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ABSTRACT

Temporomandibular arthritis will lengthen a rodent's meal duration. We hypothesized that meal duration would also lengthen after tooth pulp exposure, suggesting that this behavior could be used to measure tooth nociception. To test this hypothesis, we placed rats in feeding units and subjected 4 anterior mandibular molars to pulp exposure, with and without pre-treatment with the analgesic buprenorphine-HCl. In the first study, male Sprague-Dawley rats were placed in computerized sound-attenuated feeding modules, the pulp of 4 molars on the mandible were exposed, and meal duration was measured for 13 days. In a second study, rats were injected with either the analgesic buprenorphine-HCl or saline every 12 hrs; injections were started one day before pulp exposure. Meal duration was determined before and after treatment. In the first study, pulp exposure significantly increased daily meal duration for 8 days. In the second study, pulp exposure lengthened daily meal duration, but the group that was treated with buprenorphine-HCl showed no significant difference compared with control rats without pulp exposure. Evidence supports that a lengthening in meal duration is a response to tooth nociception and that this nociception can be measured for over a week.

KEY WORDS: orofacial, pain, meal duration, toothache, necrotic, persistent.

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A Non-invasive Model for Measuring Nociception after Tooth Pulp Exposure

INTRODUCTION

The pain or hypersensitivity associated with damage and or inflammation of the tooth has been under active investigation (Khan and Hargreaves, 2010; Fried *et al.*, 2011), but our understanding of the mechanisms involved is incomplete, in part, due to a lack of appropriate animal models.

Several models have been used to investigate pulp pain, including: biting duration (Foong *et al.*, 1982); bite force (Khan *et al.*, 2008); dental injury and weight loss (Byers and Närhi, 1999); jaw-opening reflex (Rajaona *et al.*, 1986); jaw electromyographic activity (Sunakawa *et al.*, 1999); tongue protrusion directed to the injured tooth (Chattipakorn *et al.*, 2002); behavioral changes in abnormal head movement, shaking of lower jaw, and rubbing of the mouth (Chidiac *et al.*, 2002); exploring, freezing, and yawning (Chudler and Byers, 2005); and indirect measures of nociception with Von Frey filaments or heat (Le Bars *et al.*, 2001).

These models have given us insights into the mechanisms of tooth pain, but they also have limitations. One of the original models utilized, the jaw-opening reflex, has been suggested to be unreliable (Mason *et al.*, 1985) or imprecise (Rajaona *et al.*, 1986). The bite duration model required restraining the animal from normal activity (Foong *et al.*, 1982), which can cause stress and thus produce findings with questionable physiological significance. Other models give gross effects, *i.e.*, body weight changes (Byers and Närhi, 1999). The electromyographic activity method (Sunakawa *et al.*, 1999) typically requires that the animal be unconscious, although one study utilized freely moving rats (Boucher *et al.*, 1993). Most behavioral models used to date had a limited daily window of nociception recording, *i.e.*, from min to hrs *per day*, with the nocturnal rats being placed in a test chamber during the light phase, which can cause stress in the animal (Chattipakorn *et al.*, 2002; Chidiac *et al.*, 2002; Chudler and Byers, 2005). In both conscious humans and rats, bite force has been used as a reliable measure for tooth pain and nociception, but the rat model requires training and/or restraint (A Khan *et al.*, 2007a,b; J Khan *et al.*, 2008). Measures of surface nociception by Von Frey filaments or heat may not be related to the deep orofacial pain associated with pulp exposure.

Over the past 12 yrs, Bellinger's group has developed a non-invasive animal model to study deep temporomandibular joint (TMJ) nociception. This model uses unrestrained rats that are continuously kept in sound-attenuated, computerized feeding modules for days to weeks to record meal patterns (Kerins *et al.*, 2005; Kramer *et al.*, 2010). We have shown, in male and female rats and male mice, that meal duration can be used as a non-invasive biological marker for TMJ nociception for up to 19 days in rats and 6 days in mice (Kerins *et al.*, 2003b, 2004; Bellinger *et al.*, 2007; Kramer *et al.*, 2010). Acute or more persistent TMJ arthritis was shown to affect the rat/mouse, such that when the animal initiated a meal, the animal ate more slowly, which significantly lengthened the

meal duration. When the TMJ arthritis was reduced by pharmacological intervention, meal duration returned to normal (Kerins *et al.*, 2003b, 2004; Bellinger *et al.*, 2007). Patients experiencing TMJ pain also have longer chewing cycles, and the cycle length is attenuated when TMJ pain is diminished (Hansdottir and Bakke, 2004; Bakke and Hansdottir, 2008; Pereira *et al.*, 2009). The lengthening of meal duration during TMJ pain is a “guarding behavior”, which, it can be argued, is an operationally defined nociceptive behavior (Sternberg and Wachterman, 2000).

Here we tested the ability of this non-invasive, quantitative model to measure tooth nociception. In this study, the pulp was exposed bilaterally on mandibular molars in the rat, and the change in meal duration was measured to determine if meal duration lengthened. The time-course of the response was also recorded and compared with known changes in exposed pulp morphology with time. Last, we assessed the effect of an analgesic on the nociceptive response after pulp exposure.

MATERIALS & METHODS

General

The Baylor College of Dentistry Institutional Animal Care and Use Committee approved the experimental protocol. Male (250 g) Sprague-Dawley rats (Harlan Industries, Houston, TX, USA) were kept on a 12:12-hour light/dark cycle, with lights on at 0800 hrs. They were acclimated to the feeding modules and gentled by being handled for 7 days before surgery. The rats were given food and water *ad libitum*.

Meal Duration Assay

The rats were housed individually in sound-attenuated chambers equipped with photobeam computer-activated pellet feeders (Med Assoc. Inc., East Fairfield, VT, USA) and given 45 mg rodent chow pellets (Product No. FO 165, Bioserv, Frenchtown, NJ, USA). When a rat removed a pellet from the feeder trough, a photobeam placed at the bottom of the trough was no longer blocked, signaling the computer to drop another pellet. The computer recorded the date and time and kept a running tally of the total daily food consumption. In these analyses, a meal was defined based on a 10-minute end-of-meal criterion (*i.e.*, a meal was bracketed before and after by a 10-minute period of no pellets being taken), and the minimum meal size was set at 135 mg [*i.e.*, 3 pellets] (Castonguay *et al.*, 1986). The average daily meal duration, food intake, and meal size were then calculated. The computer record of pellets dropped over time established the meal duration, which is a continuous non-invasive biological marker of orofacial nociception (surface and deep) in undisturbed male and female rats (Kerins *et al.*, 2003a, 2005).

Trial 1 — Nociceptive Measurement for 13 Days

Meal patterns and body weights were recorded on the day prior to surgery and thereafter. On the day of surgery, all rats were lightly anesthetized with ketamine/xylazine at 60% of a surgical dose (54 mg/kg; 0.5 mg/kg, respectively). A bilateral pulp exposure was performed in the mandibular first and second molars of 7 rats. Briefly, we used surgical loupes to expose the pulp bilaterally on

the occlusal surface, using a #1/4 tungsten round bur (0.5 mm diameter) without water (Type III injury; Byers and Närhi, 1999) and confirmed exposure by the presence of hemorrhage from the cavity (observations were aided by magnification). Eight anesthetized rats served as controls. The rats were returned to the modules, and meal duration was recorded for 13 days, a time frame in which complete necrosis of the pulp can be observed after exposure (Kakehashi *et al.*, 1965; Khayat *et al.*, 1988).

Trial 2 — Analgesic Treatment

Meal patterns were recorded on the day prior to drug injection (day-1) and for a day after buprenorphine or saline injection (day 1) and for a day after pulp exposure (day 2). Twenty-four hrs prior to pulp exposure (beginning of day 1), the rats were removed from the feeding modules and received a subcutaneous injection of 0.3 mg/kg of buprenorphine base (0.3 mg/mL, Hospira Inc., Lake Forest, IL, USA) or the same volume of saline. These buprenorphine or saline injections were completed every 12 hrs over the next 48 hrs of the experiment (days 1 and 2). Twelve hrs after the onset of administration of buprenorphine or saline (beginning of day 2), the rats were subdivided so that half received pulpal exposures created in the mandibular first and second molars as described previously (*i.e.*, Trial 1) or were untreated (*i.e.*, control). The rats were then placed in the feeders, and the meal duration was recorded over the following 24 hrs (day 2). All rats underwent general anesthesia. There were 5 animals *per* treatment group.

Tissue Preparation

The mandibles of 2 rats that were not treated and the mandibles of 2 rats that had pulp exposure in Trial 1 and the mandibles of 2 rats that had pulp exposure in Trial 2 were isolated and stained. To complete the staining, we removed the mandibular region after the animals' death, placed it in 10% buffered formalin for several days, demineralized it in 0.5% EDTA, and then embedded it in paraffin. The tissues were processed into serial 6- μ m sections on superfrost plus slides (StatLab, Lewisville, TX, USA) and stained with hematoxylin and eosin.

Statistics

To determine changes in meal patterns after pulp exposure, a two-way ANOVA was performed. The independent variables were pulpal exposure and time. The dependent variable for this experiment was either meal duration, food intake, meal size, or body height. The analgesia data were analyzed by three-way analysis of variance, with the independent variables being drug treatment, pulpal exposure, and time. The dependent variable was meal duration. The data found to have significant main effects were further analyzed by Bonferroni correction.

RESULTS

Histology

The pulp from Trial 1 showed inflammation, abscesses, and extensive pulp necrosis 13 days after exposure that extended into the apical areas, and foreign material was present in the area

of the perforation (Fig. 1A). The pulp from Trial 2 showed the same characteristics, except the inflammation and pulp necrosis did not extend into the apical areas (Fig. 1B). Fig. 1C is from a control animal.

Trial 1 — Nociceptive Measurement for 13 Days

Meal duration (Fig. 2) of the experimental and control groups in Trial 1 was similar the day prior to surgery (day 1), but significantly increased in the experimental group starting the day after surgery (day 1), and this increase continued through day 8 [group effect, $F(1,13) = 7.95$, $p < 0.02$]. Food intake [$F(1,13) = 0.10$, $p = 0.75$], meal size [$F(1,13) = 0.55$, $p = 0.46$], and body weight [$F(1,13) = 1.35$, $p = 0.26$] were not significantly affected by pulp exposure in Trial 1 (Figs. 3A, 3B, and 3C, respectively).

Trial 2 — Analgesic Treatment

Meal duration was similar in all rats prior to pulp exposure in Trial 2 (Fig. 4, day 1 and day 1). Administration of the drug buprenorphine did not significantly affect meal duration in Trial 2 (Fig. 4, day 1). Following surgery (post-surgery, day 2), the group that received saline showed a significant increase in meal duration, but the group that received buprenorphine was not significantly different from the group without pulp exposure (Fig. 4).

DISCUSSION

Analysis of the data from Trial 1 shows that meal duration was significantly increased for 8 days following bilateral mandibular pulp exposure of 4 molars before returning to control levels. These data fit well with those from previous studies showing dramatic pulpal necrosis within 8 days after pulp exposure (Kakehashi *et al.*, 1965; Khayat *et al.*, 1988). In Trial 2, we demonstrated that giving the analgesic buprenorphine prevented the increase in meal duration following pulp exposure in the molars. At high doses, buprenorphine increases food intake (Rudski *et al.*, 1995). Because of this known effect of buprenorphine on feeding, we had previously performed a dose-response study, and it was shown that buprenorphine did not appreciably affect meal duration at the dosage we administered in this study (Marr *et al.*, 2003). In addition, we included a buprenorphine control group that showed no effect of buprenorphine on meal duration.

The finding that buprenorphine analgesic normalized meal duration supports the conclusion that the lengthened meal duration in the saline-treated animals was due to pulp nociception and not some mechanical alteration. The analgesia effect is consistent with reports from previous pulp studies (Foong *et al.*, 1982; Sunakawa *et al.*, 1999) and somewhat analogous to longer chewing cycles in patients with TMJ disorders that were attenuated when the TMD pain was decreased (Bakke and Hansdotir, 2008).

Analysis of the data from Trials 1 and 2 indicates that meal duration measured nociception created by molar pulp exposure.

Figure 1. Hematoxylin and eosin stain of a sagittal section from a rat molar with and without a defect. The mandible was isolated, decalcified, sectioned, and stained. (A) An image of a molar from a representative rat in Trial 1 13 days after the defect was produced. (B) An image of a molar 48 hrs after the defect was produced, from a representative rat from Trial 2. (C) An image of an intact molar. Bar = 1 mm.

Figure 2. Daily meal duration before and after exposure of the rat mandibular first and second molars (Trial 1). Day 1 represents the meal duration 1 day before pulp exposure, and day 1 represents the meal duration for the first day after pulp exposure. Measurements were continued for 13 days after exposure. The "control" group was given general anesthesia on the beginning of day 1, but the pulp was not exposed. Means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, control vs. defect. Seven rats received the defect, and 8 were control rats.

The analysis also showed that tooth/pulp nociception can be measured over the course of days. Therefore, future investigators can take tissue samples from a rat at various times after pulp exposure knowing the nociceptive response (*i.e.*, lengthened meal duration) at that point in time. This should allow one to identify molecular mechanisms contributing to the nociceptive response for at least a week after exposure. With the level of nociception created in the present study, the results indicated that food intake, meal size, and body weight may not be reliable indicators for tooth nociception, because there were no significant changes in these feeding patterns.

It has been reported that the pain of TMJ patients increases with chewing in a time-dependent manner, and that the chewing cycle duration is lengthened (Stegenga *et al.*, 1993; Dao *et al.*, 1994; Gavish *et al.*, 2002; Karibe *et al.*, 2003; Bakke and

Figure 3. Food intake, meal size, and body weight of rats from Trial 1. In panels **A** and **B**, Day 1 represents the food intake and meal size 1 day before pulp exposure, and day 1 represents the food intake and meal size for the first day after pulp exposure. Measurements were continued for 13 days after exposure. We calculated the bodyweight values shown in panel **C** by subtracting the body weight of the rats after pulp exposure (day 1 or 2 or 3, etc.) from the pre-day (day-1) body weight. The "control" group was given general anesthesia on the beginning of day 1, but the pulp was not exposed. Means $\pm$ SEM. Seven rats received the defect, and 8 were control rats.

Figure 4. Histogram representing daily meal duration data obtained from male Sprague-Dawley rats after pulp exposure, with and without injection of buprenorphine-HCl (Trial 2). Rats were injected with buprenorphine (0.3 mg/mL) or saline (beginning of day 1), and then 24 hrs later, bilateral pulp exposures (defect) were made in the first and second molars of the mandible (beginning of day 2). Buprenorphine or saline injections were given every 12 hrs starting on day 1, and the injections were continued throughout day 2. The "control" groups were given general anesthesia on the beginning of day 2, but the pulp was not exposed. Bar graph represents the mean $\pm$ SEM. Five rats were analyzed for each treatment group. ** $p < 0.01$, Saline + Defect vs. Buprenorphine + Defect.

Hansdotir, 2008). Our model may allow for similar testing in rats. Our feeding modules and computer programs allow us to determine inter-pellet interval during the course of eating a meal. If molar tooth pulp nociception increased during the process of chewing, the model would be able to quantitate this, since one would hypothesize that intra-pellet intervals would increase later in the meal vs. at the beginning of the meal, suggesting that nociception was increasing. If true, this would also allow for a more sophisticated way of taking samples to study the mechanisms and progression of pulp pain. This possibility will be addressed by our group in future studies using both male and female rats.

In conclusion, the use of meal duration to measure pulp nociception in rats offers several advantages over other methods in use today: (1) Our method is quantitative, while many of the other methods are subjective in nature and thus prone to errors; (2) eating is a behavior requiring cortical participation and thus distinguishes it from reflex measurements, such as, scratching, von Frey hairs, or heat; (3) the animal does not have to be trained prior to testing; (4) after pulp exposure, the animal is no longer handled or immobilized during measurement, thus reducing further compounding stresses and alternate behaviors; (5) testing continues 24 hrs a day, thereby eliminating any artifacts of testing in the light phase, when the rodent normally sleeps; (6) testing can continue in the undisturbed animal for days as

opposed to other methods that test only in short windows of time; and, importantly, (7) no artificial test-induced competing behaviors are generated. All of these factors make the meal duration measurement a powerful tool for studying the mechanisms of pain following molar pulp exposure.

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