(3)* time to which 50% of total pellets were consumed, *(4)* the slope of the cumulative frequency plot determined by fitting data with a logistic equation, and *(5)* the median feeding interval derived from the distribution of IFIs. The median feeding interval was associated with the smallest variance and therefore proved to be the most sensitive measure with which to assess inflammation-induced changes in feeding behavior. As shown in Fig 1, it was possible to generate a frequency distribution of feeding behavior with IFIs binned at 5-second intervals, normalized with respect to the total number of pellets consumed and then pooled. SigmaPlot (V. 8.02, SPSS software) was used to fit CFA dose-response data with a modified Hill equation: IFI difference (or Evans blue/joint) = $[MAX_{effect}/(dose + EC_{50})]^n$ where MAX_{effect} = maximal increase in IFI difference (or Evans blue/joint); dose = µg of CFA; EC_{50} = Dose producing a half maximal effect; n = Hill coefficient.

Groups. Twenty groups of rats were studied in 5 different experiments. These experiments are summarized in Table 1.

Statistical Analysis. Pooled median IFIs both before and after inflammation for each group of animals were both normally distributed and of equal variance. Consequently, parametric statistics such as the Student's *t* test or a 1-way analysis of variance (ANOVA) were used to assess differences between groups. A Tukey post-hoc test was used if a significant main effect was revealed with a 1-way ANOVA. Paired *t* tests were used to assess within animal changes from baseline. Data were analyzed as a mean of median IFIs or as a mean IFI-difference score (ie, IFI difference = IFI after manipulation – IFI before manipulation). Evans blue data were not normally distributed. Consequently, nonparametric statistics such as the Mann-Whitney test or ANOVA on ranks were used to assess differences between groups. A Dunn's post-hoc test was used if a significant main effect was revealed with a 1-way ANOVA on ranks. Statistical tests were performed with SigmaStat (v 3.0, Systat Software).

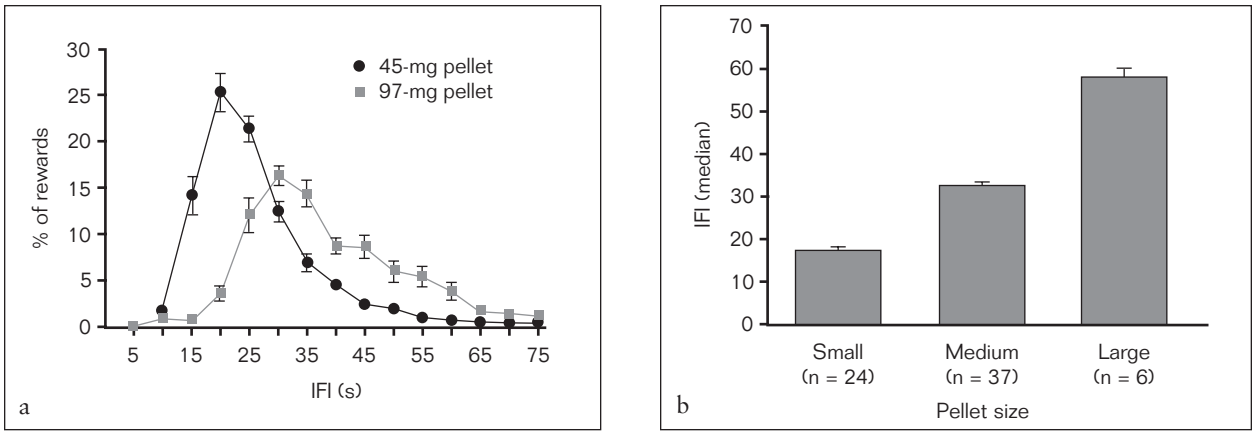

Fig 1 *(a)* Histogram of IFI normalized as a percentage of total rewards (pellets) consumed. Rats were fed either 45- or 97-mg pellets. The IFI (ie, time between the delivery of each pellet) was determined for each pellet consumed over the entire feeding period (1 hour). A 5-second bin was used to generate a histogram showing IFIs from 5 to 75 seconds. The histogram for each rat was normalized with respect to the total number of pellets consumed, so that data from each rat in the group could be pooled and plotted as a mean (±SEM). *(b)* Shift in IFI with increasing pellet size. The median IFI for each rat is shown according to pellet size. The labels *small, medium,* and *large* refer to pellet sizes of 45 mg, 97 mg, and 190 mg, respectively. The number of rats in each group is shown.

Results

Increasing Pellet Size Increased IFI

IFI increased as the size of the pellet presented increased from 45 to 97 to 190 mg. In Fig 1a, baseline IFIs of rats responding for 45-mg pellets are compared to IFIs of rats responding for 97-mg pellets. The median IFI shifted to the right with the increase in pellet size. Pooled data illustrate the increase in IFI associated with the increase in pellet size for all 3 sizes of pellets (Fig 1b).

There was no significant weight loss between baseline and test days in rats receiving any of the pellet sizes (Table 2). There also was no significant weight loss in any group of rats over the entire training and testing procedure. The number of pellets consumed on baseline and test days in rats treated with saline was not significantly different. Furthermore, although the number of pellets consumed per session was reduced as the pellet size was increased, the total weight of food consumed remained constant and independent of the pellet size.

TMJ Inflammation Induced an Increase in IFI

Twenty-four hours after CFA injection into the TMJ, the IFI was increased (Fig 2). While saline-injected rats displayed no change in IFI, all groups of CFA-injected rats, no matter what pellet size they were fed, displayed a significant increase in IFI compared to baseline ($P < .05$, paired t test). However, the inflammation-induced increase in IFI was dependent on pellet size such that the IFI differences observed in rats receiving 97- and 190-mg pellets were significantly ($P < .01$, 1-way ANOVA) greater than the IFI difference observed in rats receiving 45-g pellets (Fig 2). Of note, there was no difference in total number of pellets consumed on the test day between TMJ-inflamed rats and noninflamed control rats (Table 2). Further, there was no difference in weight change of control and inflamed rats (Table 2). Based on the results of this experiment, 97-mg pellets were used for all subsequent experiments.

Inflammation-induced Increase in IFI Associated with an Increase in Feeding Time

While it was hypothesized that the inflammation-induced increase in IFI would be associated with an increase in feeding time, it is possible that TMJ inflammation resulted in a change in the pattern of food consumption such that pellets were consumed at the same rate but that more time was spent on behaviors such as grooming, exploring, or sleep-

Table 2 Feeding Statistics

Injection	Site	Pellet size (mg)	n	Rat weight (g) BL	Rat weight (g) Test	Pellets consumed BL	Pellets consumed Test
CFA - Uni	TMJ	45	24	193 ± 4.4	188 ± 4.2	140.8 ± 5.9	143.3 ± 6.4
CFA - Uni	TMJ	97	10	186 ± 3.5	183 ± 3.1	73.0 ± 4.1	70.4 ± 6.3
CFA - Uni	TMJ	190	6	145 ± 3.0	144 ± 2.7	37.0 ± 3.23	38.4 ± 1.6
CFA - Bi	TMJ	97	6	186 ± 4.4	180 ± 5.6	74.8 ± 4.3	69.3 ± 1.2
CFA - Uni	Musc	97	6	176 ± 3.1	177 ± 3.1	72.8 ± 5.6	64.5 ± 3.1
CFA - Bi	Musc	97	8	175 ± 1.3	173 ± 1.0	68.8 ± 7.6	70.8 ± 1.5
CFA - Uni	Skin	97	6	183 ± 4.3	178 ± 4.2	77.0 ± 5.7	80.2 ± 6.2
CFA - Bi	Skin	97	6	183 ± 4.0	181 ± 3.3	75.2 ± 4.2	72.5 ± 3.4
CFA - Bi NSAID	TMJ	97	8	174 ± 4.7	173 ± 5.1	77.9 ± 3.9	85.0 ± 3.5
CFA - Bi $NaCO_3$	TMJ	97	8	174 ± 5.0	175 ± 4.5	73 ± 0.8	64.6 ± 3.5
Sal - Bi	TMJ	97	6	177 ± 2.5	172 ± 3.7	71.6 ± 1.6	86.4 ± 2.1
None	-	97	5	162 ± 1.2	163 ± 2.2	78.7 ± 2.9	87.7 ± 4.4

Injections were either CFA or sterile physiologic saline (Sal) in a volume of 50 mL administered either unilaterally (Uni) or bilaterally (Bi). The site of injection was either the TMJ, the masseter muscle (musc), or the skin overlying the TMJ (skin). One group of rats (CFA-Bi-NSAID) received an intraperitoneal injection of indomethacin 4 mg/kg 30 minutes prior to the assessment of feeding behavior. Another group of rats (CFA-Bi-$NaCO_3$) received an intraperitoneal injection of the indomethacin vehicle (1% $NaCO_3$) 30 minutes prior to testing. BL = baseline. Data are presented as mean ± SEM.

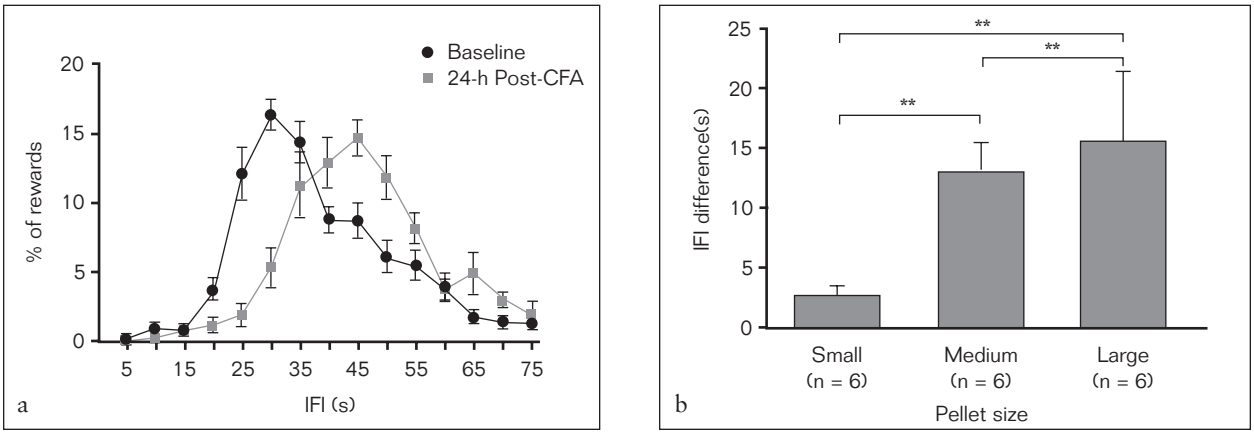

Fig 2 *(a)* Frequency plot of IFI versus percent of total pellets consumed before and after inflammation. Data are for a 97-mg pellet (medium). *(b)* The inflammation-induced shift in IFI was calculated for each rat as the difference between median baseline IFI and the median inflammation IFI. Inflammation was induced with a unilateral injection of CFA into the TMJ. Pooled data (mean ± SEM) are plotted against pellet size. ** Indicates significant differences between groups with $P < .01$ (1-way ANOVA followed by Tukey post-hoc tests).

ing. In order to explore this possibility, 2 groups of 4 rats, TMJ inflamed and saline control, were videotaped on baseline and test days. An observer blinded to experimental manipulations scored the amount of time rats spent in feeding associated behaviors (bar pressing and feeding) compared to behaviors unrelated to feeding (grooming, exploring, sleeping). The total amount of feeding time was determined, and data were analyzed as a percentage of change in feeding behavior (ie, % change in feeding time = [test day feeding time – baseline day feeding time] / baseline day feeding time). As shown in Fig 3, TMJ inflammation was associated with a significant increase in feeding time ($P < .01$, paired t test), while no change was observed in saline-treated controls. This increase in feeding time was associated with a compensatory decrease in other behaviors.

Increasing the Concentration of Inflammatory Agent Increased Both Inflammation and IFI

Since there is evidence of a positive correlation between the magnitude of the inflammatory response and the degree of hyperalgesia associated with CFA-induced inflammation of the rat hindpaw, it was predicted that the same relationship would exist for CFA-induced inflammation of the TMJ and the shift in IFI. In order to test this prediction, 6 groups of rats were studied: Intact, saline

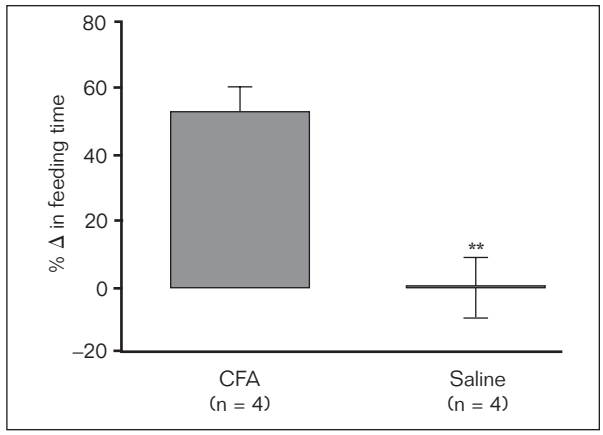

Fig 3 Rat behavior (grooming, exploring, sleeping, feeding) was quantified during baseline and test-day feeding sessions. Data were analyzed as a percent change in the amount of time spent feeding on the test day relative to the baseline day (% Δ in feeding time). Two groups of rats were studied: 1 received a unilateral injection of CFA into the TMJ, and the second received a unilateral injection of physiologic saline. ** $P < .01$ (Student t test).

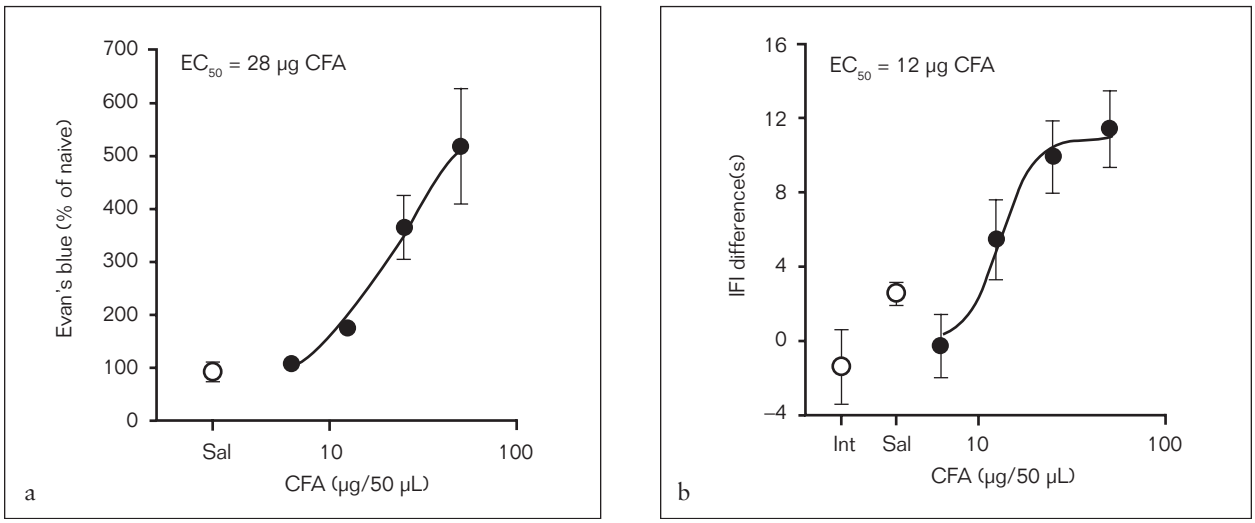

Fig 4 *(a)* 50 µL of CFA was injected unilaterally into the TMJ. Increasing the concentration of CFA increased the magnitude of inflammation as assessed with the Evans blue PE assay. Data plotted are the amount of Evans blue extracted per joint 24 hours after injection versus the amount of CFA injected. Data from saline (Sal) -injected rats are shown for comparison; each data point represents data for 3 to 6 rats. Data were fitted with a modified Hill equation, yielding an EC_{50} of 28 µg CFA. *(b)* An increase in the magnitude of TMJ inflammation resulted in an increase in the IFI difference. Data were analyzed to obtain a median IFI difference score for each rat. Pooled data are plotted as means ± SEMs. Pooled data were fitted with a modified Hill equation, yielding an EC_{50} of 12 µg CFA. Data from intact (Int) and saline (Sal) -injected rats are shown for comparison; number of rats is 5 to 6 per data point.

injected, and CFA injected with either 50, 25, 12.6, or 6.3 µg/50 µL. At the end of the test day, the degree of inflammation was independently assessed with the Evans blue PE assay. As shown in Fig 4a, CFA induced a dose-dependent increase in PE. Concomitant with the CFA-induced increase in PE, there was a dose-dependent increase in IFI (Fig 4b). Data were fitted with a modified Hill equation in order to derive an EC_{50}, which was 28 µg CFA in the Evans blue assay and 12 µg in the feeding assay.

Inflammation-induced Increase in IFI Depended on the Site of Inflammation

In order to determine whether the inflammation-induced increase in IFI was sensitive to the site of inflammation, additional groups were studied in which CFA was injected either unilaterally or bilaterally into the masseter muscle or the skin overlying the TMJ. The results of this experiment are plotted in Fig 5. While CFA injection into the

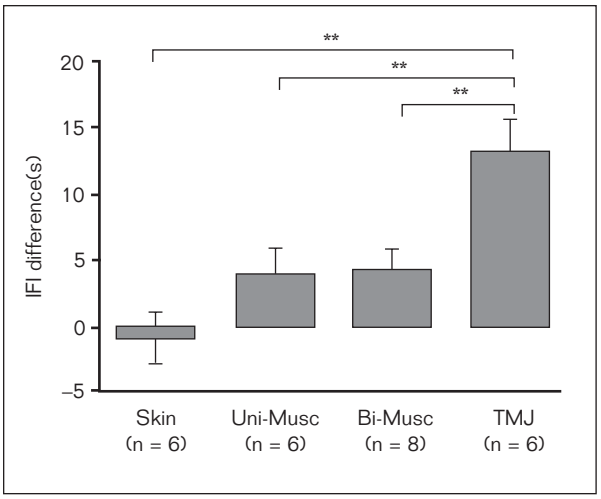

Fig 5 Inflammation of the TMJ, but not skin or masseter muscle, resulted in a shift in the IFI. Skin over the TMJ was injected unilaterally; the masseter muscle was injected either unilaterally (Uni-Musc) or bilaterally (Bi-Musc). All injections consisted of 50 µg of CFA in 50 µL. IFI-difference scores were obtained for each rat and in each group. Pooled data are plotted as a mean ± SEM. One-way ANOVA revealed a significant group effect ($P < .01$). Post-hoc analysis (Tukey test) indicated that the mean IFI difference score for rats injected in the TMJ was significantly greater than that associated with skin injections ($P < .01$) or masseter-muscle injections ($P < .01$).

skin overlying the TMJ resulted in a robust inflammatory response, as assessed by the degree of swelling and PE (Table 3), this manipulation had no influence on the IFI when injections were made unilaterally (data not shown) or bilaterally (Fig 5). Because there is evidence suggesting there is differential loading of masseter muscles during unilateral chewing,[24] and therefore chew relatively normally on the side of the mouth contralateral to the site of inflammation, the impact of both unilateral and bilateral injections of CFA into the masseter muscle was assessed (Fig 5). While both manipulations resulted in a significant increase in IFI over baseline, the magnitude of the inflammation-induced increase in IFI was significantly smaller ($P < .01$, 1-way ANOVA) than that induced by either unilateral TMJ injection (Figs 2 and 4) or bilateral TMJ injections (data not shown).

While the Evans blue assay was used to assess the magnitude of inflammation associated with CFA injection into the skin, muscle, and TMJ, this assay was also used to estimate the "spread" of the inflammation into surrounding tissue. Toward that end, muscle and skin overlying the TMJ were assayed when the TMJ was inflamed, skin and TMJ were assayed when the muscle was inflamed, and the muscle and TMJ were assayed when the skin was inflamed. This analysis revealed the following: *(1)* CFA injections into the TMJ resulted in a significant ($P < .01$, 1-way ANOVA) increase in PE in the TMJ, which was not associated with detectable increases in PE in the muscle or skin overlying this structure (Table 3). *(2)* CFA injection into the muscle resulted in significant ($P < .01$, 1-way ANOVA) increases in PE in the muscle but no detectable increases in PE in the TMJ or the skin overlying the muscle (Table 3). *(3)* In contrast, while CFA injections into the skin resulted in a significant ($P < .01$, 1-way ANOVA) increase in PE in the skin, inflammation of the skin appeared to spread to the muscle resulting in a significant ($P < .01$, 1-way ANOVA) elevation in PE in the muscle as well (Table 3).

Indomethacin Reversed Inflammation-induced Increase in IFI

In order to determine whether the TMJ inflammation-induced increase in IFI is sensitive to NSAIDs, 2 additional groups of rats were studied, both of which received a bilateral TMJ injection of CFA after baseline testing. Thirty minutes before initiation of the feeding session on the test day, 1 group received an injection of 4 mg/kg indomethacin, and the second group received an injection of the indomethacin vehicle (1% $NaCO_3$). Results of this experiment are plotted in Fig 6. While the injection of vehicle had no influence on the inflammation-induced increase in IFI (compare IFI difference of vehicle-treated rats to similar groups in Figs 2 and 4), indomethacin significantly attenuated the inflammation-induced increase in IFI. Interestingly, indomethacin had no detectable influence on Evans blue PE assessed at the end of the feeding session (Table 4).

Discussion

The goal of the present study was to develop and validate an animal model of TMD pain that was both sensitive and relatively easy to use. Towards that end, an operant conditioning paradigm was employed that enabled the researchers to monitor rat feeding behavior. Through the use of a modified Skinner box in which rats were trained to bar-

Table 3 Evans Blue Data for Skin, Muscle, and Joint Injections

Site of inflammation	n	Evans blue (μg/g dry weight) TMJ	Muscle	Skin
TMJ	9	71.7 ± 9.4	14.9 ± 2.2	19.2 ± 3.3
Muscle	5	23.7 ± 7.2	63.2 ± 8.2	10.9 ± 4.5
Skin	5	11.5 ± 2.7	56.4 ± 27	82.6 ±18.0
Intact	6	13.6 ± 2.8	8.9 ± 2.0	5.2 ± 1.0

Total Evans blue extracted from each tissue type (μg) was normalized to the dry weight (g) of the tissue from which it was extracted. Intact rats received no injections. Data are presented as mean ± SEM. For TMJ tissue, 1-way ANOVA revealed a significant difference between groups ($P < .01$) with post-hoc analysis (Dunn's test), indicating that mean Evans blue in the TMJ in response to inflammation of the TMJ was significantly greater than Evans blue in the TMJ in response to inflammation of the muscle ($P < .01$) or skin ($P < .01$) as well as the Evans blue in the TMJ of intact rats ($P < .01$). For muscle tissue, 1-way ANOVA revealed a significant difference between groups ($P < .01$) with post-hoc analysis (Dunn's test), indicating that mean Evans blue in the muscle in response to inflammation of the muscle was significantly greater than Evans blue in the muscle in response to inflammation of the TMJ ($P < .01$) or the Evans blue in the muscle of intact rats ($P < .01$). In addition, Evans blue in the muscle following inflammation of the skin was significantly greater than Evans blue in the muscle in response to inflammation of the TMJ ($P < .01$) or the Evans blue in the muscle of intact rats ($P < .01$). For skin tissue, 1-way ANOVA revealed a significant difference between groups ($P < .01$) with post-hoc analysis (Dunn's test) analysis indicating that mean Evans blue in the skin in response to inflammation of the skin was significantly greater than Evans blue in the skin in response to inflammation of the TMJ ($P < .01$) or muscle ($P < .01$) as well as the Evans blue in the skin of intact rats ($P < .01$).

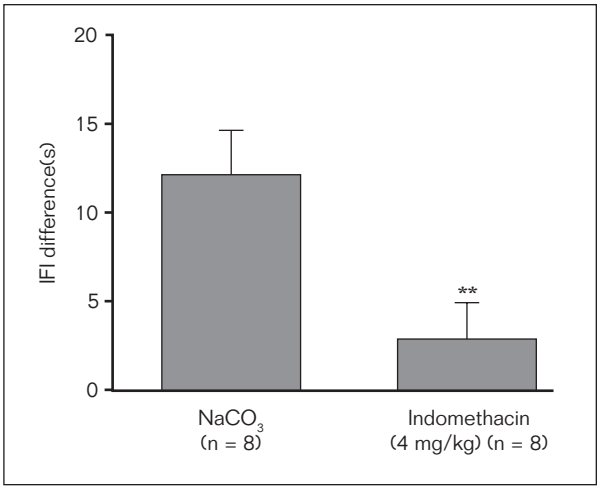

Fig 6 Indomethacin attenuated the CFA-induced shift in the IFI. Both groups of rats received a bilateral injection of CFA into the TMJ immediately after the baseline feeding behavior was assessed. Thirty minutes before feeding behavior was assessed the next day, rats received an intraperiotoneal injection of indomethacin (4 mg/kg) or vehicle (1% $NaCO_3$). Median IFI was determined for each rat for the baseline and test-day feeding sessions, and IFI difference scores were calculated. Pooled data are plotted as means ± SEM. ** indicates $P < .01$, Student *t* test.

Table 4 Evans Blue Data from Indomethacin and Vehicle Groups

Injection site/ IP injection	n	Evans blue (μg/g dry weight) TMJ	Muscle	Skin
TMJ/Indomethacin	8	148.2 ± 31.2	15.4 ± 2.6	20.0 ± 3.9
TMJ/$NaCO_3$	8	126.7 ± 33.5	17.7 ± 4.3	14.5 ± 3.5

Total Evans blue extracted from each tissue type (μg) was normalized to the dry weight (g) of the tissue from which it was extracted. Data are presented as means ± SEM. TMJ were injected bilaterally in both groups of rats with CFA (50 μL of a 1:1 emulsion) 24 hours before feeding behavior was assessed. Indomethacin (4 mg/kg) or its vehicle (1% $NaCO_3$) was injected intraperitoneally 30 minutes prior to assessment of feeding behavior. Evans blue content was assessed within 15 minutes of the end of the feeding session. Indomethacin had no detectable influence on Evans blue in the TMJ, muscle, or skin ($P > .05$; Mann-Whitney test). There was also no significant difference ($P > .05$; 1-way ANOVA) between Evans blue in the TMJ of rats injected with indomethacin or vehicle and rats that did not receive an injection (ie, TMJ inflammation in Table 2).

press for food, it was possible to monitor the timing and duration of a meal by recording the timing of the delivery of each pellet of food. Importantly, it was possible to obtain a good estimation of the rate at which each pellet of food was consumed. This rate, quantified as an IFI, turned out to be a sensitive parameter with which to assess changes in feeding behavior. Using the IFI as a dependent variable, it was possible to perform stimulus response functions (by varying the size of the food pellet), as well as to assess the relative impact of both the magnitude and site of inflammation on feeding behavior.

The results of the present study suggest that inflammation-induced increase in IFI is a manifestation of hyperalgesia. This suggestion is based on the following observations: *(1)* There was a pellet size–dependent increase in IFI, with an increase in pellet size resulting in an increase in the IFI. This correlation is consistent with behavioral changes observed in TMD patients who avoid hard foods and other stimuli that induce excessive jaw activity.[25] *(2)* The inflammation-induced increase in IFI was not associated with a significant decrease in the number of pellets consumed, suggesting that this feeding deficit was not due to a generalized change in appetite, alterations in a set-point regulating satiety, or a stress response, which has been documented to result in a decrease in food consumption.[16] *(3)* Further evidence that the shift in IFI was not due to a generalized illness response comes from the observation that the shift in behavior was specific to inflammation of the TMJ; profound inflammation of the skin overlying the TMJ had no detectable influence of IFI, and inflammation of the masseter muscle was associated with a significantly smaller increase in IFI. *(4)* The inflammation-induced increase in IFI was dependent on the magnitude of inflammation. Concentrations of CFA that did not produce a significant increase in PE, an independent measure of inflammation, were not associated with an increase in IFI. However, over a limited range, increasing concentrations of CFA were associated with increasing shifts in IFI. *(5)* The inflammation-induced increase in IFI was attenuated by an NSAID. Importantly, acute application of indomethacin has been shown to reverse inflammatory hyperalgesia in models of inflammatory pain.[26] Furthermore, the increase in IFI was associated with an increase in feeding behavior, suggesting that in the presence of inflammation, rats were eating more slowly. This change in behavior is consistent with the presence of hyperalgesia, where animals would be expected to take smaller, more frequent bites and consequently spend more time with a pellet; however, other behavioral adaptations to TMJ inflammation, such as eating in bursts, might have been expected. In summary, results of the present study are consistent with the suggestion that the inflammation-induced increase in IFI reflects the presence of hyperalgesia and not other factors that influence feeding behavior. As such, it may be possible to use this operant conditioning paradigm as a model system to study underlying mechanisms associated with TMD pain.

It is interesting to note that while pretreatment with indomethacin significantly attenuated the inflammation-induced increase in IFI, there was no significant influence of this compound on PE in the TMJ. This result was surprising in light of previous results indicating that indomethacin attenuates inflammation-induced PE (ie, see Xie et al[27]). Furthermore, while there is evidence that indomethacin fails to attenuate histamine- and formalin-induced PE,[28,29] a dose of 4 mg/kg has been shown to attenuate CFA-induced PE in the knee joint by ~30% 2 hours after administration.[30] While the basis for this failure is not immediately clear, it is possible that the activity in the TMJ associated with feeding for an hour prior to assessment of PE overwhelmed the anti-inflammatory influence of the indomethacin.

The operant conditioning paradigm used in the present study is a modification of meal-pattern analysis proposed by previous investigators.[16,18,19] Meal pattern analysis involves monitoring rat feeding behavior over a 24-hour period and enables determination of meal duration, meal number, intermeal interval, and food intake. With this method of analysis, TMJ inflammation results in a decrease in food intake that reflects an increase in meal number. Consistent with results of the present study, the meal size remained the same in the presence of inflammation, but meal duration was increased.[16,19] A decrease in food intake was not observed in the present study, which likely reflects the fact that food intake was essentially reduced to 1 meal per day. Interestingly, results from a recent meal pattern analysis study indicate that the increase in meal duration, which reflects a slower rate of food consumption, is the change in meal pattern specific to TMJ inflammation.[16] Inflammation of knee joints resulted in a decrease in food intake that reflected a decrease in meal number in the absence of a change in meal duration.[16] That the increase in meal duration is also sensitive to anti-inflammatory interventions[16,18] lends further support to the suggestion that the inflammation-induced increase in IFI reflects the presence of hyperalgesia.

Meal pattern analysis clearly has a number of advantages over other models developed for the study of TMD pain. Rats are unrestrained, freely moving, and able to perform all natural behaviors. This approach requires no training. Behaviors analyzed are directly relevant to TMD pain, given the primary complaint of this disorder is loss of normal oral-motor function. In addition, the changes in behavior are relatively robust. Other models involve restraint[7,15] or anesthesia,[14] which are potential confounders, and it is unclear to what extent the response to algesic compounds[11–14] reflects processes underlying TMD pain. The model described in the present study has all the advantages of meal pattern analysis, except the training requirement. However, even though it can take up to 2 days to train rats, there are at least 2 potential advantages to the operant conditioning model over meal pattern analysis. First, use of IFI as a dependent variable confers a relatively high degree of sensitivity to the approach, given that statistically significant changes in IFI were detectable with as few as 6 rats per group. Second, and potentially more important, behavioral endpoints are assessed in a relatively small amount of time. This enables screening of interventions that may have a relatively short half life.

However, the model described in this study is not without its limitations. These include the following. First, as noted, the model is associated with a significant amount of training time. Second, although statistically significant differences between groups were detectable with as few as 6 animals per group, the effect size is relatively small. Third, while a shift in feeding behavior might have been detectable with larger injection volumes in the masseter muscle, the most pronounced changes in behavior were observed following injections into the TMJ, and the TMJ is a relatively difficult target to hit. Fourth, on average, physiologic saline injections into the TMJ had no significant influence on feeding behavior. However, within a 24-hour window, even minor TMJ trauma such as that associated with a physiologic saline injection may result in a small increase in IFI, as was observed in several animals following TMJ injections with saline. And fifth, the model is still based on an indirect measure that involves a motivational component which, under certain conditions, may serve as a potential confounder.

There are several reasons to suggest that the model presented may be a valid tool for assessing the efficacy of therapeutic interventions for the treatment of TMD pain. First, the model is based on overt inflammation of the TMJ, and there is evidence to suggest TMD pain may reflect inflammation of the TMJ.[25,31] Inflammatory TMD pain may be evoked by localized phenomena such as synovitis, capsulitis, and retrodiscitis, as well as systemic disorders, including rheumatoid arthritis, osteoarthritis, and Lyme disease.[25,32–34] Second, while the model is associated with a quantifiable loss of joint function, inflammatory disorders of the TMJ are characterized by constant dull aching pain that is accentuated by joint movement or loading the joint. Importantly, movement of the joint, particularly that associated with loaded movements (eg, chewing food) is avoided by patients suffering from pain associated with inflammation of the TMJ.[25] Third, there was a correlation between the severity of the inflammation and the magnitude of the feeding deficit in the present model, while there is a correlation between the extent of inflammation and the amount of TMD pain observed in patients.[25,35,36] Fourth, the loss of function associated with TMJ inflammation in the present model was responsive to an NSAID, and NSAIDs have some analgesic efficacy in humans.[37,38]

Acknowledgments

This work was supported by National Institutes of Health grant no. AR049555 (MSG). The authors would also like to thank Maryna Petrasheuskaya for her expert technical assistance.

References

1. Sarlani E, Greenspan JD. Why look in the brain for answers to temporomandibular disorder pain? Cells Tissues Organs 2005;180:69–75.
2. Svensson P, Arendt-Nielson L. Clinical and experimental aspects of temporomandibular disorders. Curr Rev Pain 2000;4:158–165.
3. Greene CS. The etiology of temporomandibular disorders: Implications for treatment. J Orofac Pain 2001;15: 93–105.
4. Di Paolo C, Panti F, Crocitto P, De Luca M. Long term follow-up of patients affected by temporomandibular joint dysfunction treated with orthodontic and/or prosthetic occlusal therapy. Minerva Stomatol 2004;53:5555–5570.
5. Suvinen TI, Reade PC, Kemppainen P, Kononen M, Dworkin SF. Review of aetiological concepts of temporomandibular pain disorders: Towards a biopsychosocial model for integration of physical disorder factors with psychological and psychosocial illness impact factors. Eur J Pain 2005;9:613–633.

6. Gesch D, Bernhardt O, Mack F, John U, Kocher T, Alte D. Association of malocclusion and functional occlusion with subjective symptoms of TMD in adults: Results of the Study of Health in Pomerania (SHIP). Angle Orthod 2005;75:183–190.
7. Ren K. An improved method for assessing mechanical allodynia in the rat. Physiol Behav 1999;67:711–716.
8. Imbe H, Iwata K, Zhou QQ, Zou S, Dubner R, Ren K. Orofacial deep and cutaneous tissue inflammation and trigeminal neuronal activation. Implications for persistent temporomandibular pain. Cells Tissues Organs 2001;169: 238–247.
9. Schutz TC, Andersen ML, Tufik S. Influence of temporomandibular joint pain on sleep patterns: Role of nitric oxide. J Dent Res 2004;83:693–697.
10. Schutz TC, Andersen ML, Tufik S. Sleep alterations in an experimental orofacial pain model in rats. Brain Res 2003;993:164–171.
11. Roveroni RC, Parada CA, Cecilia M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: The TMJ formalin test. Pain 2001;94:185–191.
12. Clemente JT, Parada CA, Veiga MC, Gear RW, Tambeli CH. Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett 2004;372:250–255.
13. Hartwig AC, Mathias SI, Law AS, Gebhart GF. Characterization and opioid modulation of inflammatory temporomandibular joint pain in the rat. J Oral Maxillofac Surg 2003;61:1302–1309.
14. Ro JY, Capra N, Masri R. Development of a behavioral assessment of craniofacial muscle pain in lightly anesthetized rats. Pain 2003;104:179–185.
15. Ro JY. Bite force measurement in awake rats: A behavioral model for persistent orofacial muscle pain and hyperalgesia. J Orofac Pain 2005;19:159–167.
16. Kerins CA, Carlson DS, Hinton RJ, et al. Specificity of meal pattern analysis as an animal model of determining temporomandibular joint inflammation/pain. Int J Oral Maxillofac Surg 2005;34:425–431.
17. Kerins C, Carlson D, McIntosh J, Bellinger L. A role for cyclooxygenase II inhibitors in modulating temporomandibular joint inflammation from a meal pattern analysis perspective. J Oral Maxillofac Surg 2004;62:989–995.
18. Kerins CA, Carlson DS, McIntosh JE, Bellinger LL. Meal pattern changes associated with temporomandibular joint inflammation/pain in rats; analgesic effects. Pharmacol Biochem Behav 2003;75:181–189.
19. Harper RP, Kerins CA, Talwar R, et al. Meal pattern analysis in response to temporomandibular joint inflammation in the rat. J Dent Res 2000;79:1704–1711.
20. Thut PD, Flake NM, Hermanstyne TO, Gold MS. An operant conditioning model with which to assess temporomandibular pain. Abstract presented at the 29th Scientific Annual Meeting of the American Academy of Orofacial Pain, San Francisco, 25–28 March 2004.
21. Flake NM, Bonebreak DB, Gold MS. Estrogen and inflammation increase the excitability of rat temporomandibular joint afferent neurons. J Neurophysiol 2005;93:1585–1597.
22. Zhang RX, Lao L, Wang X, Ren K, Berman BB. Electroacupuncture combined with indomethacin enhances antihyperalgesia in inflammatory rats. Pharmacol Biochem Behav 2004;78:793–797.
23. Carr J, Wilhelm DL. The evaluation of increased vascular permeability in the skin of guinea pigs. Aust J Exp Biol Med Sci 1964;42:511–522.
24. Dessem D. Interactions between jaw-muscle recruitment and jaw-joint forces in Canis familiaris. J Anat 1989;164:101–121.
25. Okeson J. Management of TMD and Occlusion. St Louis: Mosby, 2003.
26. Schaible HG, Ebersberger A, Von Banchet GS. Mechanisms of pain in arthritis. Ann N Y Acad Sci 2002; 966:343–354.
27. Xie G, Wang Y, Sharma M, et al. 5-Hydroxytryptamine-induced plasma extravasation in the rat knee joint is mediated by multiple prostaglandins. Inflamm Res 2003;52:32–38.
28. Gimeno G, Carpentier PH, Desquand-Billiald S, Finet M, Hanf R. Respective role of lipoxygenase and nitric oxide-synthase pathways in plasma histamine-induced macromolecular leakage in conscious hamsters. Br J Pharmacol 1999;126:1801–1809.
29. Damas J, Liegeois JF. The inflammatory reaction induced by formalin in the rat paw. Naunyn Schmiedebergs Arch Pharmacol 1999;359:220–227.
30. Campbell EA, Gentry C, Patel S, et al. Oral anti-hyperalgesic and anti-inflammatory activity of NK(1) receptor antagonists in models of inflammatory hyperalgesia of the guinea-pig. Pain 2000;87:253–263.
31. McNeill C, Dubner R. What is pain and how do we classify orofacial pain? In: Lund JP, Lavigne GJ, Dubner R, Sessle BJ (eds). Orofacial Pain: From Basic Science to Clinical Management. Chicago: Quintessence, 2001:3–14.
32. Broussard JS Jr. Derangement, osteoarthritis, and rheumatoid arthritis of the temporomandibular joint: Implications, diagnosis, and management. Dent Clin North Am 2005;49:327–342.
33. Dworkin SF, LeResche L (eds). Research Diagnostic Criteria for Temporomandibular Disorders: Review, criteria, examinations and specifications, critique. J Craniomandib Disord 1992;6:301–355.
34. Lobbezoo F, Drangsholt M, Peck C, Sato H, Kopp S, Svensson P. Topical review: New insights into the pathology and diagnosis of disorders of the temporomandibular joint. J Orofac Pain 2004;18:181–191.
35. Pereira FJ, Lundh H, Eriksson L, Westesson PL. Microscopic changes in the retrodiscal tissues of painful temporomandibular joints. J Oral Maxillofac Surg 1996;54:461–468.
36. Murakami K, Segami N, Fujimura K, Iizuka T. Correlation between pain and synovitis in patients with internal derangement of the temporomandibular joint. J Oral Maxillofac Surg 1991;49:1159–1161.
37. List T, Axelsson S, Leijon G. Pharmacologic interventions in the treatment of temporomandibular disorders, atypical facial pain, and burning mouth syndrome. A qualitative systematic review. J Orofac Pain 2003;17:301–310.
38. Ta LE, Neubert JK, Dionne R. Pharmacological treatments for temporomandibular disorders and other orofacial pain. In: Dionne R, Phero J, Becker D (eds). Management of Pain and Anxiety in the Dental Office. New York: Saunders, 2002:354–367.