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Distance-based permutation of inter-meal differences as a sensitive test of temporomandibular joint nociception in rats

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Abstract

Purpose—Compare non-parametric permutation method using intra-meal rate as endpoint to existing ANOVA method that uses average daily meal duration as an endpoint for detection of chronic pain in Sprague-Dawley rats.

Methods—Nociception following bilateral temporomandibular joint (TMJ) injection of high-dose of Complete Freund's Adjuvant (CFA, 250 µg/50 µL per side) could be detected in young adult male Sprague-Dawley rats using average daily meal durations as a measure of nociception for up to 19 days (Kramer, Kerins, Schneiderman, & Bellinger, 2010) using ANOVA and multiple comparisons range tests. In this study, we reanalyzed the data using a non-parametric permutation procedure based on absolute differences between intra-meal feeding rate curves. In addition, to that experiment, we injected bilaterally the TMJ of naive rats with either a low-dose CFA (15 µg/50 µL per side, n=6) or saline (50 µL of 0.9%, n=4) and monitored the animals for 7 days.

Results—The permutation test of the intra-meal feeding rate detected the presence of nociception in the high-dose CFA treatment group for up to 40 days or twice as long as when using ANOVA on average daily meal durations. The permutation method also detected the low-dose CFA induced nociception with ten-times lower *p*-values and for several days longer than ANOVA of changes in meal durations. CFA-induced injury resulted in even reduction of intra-meal feeding rate and lengthening of the meals in both high- and low-dose CFA-injected animals. The rate analysis also showed when the rats first started a meal they were experiencing the same level of nociception as at the end of the meal. This demonstrated that intra-meal chewing itself did not alter the level of nociception.

Conclusions—These results suggest that permutation tests based on differences in intra-meal feeding rates can be used as a sensitive test to determine and study the temporal patterns of TMJ nociception.

Keywords

feeding rate; meal analysis; nociception; permutation test; rat; temporomandibular joint; pain

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Introduction

Determination of the physiological state of an animal following an intervention usually involves comparison of a physical or behavioral parameter of interest using at least two groups, i.e., a control and treatment. When the response to a treatment is variable, some kind of statistical testing procedure is used to qualify and quantify the comparison. For example, the determination if an animal is experiencing nociception is extremely important and at times difficult to measure. Various techniques have been employed to determine whether an animal is experiencing nociception (Kramer, He, Puri, & Bellinger, 2012; Mogil, 2009). Some nociception measurements, such as, using von Frey filaments, hot plate or other methods involve handling the animals, which induces stress and can affect the test outcome. Thus, methods utilizing natural behaviors on undisturbed animals are more desirable.

Meal pattern analysis have been used in studying neuronal circuits affecting feeding behavior (Clifton, 2000; Vickers & Clifton, 2012) and more recently has been employed to measure orofacial nociception for some time (Kerins et al., 2005; Kerins, Carlson, JE, & Bellinger, 2003; Kramer et al., 2012, 2010). This method uses the computer measure of consumption of precision weighed pellets over time to determine meal durations. As also occurs in human when the subject is experiencing orofacial pain (TMJ, masseter, tooth pain) the mice and rats eat more slowly and significantly increase their meal duration (Kerins et al., 2005, 2003; Kramer & Bellinger, 2014; Kramer et al., 2012, 2010; Kramer, Umorin, & Bellinger, 2015). The benefits of using a record of pellet consumption of animals in sound attenuated home chambers for determination of nociception is that feeding is a natural behavior and that the animals are undisturbed when they are evaluated. Thus, after the initial intervention the animals are left undisturbed so they do not experience any competing behaviors or are handled which can interfere with the measurement of nociception.

A problem a researcher faces when correlating meal patterns to changes in TMJ and orofacial nociception is identifying meals. While in humans the meals taken are often quite discreet, animals, such as rodents, have very diverse patterns ingestion. This problem can be overcome, as noted above, by using computerized pellet feeding devices that record the precise timestamps of an individual's intake of the food pellets and by this the determination of the positive correlated to nociception, i.e., meal duration. However, we asked the question whether using meal duration as measure of TMJ and orofacial nociception could be further refined. We hypothesized that within a single meal of a particular meal duration control and rats experiencing TMJ orofacial nociception might consume the precision made pellets at different rates. For example, we assumed that a rat experiencing pain might consume the pellets at varying rate over the course of the meal duration, whereas a control rat would take pellets at a constant rate over the meal. We also wanted to determine whether the nociception level changed during a single meal; i.e., did the act of chewing a pellet affect subsequent experience of nociception.

If this assumption is true then another problem faced would be choosing the appropriate statistical testing procedure. Permutation methods have long been used in life sciences to test hypotheses when assumptions of the normality of data distribution and homogeneity of variance do not hold, which is the majority of cases. Various permutation techniques provide

robust estimates and hypothesis testing that is conditional on the observed data. A consideration when using permutation analysis is the significant computational time required. Nevertheless, distance-based permutation methods based on the differences between objects rather than absolute values of measured parameters may provide for superior sensitivity of analyzing data. These tests have been used to study various environmental and life-science phenomena (Mielke & Berry, 2007). When comparing multivariate or vector data, how a distance between objects or observations is calculated becomes important, with metric distances preferred due to intuitive interpretation of the test statistics.

Previous work of the Bellinger's group established that meal duration can be used as a sensitive specific non-invasive marker of TMJ and orofacial nociception in rats for up to 19 days following a CFA injection (Kramer et al., 2010). In the present study, it was investigated if intrameal meal pattern analysis might be a finer determinant of the presence (length) of TMJ nociception. Specifically, intra-meal pellet consumption rates as functions of intra-meal time were analyzed to determine if differences in feeding rates would prove to be more sensitive than differences in meal duration alone in determining TMJ nociception and whether this analysis could detect the presence of TMJ nociception over longer periods of time. Thus it was determined whether statistical testing using permutation of feeding rats could offer a more precise measure of the "fast-and-quick" meal consumption of the control rats and "slow-and-long" meal consumption of the CFA-injected rats.

Methods

Feeding Data

We are using the data from a rat feeding experiment originally described in Kramer et al. (2010) – Experiment 1, and from a small pilot experiment – Experiment 2, unpublished data. These studies were approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee and conducted in accordance with the USDA and National Institutes of Health guidelines (National Research Council, 2011). The designs for Experiments 1 and 2 was identical except for the quantity of CFA injected and the duration of monitoring. Young adult male Sprague-Dawley Harlan Industries, Houston, TX rats were caged individually in sound-attenuated modules equipped with photobeam computer-activated pellet feeders (Med Assoc. Inc., East Fairfield, VT) and given food and water ad libitum. The animals were kept on a 12 h/12 h light/dark cycle with lights on at 08:00. All rats were given precision made 45 mg rodent pellet diet (Bioserv, Frenchtown, NJ). When a rat removed a pellet from a trough of the feeder this signaled the computer to drop another pellet into the trough. The computer recorded each time the pellet was released with 1 s precision. The computer record of pellet drop times provided a raw data for meal pattern analyses. It should be noted that the rats on average drop less than five pellets per day. In Experiment 1, after acclimating in the feeders for at least two days the rats received either bilateral CFA injections (250 µg/50 µL per side) or physiological saline injections (50 µL of 0.9%) into TMJ. The day of injection was taken as day 0 when 15 rats were injected with CFA and 13 with saline solution. At day 21 one saline-injected rat and two CFA-injected rat were sacrificed for tissue analysis in the previous study (Kramer et al., 2010) thus reducing the number of animals monitored. On day

22 the saline-injected animals were injected CFA (n=6) or saline (n=6) (same amounts as for TMJ injection) into the knee. The animals were monitored for a period of up to 41 days after the TMJ injection. One CFA-injected animal was removed from analysis because the animal experienced weight loss due to poor feeding and thus was not representative of the population of interest. The total number of meals per day varied from 48 to 147 in the saline group and from 54 to 126 in the CFA group. For further details of this experiment see Kramer et al. (2010). In experiment 2, ten rats were injected with either 15 µg/50 µL per side of CFA (n=6) or 50 µL physiological saline (n=4) solution into the TMJ. The animals were monitored for eight days post-injection. Other conditions were the same as in Experiment 1. The total number of meals per day varied from 16 to 28 in the saline group and from 16 to 32 in the CFA group.

Permutation Procedure

We used a statistical analysis of permutation of the objects, in our case meals, between groups as a means of testing for the significant difference between the groups. As in parametric testing, permutation testing is a statistical form of comparison of between-group to within-group distances and as such, it is sufficient to know the distances between individual objects and not the actual coordinates of the individual objects as in the parameter space. In our case, we sought to uncover differences between two groups based on the feeding patterns that animals exhibited, and thus, we somehow needed to identify meals, quantify meals and determine the distances between them.

The meals were identified based on a point process model (Perkel, Gerstein, & Moore, 1967) in a way similar to identification of spike bursts in electrophysiology (Cocatre-Zilgien & Delcomyn, 1992). The series of pellet drop times was converted into a series of inter-pellet intervals by subtracting the consecutive pellet drop times. The inter-pellet intervals were inverted giving the pellet rate which was assigned to the interval between two pellet drops that defined that interval. In this study we used an inter-meal interval 600 s long. This particular duration was chosen, because the original study used 10 min threshold to define the end of a meal, i.e., a ten minute period of no intake denoted the end of the previous meal. This ten minute end of meal definition was previously recommended for Sprague-Dawley rats (Castonguay, Kaiser, & Stern, 1986). It is smaller than the end of drinking bout threshold suggested for Wistar rats, which is 14 to 17.7 min (Zorrilla et al., 2005). However, meal and drinking patterns can be dramatically different among different strains of rats (Glendinning & Smith, 1994). The chosen inter-meal interval was inverted giving the maximum pellet rate ($1/600$ s) that would correspond to a period between meals. This rate was used to threshold the series of pellet rates. The rates above the threshold ($> 1/600$ s) would correspond to meals; the rates below ($< 1/600$ s) would correspond to inter-meal intervals. The continuous sequence of pellet rates above the threshold would constitute a sequence of rates within a single meal. Thus, the choice of a particular inter-meal interval in the present study would ensure that pellets that were separated by an interval of > 10 min would fall into different meals consistent with those previous studies. The over-threshold sequences had to be of a minimum size to be counted as meals. This minimum meal size requirement was due to the fact that rats occasionally eat several pellets, but not go into a bout of eating that one could consider as a meal. This minimum meal size value was set to

three pellets, i.e., 135 mg. Furthermore, meals consumed during the light phase of the cycle were excluded. The above selection criteria (Kramer et al., 2010) did not affect the outcomes of the analysis as very few meals occurred during light phase of the light/dark cycle and on only a few occasions were less than three pellets in size were taken: these non-meal, by definition, feeding bouts were excluded from further analysis. This is very consistent with previous findings in our laboratory (Kerins et al., 2005, 2003; Kramer & Bellinger, 2014; Kramer et al., 2012, 2010, 2015).

Meals were quantified in the following way. Within each meal the first pellet meant the initiation of a meal and the intra-meal time was set to 0 at that pellet drop. Consecutive pellet drop times for that meal were adjusted to the intra-meal times by subtracting the drop time of the first pellet in the meal. Continuous set of rates within a single meal would present as a step function where pellet rate would change at the time each pellet was taken. For comparison purposes the step function for each meal was sampled at 1 s intervals for the duration of the meal. Thus, each meal was represented by a discrete set of values – an intra-meal feeding rate function, f_k , where $k = 0, \dots, n$, n being the meal duration. By necessity for meal comparison, n was fixed and set to 1800 (= 30 min) for all meals. For meals shorter than n the trailing values were set to 0. For meals longer than 1800 s only the first 1800 s were used for analysis; this truncated 2%, or 160 meals. Meal duration was calculated separately from untruncated and unpadded sequence of pellet rates for each meal.

We approached calculating distances between meals in the following way. We used a norm of a difference between intra-meal feeding rate functions as distance between two distinct meals, i and j :

$$\Delta_{ij} = \|f^i - f^j\|$$

more specifically,

$$\Delta_{ij} = \sum_{k=1}^n |f_k^i - f_k^j|$$

that is, we chose a city-block metric, and while Euclidean and other metrics would be possible, we deemed them not as advantageous.

The average within-group distance for group g is the average of all the between-meal distances in that group:

$$\xi_g = \left(\binom{n_g}{2} \right)^{-1} \sum_{i,j \in g, i \neq j} \Delta_{ij}$$

where n_g is the number of meals in group g .

The permutation statistic then was calculated as:

$$\delta = \sum_{g=1}^G C_g \xi_g$$

where $g = 1, \dots, G$, G is the number of groups, and ξ_g is the average within-group distance for group g ; C_g is the group weight, usually n_g/n_{total} , n_{total} being the total number of meals. While parametric statistical tests, such as ANOVA, or Student's test, use between-group-related value in the statistic, we used the sum of within-group distances to simplify the calculations. The sum of all the distances in a sample is equal to the sum of all within-group and all between-group distances and is fixed under permutation for a fixed sample. Thus, by testing for within-group distances it by necessity also tests for between-group distances. The distribution tail that is used to calculate the Type I error is simply switched from upper to lower: while the greater between-group-based statistic indicate the greater difference between groups (like in ANOVA), the smaller within-group-based statistic (like the one we used) would indicate the same. Thus, we looked at the lower tail of the distribution of δ under permutation to calculate Type I error.

Permutation testing consists of switching, or permuting, the meals between the groups and then recalculating δ . The number of permutations where δ is less than the statistic for the original placement of meals into groups divided by the total number of permutations would give the probability of Type I error under the null hypothesis of no difference between the groups. Usually the number of possible permutations far exceeds researcher's ability to generate them all in order to assemble a complete population of δ s. Thus, there are two main approaches in estimating the population distribution of δ : calculation of Pearson type III distribution as an approximation of the distribution of δ under permutation and Monte-Carlo sampling. Here we applied the Monte-Carlo sampling approach to estimating the Type I error. Because inexhaustive sampling of permutations from the population of all permutations is associated with an error of its own, the number of permutations used the estimate Type I error has to be large to minimize the sampling error. Specifically, we used 40,000,000 permutations to achieve precision to the fifth decimal place assuming Type I error was around 0.001, according to Mielke and Berry (2007).

Results

For permutation analysis to be valid data must satisfy exchangeability requirement (Draper, Hodges, Mallows, & Pregibon, 1993; Hayes, 1996). To verify exchangeability of the intra-meal feeding rate functions, a linear model was fitted to meal sizes with day of the experiment and meal order within the night (rats mostly eat at night, and consume between 10 and 20 meals) as independent variables; the data were taken from the day -2 to 20 from control animals to correspond to null hypothesis of no differences between meals. Even though the slopes for each variable were significant ($p < 2 \cdot 10^{-16}$ for meal order and $p = 0.0827$ for day of the experiment, $df(\text{residuals}) = 2978$), the slopes were small in absolute value (-1.5024 for meal night order and -0.0956 for day of the experiment). Because of the variability of meal size around mean ($mean = 34.16$, $\sqrt{MSE} = 19$) the trends were considered of no practical significance leading to the conclusion that all meals from control rats were

similar. From similarity of the meal size parameter across meals we inferred the exchangeability of the intra-meal feeding rate functions following exchangeability discussion in Draper et al. (1993).

Treatment versus control permutation tests were made separately for each of the two days preceding the injection and for 40 days post-injection for Experiment 1 (Figure 1). For Experiment 2, the comparisons of control to treatment groups using permutation tests were made for two days preceding injection and for each of the six days following the injection (Figure 3). The meals from the same rat were treated as independent in both experiments. Meals from different rats were also considered as independent and were pooled into control and treatment groups correspondingly. The program to perform the permutation tests was written by the first author in R programming language with parts of the code written in C and compiled into R functions and was executed on HPC cluster. The code is available in the Supplement.

On all figures the first night after saline or CFA injection is presented as day 0. Thus, the last night of observation in Experiment 1 or the last night in Experiment 2 are presented as day 40 and day 7 respectively. Meals for the saline injected and CFA-injected groups were compared based on the distance between the corresponding feeding rate curves. We chose the absolute (also known as Manhattan, or city-block) distance calculated by summing absolute differences between the rates for the corresponding time points for meals in question. The choice was dictated by the intuitive interpretation of the distance since it has metric properties and consequent intuitive interpretation of statistical differences, should such be discovered in the data.

We chose Type I error rate of $\alpha = 0.01$ for more stringent analysis of our experiments. To determine significance of individual tests during multiple comparison procedure within each experiment, Bonferroni correction was applied that controlled per-family Type I error rate (Kirk, 2013). The per-family error rate was divided by the number of individual tests in experiment. Thus, for Experiment 1 individual tests had to have a p -value at or below $\alpha' = 0.01/43 = 0.00023$ to be deemed significant. For Experiment 2, individual tests had to have a p -value at or below $\alpha' = 0.01/10 = 0.001$ to be deemed significant. There was no effect of treatment on animal weight (repeated measures ANCOVA with animals nested within treatment and time as covariate, $p(\text{treatment}) = 0.994$, $df = 23$; data not shown). Meal size vs day of the experiment trend was of varying sign and often insignificant for individual animals. As mentioned above, the time trend of meal size for pulled meals was significant but the slope value was small and given the variation of meal size the time trend of meal size was considered of no practical significance.

The daily course of p -values based on permutation test on meal distances of individual meals between control and treatment rats covering days -2 through 40 days of observation in Experiment 1 is presented in Figure 1. After Bonferroni correction for multiple comparisons the results showed the two groups of rats were not significantly different on two days preceding the saline or CFA injection; the differences became highly significant ($p < 10^{-7}$) immediately after the injection and the groups remained significantly different ($p \approx 10^{-6}$) for 40 days of the experiment.

The feeding rate curves were estimated as described above. The median intra-meal feeding rate and the feeding rate upper and lower quartiles for treatment and control groups in Experiment 1 for the days -2, 5, 19, and 40 are presented on Figure 2. Before injection (Figure 2, day -2) meals would start at a rate approximately 0.1 pellets/s which would continue to approximately 100 s into the meal and then gradually decrease to zero at 300 s into the meal. At the onset of treatment (Figure 2, day 5 – we chose to present this day because daily food intake for CFA injected rats returned to the same level as for the saline-injected animals), the CFA-injected rats exhibited a significantly reduced rate of uptake, 0.05 pellets/s, at the beginning of each meal. Their intake of pellets also continued for longer periods (> 500 s) than saline-injected animals (i.e. 300 s). Towards the end of the experiment (Figure 2, day 40) the median pellet rate curve of the CFA-injected animals became close to the median pellet intake rate curve of the control group, but never matched it like before the injection. This change with time was presumably because nociception showed some abatement with time.

Results for the permutation tests for days -2 through 7 from Experiment 2 are presented in Figure 3. For comparison, we also present test results for a set of daily *t*-tests based on average daily meal durations, similar to the original high CFA dose study (Kramer et al., 2010), which used a related multiple range test for *post-hoc* daily comparisons. After Bonferroni correction for multiple comparisons which makes the set of tests more conservative than multiple range test (Kirk, 2013), the tests revealed significant differences between control and treatment groups. The permutation test was significant on days -1, 0, 1, 2, 3. Examination of the feeding patterns of individual rats revealed that two of the rats in the group that later became treated with saline injection into TMJ ate all of their meals within 200 s on day -1 while other unaffected rats in both groups had meals that went beyond 200 s. Given that the number of rats in the saline group was four, this skewed the distances between control and treatment groups and consequently the test statistic into a large enough value to become significant on that day when no physiological difference between the groups existed. The omnibus repeated measures ANOVA on individual meal durations with injection treatment and time as factors yielded non-significant injection ($p = 0.0129$) and time ($p = 0.5787$) effects ($df(\text{injection treatment}) = 1$, $df(\text{time}) = 7$, $df(\text{interaction}) = 7$) and a non-significant time-injection interaction ($p = 0.0243$, $df(\text{residuals}) = 334$) term which was used to test the main effects; multiple comparison daily *t*-test was not significant on any day of the experiment ($df = 23$ to 56) at family-wise $\alpha = 0.01$ and Bonferroni correction.

The median intra-meal feeding rate and feeding rate the upper and lower quartiles for treatment and control groups in Experiment 2 for the days -2, 0, 5, and 7 are presented on Figure 4. Before injection (Figure 2, day -2) meals would start at a rate approximately 0.125 pellets/s which would continue to approximately 100 s into the meal and then gradually decrease to zero at 200 s into the meal. At the onset of treatment (Figure 4, day 0), CFA-injected rats exhibited significantly reduced rate of uptake, 0.05 pellets/s, at the beginning of each meal and also continued to take pellets for longer periods (> 400 s) for majority of meals compared to saline-injected animals. Towards the end of the experiment (Figure 4, day 7) the median rate curve of the CFA-injected animals became almost identical to the

median rate curve of the saline-injected animals: starting at 0.125 pellets/s and continuing at that level to 100 s and then decreasing to zero by approximately 200 s into the meal.

Discussion

In this study we analyzed the intra-meal feeding rate by a permutation method and found that the method is twice as sensitive as a previously reported indicator of TMJ nociception (Kramer et al., 2010). The current study and the previous study placed rats individually in sound attenuated computerized pellet feeders in which precise meal patterns, i.e., meal size, frequency and duration could be calculated. The original work by our laboratory (Kramer et al., 2010) in which this study was compared, used daily averages for meal duration, meal size, meal frequency for each rat to compare treatment effects using traditional analysis of variance and concluded that the average daily meal duration was the best predictor of TMJ nociception. Using this endpoint and statistical analysis of ANOVA and a range test we were able to detect the presence of nociception for 19 days following TMJ CFA injection. In the present study we used feeding rate, i.e., 45 mg pellets dropped per time, within individual meals to determine nociception. The premise of the calculation was rats experiencing TMJ nociception would take pellets at a slower rate than control rats. Thus the basis for the permutation calculation was the distance between pellets consumed and then additionally the distances between meals for each day. Using rate of eating as a measure of TMJ nociception we were able to determine that the animals of the previous study (Kramer et al., 2010) where meal duration showed the animals experiencing nociception for 19 days were actually manifesting nociception up to 40 days after inflammation of the TMJ began, Figure 1. In fact the rats were still experiencing nociception when the measurements stopped and thus might have been in nociception for an even longer time period.

In the second Experiment of the present study in which a low-dose of CFA was injected into the TMJ, p -values from the permutation test were much lower than when the data were calculated as meal durations and an ANOVA and range test were used to calculate significance. Thus, when the CFA group was compared to the control animals using the permutation test it was shown that the treated rats were experiencing nociception for several days while no nociception could be observed using meal duration alone (Figure 3).

We attribute the general trend toward higher p -values in both Experiments after CFA injection to a gradual abatement of nociceptive effect. Thus, the permutation test allowed detection of nociception for a longer period of time in the rat feeding data. Even at low dose it was superior to previously used measure of nociception. Thus, analyzing meal eating rate can reveal both chronic TMJ nociception in case of severe TMJ inflammation and also reveal the return to normal functioning in case of mild TMJ injury.

The current analysis was more sensitive than measuring meal duration alone to detect nociception for longer periods of time as the current analysis refined the meal duration measurement by determining the rate at which the rats took pellets with the meal. Thus meal duration only measured the total meal duration, i.e., lengthened by nociception, whereas the current analysis measured meal duration and within the meal the rate at which the rate took the pellets. Using individual meal rates over the daily averages used in the original study

(Kramer et al., 2010) improved the power of the permutation test partly due to increased degrees of freedom (Kirk, 2013). There are more objects (individual meals) used in permutation test than that used in the ANOVA test of daily averaged meal durations. And, while the performance of a parametric test would improve if individual meal durations were used, the test would also suffer from the presence of outliers in non-normal data: the distribution of individual meal durations was heavily skewed, especially for the CFA injected group (data not shown). The extreme values in either control or treatment group would unevenly contribute to either smaller or larger p -values on different days. In general, ANOVA often performs as well (or as badly) as the permutation test, even when data severely deviate from normal (Hayes, 1996). However, multivariate distance-based version of the permutation test usually outperforms the multivariate ANOVA (Mielke & Berry, 1999).

Most importantly, since our vector-based distance determination is similar to multivariate case we think that the choice of distance function as a difference between vector (i.e. multivariate)-represented meals was the factor that contributed superior performance of the permutation test compared to t -test in this study or the range test in the original study.

The choice of vector meal rate quantification and distance between meals function would, thus, affect the performance of the test. One can use binning (Shimazaki & Shinomoto, 2007) or kernel density estimation (Cunningham, Gilja, Ryu, & Shenoy, 2009; Shimazaki & Shinomoto, 2010) instead of inverse inter-pellet interval to identify and quantify meal rates. When applied to the original data the inverse inter-pellet interval meal quantification method proved to be more sensitive than meal duration alone. It would be interesting to explore the effect of other meal identification methods: deviation from expected Poisson distribution (Legendy & Salcman, 1985) or rank-surprise criteria (Gourévitch & Eggermont, 2007) on the performance of the permutation test. We have not tried them.

We chose to calculate distances between meals using absolute difference function, because we think it was a superior method. Additionally, while could have chosen other forms of distance functions, e.g. Euclidean, squared Euclidean, or general Minkowski distances (Mielke & Berry, 2007), we sought better interpretability of the distances and thus chose a metric distance. Euclidean and general Minkowski distances while being metric probably would provide no more benefits compared to the absolute difference distance that we used so we chose not to use them because of greater computational overhead. Therefore, according to Mielke and Berry (2007, sec. 2.8) absolute distance would provide for congruence between data and analytical spaces and would make the statistic relatively insensitive to extreme values.

Another important finding in our study was the shape of the intra-meal feeding curve and its changes with CFA administration. Given that the average meal size for CFA-injected rats was not significantly different from saline-injected rats, but the meal duration significantly increased (Kramer et al., 2010), it is obvious that the average feeding rate within a meal should decrease for injured rats. However, no one has looked into how the instantaneous feeding rate changes within a meal. Our results show that normal rats start a meal at about 0.15 pellets/s rate and they gradually decrease their feeding rate to zero (end-of-meal) within

200 to 400 s (Figure 2, day -2, Figure 4, day -2). This is consistent with current concepts on the onset of satiety during feeding. The CFA-injected rats have significantly lower specific feeding rate of 0.05 pellets/s versus the control animals at the onset of the meal (Figure 2: day 5) and > 1000 s, versus control rats in the high-CFA Experiment 1. Consistently, the low-dose CFA injection (Experiment 2) produced similar low-feeding rate and lengthening of the meals, Figure 4: day 0. The data revealed the CFA-injected rats would start eating pellets below the normal control animals' rate and would keep the same rate for a longer period. The rats would maintain this reduced rate without significant change suggesting they were experiencing nociception at the onset of the meals as well as during meals. This indicates that nociception does not get worse during meals on the scale of minutes and that there was no meal-specific nociception component that contributed to the reduced overall pellet take rate during meals. It is also possible the rats recognized the increased nociception immediately after taking the first pellet and slowed their subsequent pellet consumption. To resolve this initial pellet rate question a measurement of neural nociceptive input or chemical nociceptive markers on the scale of seconds would be required.

An important feature of the analysis presented here is the use of vector data in the form of the feeding curve to determine the distances between meals. The use of vector data to compare meals has its advantages and disadvantages. A disadvantage of using this approach to detect nociception is that the direction of change obtained by subtracting two feeding curves is a multidimensional value whose interpretation is not as straightforward as the interpretation of meal differences based on meal duration. Another disadvantage of specifically using resampling to obtain a p -value is that using a random sample of the permutations (versus complete set of all possible permutations) to obtain the distribution of a statistic requires significant computational power to compensate for additional Type I error. One can use Pearson type III moment approximations to avoid additional Type I error and reduce computational burden at the expense of losing precision in estimating p -value outside of 0.005 p 0.10 range (Mielke & Berry, 2007).

An advantage of the method for comparing meals using distances between feeding curves is that using vector data allows for a more comprehensive comparison which reveals more fine-grained distinctions as more data are used: two meals can be distinguished as long as they are not identical. Another advantage is that the method is non-parametric and, thus, does not require one to make unrealistic assumptions about data normality. In addition, the permutation method is very flexible and allows for the use of different kinds of scalar distance function and gives a choice of how the data reduction is performed. The use of greater amount of information from the experiments combined with the statistical procedure that does not make unwarranted assumptions contributed, along with strong nociceptive effects, to the extremely low p -values obtained in the test show the method has distinct advantages.

In conclusion, comparison of injured and control groups using a permutation method on intra-meal distances detects the nociception effects with much lower Type I error rates and detects the presence of injury for twice as long as the comparison of average daily meal durations alone. CFA-induced injury results in even reduction of intra-meal feeding rate and lengthening of the meals. Comparison of intra-meal feeding rates using permutation test on

distances between intra-meal feeding rate curves may prove useful in detecting chronic conditions of TMJ nociception in the rat.

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Figure 1.
P – values for permutation test on inter-meal distances (solid line) and *t*–test on meal durations (dashed line) for Experiment 1. Horizontal dotted line is the 0.01 significance level after Bonferroni correction (0.00023). Day 22 is excluded due to extensive rat handling. Note log-scale for *p*–values.

Figure 2. Median meal rates (solid line) with upper and lower quartiles (dotted line) in Experiment 1, days -2, 5, 19, and 40. x-axis: intra-meal time, s; y-axis: meal rate, pellet/s. Blue color: saline-injected animals; red color: CFA-injected animals. The median rate and quartiles were smoothed with running median filter with 55 s window.

Figure 3.
P – values for permutation test on inter-meal distances (solid line) and *t*–test on average daily meal durations (dashed line) for Experiment 2. Horizontal dotted line is the 0.01 significance level after Bonferroni correction (0.001). Note log-scale for *p*–values.

Figure 4.

Median meal rates (solid line) with upper and lower quartiles (dotted line) in Experiment 2, days -2, 0, 5, and 7. x-axis: intra-meal time, s; y-axis: meal rate, pellet/s. Blue color: saline-injected animals; red color: CFA-injected animals. The median rate and quartiles were smoothed with running median filter with 55 s window.