Title
The dolognawmeter: A novel instrument and assay to quantify nociception in rodent models of orofacial pain

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The dolognawmeter: A novel instrument and assay to quantify nociception in rodent models of orofacial pain

by

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THESIS

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[redacted]

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Dr. John Dolan's role in co-authored publication

The text of this manuscript is a reprint of the material as it appears in *Journal of Neuroscience Methods 187 (2010)207-215* as an article entitled, "The dolognawmeter: a novel instrument and assay to quantify nociception in rodent models of orofacial pain." Dr. John Dolan is the first author. The remaining co-authors of the original publication include David Lam, Stacy Achdjian, and Brian Schmidt. The manuscript describes a device and assay that Dr. Dolan invented and fabricated to quantify a behavioral index of orofacial nociception in rodents. He then performed many of the early experiments. He and I planned and directed all of the experiments. He interpreted the data and wrote the manuscript. He also wrote the revision of the manuscript. Dr. Dolan's innovation and labor in the project is comparable to the work required for a PhD thesis.

Brian L. Schmidt
Research advisor

The dolognawmeter: A novel instrument and assay to quantify nociception in rodent models of orofacial pain.

John C. Dolan

ABSTRACT

Rodent pain models play an important role in understanding the mechanisms of nociception and have accelerated the search for new treatment approaches for pain. Creating an objective metric for orofacial nociception in these models presents significant technical obstacles. No animal assay accurately measures pain-induced orofacial dysfunction that is directly comparable to human orofacial dysfunction. We developed and validated a high throughput, objective, operant, nociceptive animal assay, and an instrument to perform the assay termed the dolognawmeter, for evaluation of conditions known to elicit orofacial pain in humans. Using the device our assay quantifies gnawing function in the mouse. We quantified a behavioral index of nociception and demonstrated blockade of nociception in three models of orofacial pain: (1) TMJ inflammation, (2) masticatory myositis, and (3) head and neck cancer. This assay will be useful in the study of nociceptive mediators involved in the development and progression of orofacial pain conditions and it will also provide a unique tool for development and assessment of new therapeutic approaches.

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1. INTRODUCTION

More than 20% of adults are afflicted by orofacial pain (Lipton et al., 1993). Some of the most severe and difficult to treat forms of orofacial pain result from temporomandibular joint (TMJ) disorders, masticatory muscle inflammation and head and neck cancer. Patients with TMJ disorders or masticatory muscle inflammation report that chewing induces the highest levels of pain (Bush et al., 1989; Dworkin et al., 1990; Fricton et al., 1985; Gavish et al., 2000; Winocur et al., 2001; Zarb and Thompson, 1970). Patients with TMJ disorders experience significant and prolonged pain compared to normal subjects after chewing for an extended period of time (Karibe et al., 2003). In patients with masticatory myositis, jaw opening and closing while chewing significantly exacerbates muscle pain (Bowley and Gale, 1987; Christensen, 1976; Christensen and Radue, 1985; Dao et al., 1994; Molin, 1972; Plesh et al., 1998; Scott and Lundeen, 1980). Duration of chewing is associated with development of pain. Patients with TMJ and masticatory muscle pain experience a significant increase in pain after chewing for 9 min (Gavish et al., 2002). The American Academy of Orofacial Pain recommends resting the jaw and limiting jaw movement in patients with TMJ or masticatory muscle pain (Pain).

Like joint and muscle disorders, head and neck cancer causes functional allodynia (Connelly and Schmidt, 2004; Kolokythas et al., 2007). Patients with head and neck cancer report pain as the worst symptom (Shedd et al., 1980), and the primary determinant of a poor quality of life (Chaplin and Morton, 1999; Hodder et al., 1997). In their final months of life 85% of these patients report pain as the most common problem (Shedd et al., 1980). Half of all head and neck cancers are incurable and many patients suffer from intractable cancer pain for extended periods. Oral cancer patients experience significant debilitation of chewing and oral function secondary to pain (Connelly and Schmidt, 2004; Rogers et al., 2006). To investigate orofacial pain and

ultimately develop treatments for patients we have created an assay that elicits and measures pain-induced oral dysfunction in an animal model.

Masticatory dysfunction is one of the hallmarks of orofacial pain. An objective animal assay that quantifies nociception-induced dysfunction, as seen clinically, is not available. We hypothesize that nociception-induced gnawing dysfunction can be used as an index of orofacial nociception in an animal model. Gnawing involves incisor occlusion and molar disclusion but shares nearly all of the anatomical and physiological components of chewing in humans. To test our hypothesis we designed and validated a novel assay and device (a dolognawmeter-dolor for pain, gnawmeter for measurement of gnawing) that together objectively quantify gnawing function in mice. We quantified a behavioral index of nociception and demonstrated blockade of nociception in three models of orofacial pain: (1) TMJ inflammation, (2) masticatory myositis, and (3) head and neck cancer.

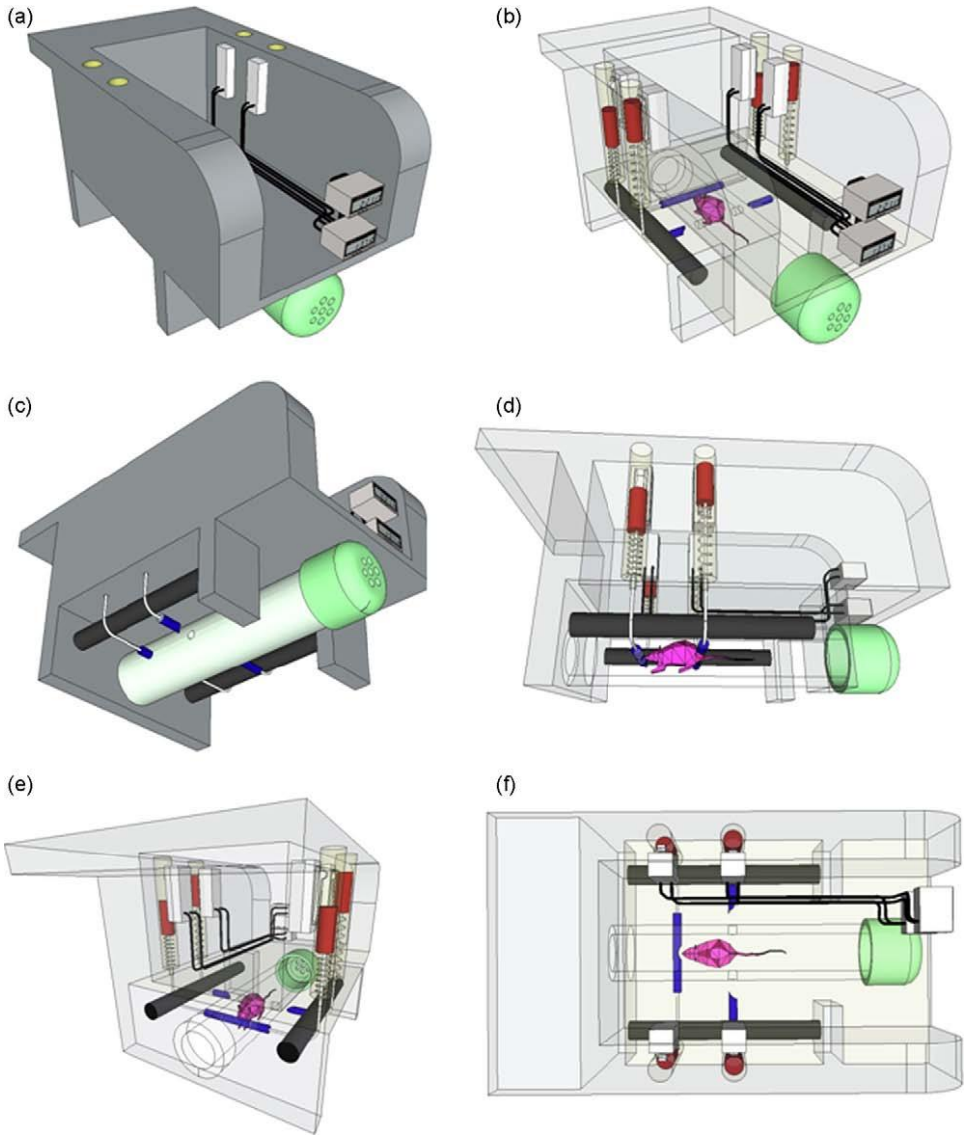

Fig. 1. Three-dimensional schematic of a dolognawmeter. (a) Opaque quarter rear view. Removal of the green cap allows loading of the mouse into the device. (b) Transparent view in quarter rear view showing the mouse gnawing the second dowel after severing the first dowel. The dowels are attached to springs and retracted once severed. The second timer automatically starts once the first dowel is severed. (c) Opaque bottom view of device. (d) Transparent side view with mouse in the confinement tube. The first dowel has been severed and the mouse immediately starts gnawing the second dowel. (e) Transparent quarter frontal view. Once the mouse gnaws through the second dowel the mouse is able to move free of the confinement tube and has access to the standard cage housing the dolognawmeter. (f) Transparent view from above.

2. MATERIALS AND METHODS

2.1. Design of the dolognawmeter

The dolognawmeter is designed for high throughput. Experimental productivity is limited only by the number of devices employed. A dolognawmeter is fabricated as described in Figure 1 and 2.

The confinement tube is manufactured from a 180 mm section of commercially available schedule-80 polyvinyl chloride (PVC) tubing with an internal diameter of 24 mm and an external diameter of 33 mm. A set of radially oriented holes is drilled entirely through the tube at 30 mm and 50 mm from the end of the tube (opposite the end-cap). These two sets of holes are oriented parallel to each other and 90° to the long axis of the tube. These cross-drilled holes are appropriately sized to allow the obstructing dowels to freely pass through.

The confinement tube is sized to accommodate the mouse but not allow the animal to turn around head-to-tail or escape over or under the obstacle (dowel) even when a portion of the dowel is gnawed away. Only when the mouse completely severs the dowel can it escape from the tube.

A removable, threadless, perforated PVC end-cap forms a friction fit over the end of the tube. The end-cap is removed to load the mouse head-first into the tube and subsequently replaced to confine it between the end-cap and dowels.

Two dowels in series are used as obstacles to confine the mouse. The dowels are inserted through the radially oriented, cross-drilled holes perforating the confinement tube wall.

The first obstacle is a cross linked polyethylene foam (XP-60, Exemplary Foam, Elkhart, IN) dowel with a diameter of 9 mm and a length of 50 mm. The foam exhibits a Shore-A durometer of 45 (Rex Gauge Co., Carpentersville, IL). Using a drill press and coring drill, the foam dowel is cored from a 50 mm thick sheet of flat-stock. The coring bit was custom manufactured from an

appropriately sized seamless, thin-walled, 304 stainless steel tube. The second obstacle is an ethylene vinyl acetate (EVA) resin dowel with a diameter of 7 mm and a length of 50 mm. The EVA resin exhibits a Shore-A durometer of 70. Round-stock material in a 7 mm diameter is available commercially as a glue stick (Ace Hardware, E. Greenwich, RI, item #2013605).

Separate spring-loaded pistons attach to the ends of the dowels with a metal hook on the end of a 1.5 mm stainless steel cable. The pistons immediately retract a dowel laterally from the tube once the animal gnaws through it. Retraction of a severed dowel permits forward movement of the mouse. Timers are actuated by the motion of the pistons once a dowel is severed. A separate timer automatically records the time required to sever each dowel.

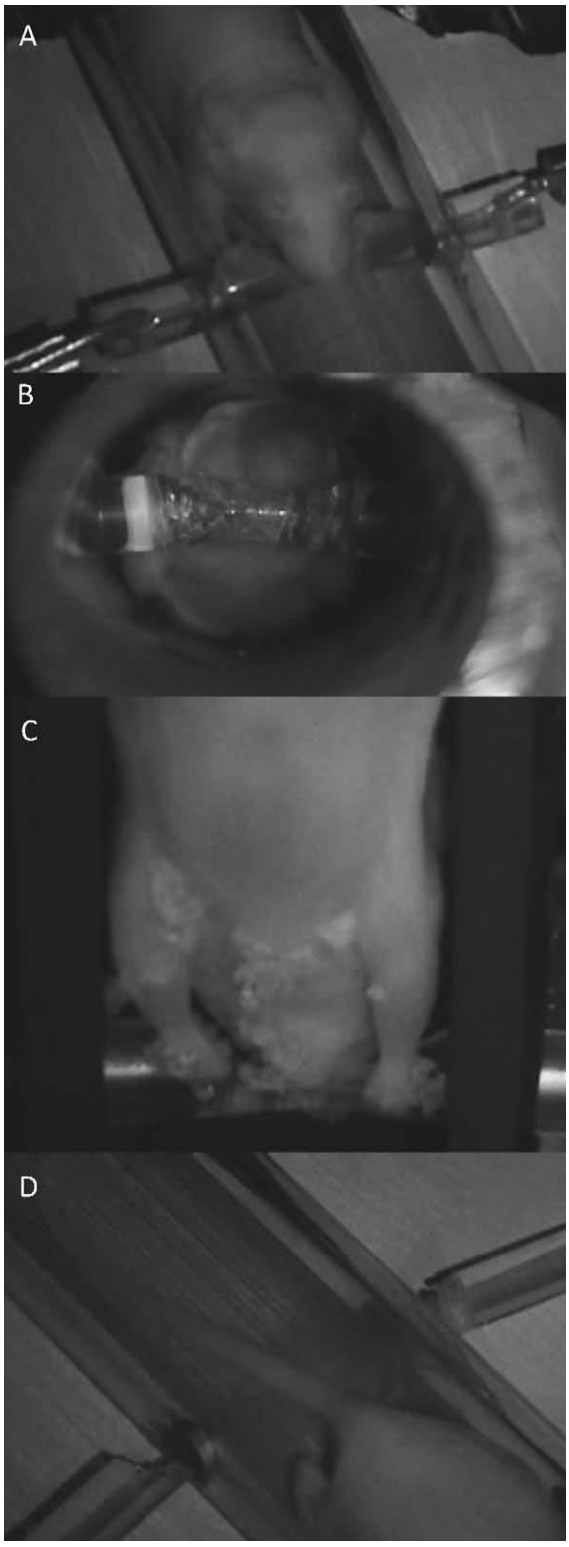

Fig. 2. Infrared photographs of mouse gnawing through the dowel and escaping from a transparent version of the dolognawmeter.

(a) Overhead view of mouse gnawing through dowel. With training, mice learn to gnaw through the dowel in a "V" shaped pattern.

(b) Frontal view of mouse in the dolognawmeter gnawing through a dowel.

(c) Inferior view of mouse gnawing through dowel. Note that the mouse does not consume the detritus (white flakes) from the dowel.

(d) Once the mouse gnaws through the dowel, springs immediately retract the severed dowel and allow the mouse to exit the dolognawmeter (see supplemental video).

The mouse dolognawmeter is designed to fit into a standard clean facility mouse cage (Super Mouse Micro-Isolator ™ 750, Lab Products, Inc., Seaford, DE) so that experiments minimize stress and decrease the risk of disease transmission in immunocompromised mice. The dimensions of the mouse dolognawmeter are approximately 17 cm wide, 13 cm high and 29 cm long. To further reduce the risk of disease transmission and to aid in clean-up, the device isolates the mouse from the timers and dowel retraction mechanism. Thus, during a gnawing session the mouse contacts only the internal surfaces of a removable confinement tube, the internal surface of the end-cap, the obstructing dowels, and the external face of the device that is perforated to accommodate the confinement tube. Once the mouse escapes from the confinement tube, it gains access to a truncated portion of its regular cage where the acrylic face of the dolognawmeter creates one of the walls. All surfaces in contact with the animal can be rapidly cleaned and disinfected. Due to the configuration of the active cage ventilation system and the design of the dolognawmeter, fresh air circulates through the confinement tube and exits behind the mouse for the duration of the experiment. This configuration prevents overheating of the mouse in the small, thermally insulated confines of the tube. If less airflow through the tube is desired, a non-perforated end-cap can be used.

A mouse is loaded into the dolognawmeter by holding the animal at the base of the tail while presenting the open confinement tube to the animal. The mouse instinctually climbs head-first into the tube. Once the mouse is loaded and the end-cap is placed on the back of the tube, the timer dedicated to the first dowel is manually started. At this stage the

entire cage containing the device is then placed into a standard cage rack and no further investigator-animal interaction occurs.

The mouse is confined anteriorly by a series of two dowels that have been placed horizontally through the tube, perpendicular to the long axis, and spaced 20 mm apart. Pistons activated by springs place each dowel under tension at right angles to the long axis of the confinement tube. The tensioning system attaches to both ends of a dowel with a separate cable and hook. When the animal severs the first dowel, pistons attached to the external ends of the dowel retract it laterally from the tube. At the same time, this set of pistons actuates a switch to stop the timer dedicated to the first dowel (the timer that was manually started when the animal was first loaded into the tube). Simultaneously, these pistons start the timer dedicated to the second dowel. Since the first dowel is now retracted, the mouse moves forward to begin gnawing on the second dowel. After the second dowel is severed, it is retracted by a second set of pistons. These pistons simultaneously actuate a switch to stop the timer dedicated to the second dowel. The mouse is then free to escape from the confinement tube and gain access to a truncated portion of the regular cage. Once the mouse is free, the trial is complete, and the time required to gnaw through each dowel has been recorded separately. Only the gnaw-time for the second dowel is used as the outcome variable for the experiments described in the present study.

2.2. Behavioral testing of the mice in the dolognawmeter

Mice were housed in a temperature-controlled room on a 12:12 h light cycle (0600–1800 h light), with unrestricted access to food and water. All procedures were approved by the University of California San Francisco Committee on Animal Research, and researchers were trained under the Animal Welfare Assurance Program.

Behavioral testing in the dolognawmeter was performed at approximately 1900 h. To allow for adequate recovery time, mice were never tested on consecutive days. The first 10 gnawing trials for an animal are termed "training" trials and allow the mice to learn to consistently gnaw through the series of two dowels in an efficient "V" pattern. Mice are often slower to escape from the device in the first few training trials. All of the mice we have tested (n = 200) begin to gnaw in a consistent "V" pattern in the first few training trials. They produce the same "V" pattern in all subsequent trials and thus accomplish the task with a quantum of gnawing effort each trial. A baseline gnaw value was established for each animal as the mean of the gnaw-times to sever the second dowel during the final three training sessions. The general experimental approach for each of the three orofacial pain models involved the following sequence:

(1)Ten training trials in the dolognawmeter over twenty days

(2)Baseline gnawing function is operationally defined as the mean gnaw-time to sever the second dowel for the last three training trials.

(3) Creation of the orofacial pain model (see below).

(4) Assessment of gnawing function relative to the baseline.

(5) Assessment of gnawing function in a separate group of animals, after creation of the model and administration of an analgesic.

All three models were produced using adult female mice that were 4–5 weeks in age (16–20 g) before training. Estrous cycles were not monitored. After baseline values were determined, the pain models were established. For all injection procedures below, mice were briefly sedated with inhalational anesthesia (O2: 1 L/min; isoflurane: 1.5–2.5%) and body core temperature was maintained within the physiological range of 37–37.5 °C.

2.3. TMJ inflammatory pain model

The TMJ inflammatory pain mouse model was produced in FVBN mice (Charles River Laboratories, Hollister, CA) and gnawing function was compared in three subgroups: (1) CFA injection into the left TMJ, (2) saline control injection into the left TMJ, and (3) indomethacin pretreatment prior to CFA injection into the left TMJ. A similar experimental paradigm was applied to all subgroups. After establishing baseline gnaw values a 27-gauge, 1/2 in. long needle (Becton Dickinson & Co., Franklin Lakes, NJ) was inserted through the facial skin. The needle was advanced superiorly and anteriorly until the zygomatic arch was identified with the tip of the needle. The needle was then walked inferiorly until it passed the edge of the arch and dropped into the joint space. Once in the joint space, 25 μL of complete Freund's adjuvant (CFA; Sigma Chemical Company, St. Louis, MO) or saline control was injected over a 5-s period into the left TMJ.

The effect of a nonsteroidal anti-inflammatory drug on CFA-induced changes in gnaw responses was also studied to confirm that the measured behavior is affected by nociception. Indomethacin was injected intraperitoneally (5 mg/kg; Cayman Chemical, Ann Arbor, MI) 30 min prior to CFA injection into the left TMJ in one subgroup of mice. Dolognawmeter measurements were then made in awake mice at 10 h post-injection of CFA and every 48 h thereafter.

In a further series of experiments, a separate group of mice was injected as described above with CFA into the left TMJ and saline control into the right TMJ for histopathological analysis of inflammation. At 10 h post-injection, these mice were euthanized with CO2 and cervical dislocation and their TMJ tissues harvested for histopathology with hematoxylin and eosin (H and E) staining. The injected TMJ was removed en bloc, placed in 10% buffered formalin and demineralized. Five micrometer sections were cut and stained. A sagittal section midway between the medial and lateral pole of the condyle was selected and reviewed for histologic evidence of inflammatory infiltrate.

2.4. Masticatory muscle inflammatory pain model

The masticatory muscle inflammatory pain mouse model was produced in FVBN mice (Charles River Laboratories, Hollister, CA) and gnawing function was compared in three subgroups: (1) CFA injection into both masseter muscles, (2) saline control injection into both masseter muscles, and (3) indomethacin pretreatment prior to CFA injection into

both masseter muscles. A similar experimental paradigm was applied to all subgroups. After baseline gnaw values were established for each animal, a 25-gauge, 5/8 in. long needle (Becton Dickinson & Co., Franklin Lakes, NJ) was inserted through the facial skin and upon entering the masseter muscle, 50 μL of CFA (Sigma Chemical Company, St. Louis, MO) or saline control was injected over a 5-s period. Both the right and left masseter muscles were injected. The effects of a nonsteroidal anti-inflammatory drug on CFA-induced changes in gnaw response was also studied by injecting indomethacin intraperitoneally (5 mg/kg; Cayman Chemical, Ann Arbor, MI) 30 min prior to CFA injection into the masseter muscles in one subgroup of mice. Dolognawmeter measurements were then made in awaken mice at 10 h post CFA injection and every 48 h thereafter.

In addition, a separate group of mice was injected as described above with CFA into the left masseter muscle and vehicle control in the right masseter muscle for histopathological analysis of inflammation. At 10 h post-injection, these mice were euthanized with CO2 and cervical dislocation and their masseter muscles harvested for histopathology. Five micrometer sections were cut and stained with H and E.

2.5. Head and neck cancer model

Squamous cell carcinoma (SCC) was produced in BALB/c (Charles River Laboratories, Hollister, CA) athymic, immunocompromised mice with the orthotopic head and neck cancer model we previously described (Ramos et al., 2002). The anatomic and functional

features of this mouse cancer model parallel those found in human patients with head and neck cancer.

Gnawing function was compared between three subgroups:

(1) SCC injected into the floor of mouth with subsequent tumor development,

(2) vehicle control injected into the floor of mouth (sham), and (3) morphine treatment of a group of animals that developed tumors in the floor of the mouth after inoculation with SCC.

A similar experimental paradigm was applied to all subgroups. After baseline gnaw values were established for each animal, mice were injected with either 3×10^5 cells of SCC in a vehicle consisting of a mixture of 60 µL of Dulbeco's modified Eagle's medium (DMEM) and 60 µL of Matrigel™ (Becton Dickinson & Co., Franklin Lakes, NJ) or (2) vehicle alone into the floor of the mouth through an extraoral approach. After induction of cancer in the tumor group, gnawing was measured on nonconsecutive days.

The sham-operated group was tested at the same time interval as the cancer group.

We evaluated morphine antinociception in the cancer group because this drug is the first-line treatment for pain in cancer patients. Once the mouse demonstrated a gnaw-time at least 2 standard deviations above its baseline systemic (intraperitoneal) morphine (20 mg/kg; Henry Schein, Indianapolis, IN) was administered 30 min before the mouse was loaded into the dolognawmeter for the next trial. Upon completion of all experimental trials, mice were euthanized with CO2 and cervical dislocation and the floor of mouth and submental tissues were harvested for histopathology with H and E staining.

2.6. Data analysis

For all three models, each animal served as its own control and gnawing function was reported as a percent change relative to baseline. Mann–Whitney U test, t-test, Log-Rank test and RM ANOVA-on-ranks were used as appropriate ($P < 0.05$ considered statistically significant).

3. RESULTS

3.1. TMJ inflammatory pain model

CFA injection into the left TMJ produced a mean 567.0 ± 273.8 (SEM) % increase in gnaw-time compared to the TMJ saline-injected model at 10 and 58 h following injection (Fig. 3). Pretreatment with indomethacin prevented the increase in gnaw-time seen in animals receiving only CFA injection into the left TMJ (Fig. 3). Histologic evaluation of the TMJ demonstrated that CFA injection produced an inflammatory infiltrate (Fig. 4a) while saline injection into the TMJ did not (Fig. 4b).

3.2. Masticatory muscle inflammatory pain model

The masseter myositis mouse model demonstrated a significant increase in gnaw-time 10 h after CFA injection (Fig. 5). The myositis model demonstrated a mean 112.8 ± 49.1 (SEM) % increase in gnaw-time compared to the saline-injected model at 10 h following injection. Pretreatment with indomethacin prevented the increase in gnaw-time seen in

animals receiving only CFA injection into masseter muscles (Fig. 5). Histologic evaluation of the masseter muscle injected with saline showed normal cytoarchitecture with no evidence of an inflammatory infiltrate (Fig. 6a). The masseter muscle injected with CFA demonstrated an inflammatory infiltrate characterized by polymorphonuclear leukocytes (Fig. 6b).

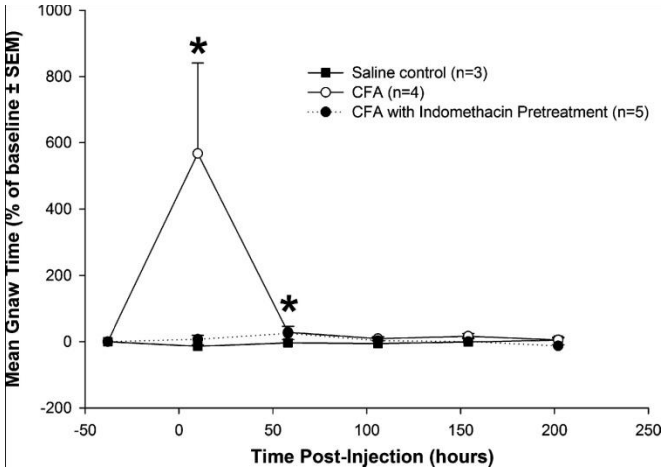

Fig. 3. Effect of temporomandibular joint (TMJ) inflammation on gnaw-time. CFA was injected into the left TMJ of mice and their gnaw-time was compared to mice with saline injected into the TMJ and indomethacin-pretreated mice receiving CFA injection into the TMJ. Gnaw-time was significantly increased at both 10 and 58 h post-CFA injection (white circles, n = 4) compared to saline injection (black squares, n = 3) into the TMJ ($^{*}P < 0.05$, Mann–Whitney U Test). Indomethacin pretreatment (5 mg/kg intraperitoneal) 30 min prior to CFA injection (black circles, n = 5) into the TMJ completely blocked CFA effects on gnaw-time increase ($^{*}P < 0.05$, Mann–Whitney U Test).

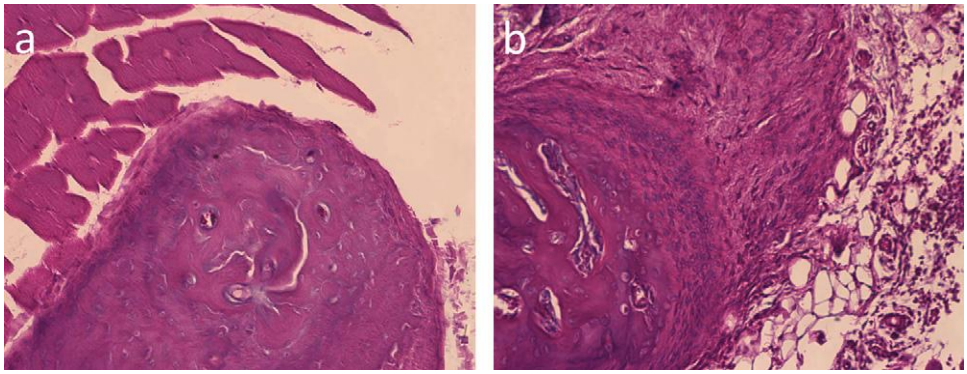

Fig. 4. Histologic evidence of CFA-induced inflammation in the TMJ. Histological sections of TMJ were obtained from mice 10 h post-injection of CFA into the left TMJ and control saline into the right TMJ (H and E stained, n = 3 per side). (a) Normal condyle and joint space of TMJ 10 h post-saline injection contrasts markedly with (b) abundant, acute and chronic inflammatory cells infiltrating the joint space and associated granulation tissue on the condylar surface. The histology results presented in (a) are representative of 3 control sides and the histology results in (b) are representative of 3 CFA-injected sides.

Mice that developed visible head and neck cancer by postinoculation day 7 (n = 4) were evaluated for behavioral changes. Mice with head and neck cancer demonstrated a significant increase in gnaw-time by post-inoculation trial #7 (Fig. 7). All animals exhibited a progressive increase in gnaw-time above baseline values. The threshold for failure to sever the second dowel (i.e. the maximum time allowed to completely sever the second dowel and escape) was operationally defined as 1000% of baseline. The University of California San Francisco Committee on Animal Research veterinarians recommended that an animal should not be confined for longer than 8 h. We used this guideline to determine the threshold for failure to sever the second dowel (i.e. the maximum time allowed to completely sever the second dowel and escape). Our preliminary studies demonstrated that the average baseline for the mice is 2620 s. We set the limit at 1000% because this duration is approximately equal to the maximum time animals are allowed to remain in the confinement tube (8 h). Animals were removed from the device if they reached this threshold. We evaluated the temporal relationship between tumor formation and gnaw failure (Fig. 8). All mice failed by post-inoculation gnaw trial 12. Histologic examination of the sham (control) animals, that received inoculation of SCC vehicle only, showed no evidence of carcinoma (Fig. 9a). Mice receiving inoculation of SCC showed carcinoma involvement of the dermis and muscle (Fig. 9b and c). Morphine demonstrated that the dolognawmeter could detect attenuation of nociception in the head and neck cancer model. When the mouse gnaw-time reached two standard deviations or more above its baseline gnaw-time, systemic morphine was administered 30 min before the mouse was loaded into the dolognawmeter for the next trial. Systemic morphine produced a significant decrease in mean gnaw-time (mean 293.2 ± 180.0 (SEM) %, $P < 0.05$, Mann–Whitney U test) (Fig. 10).

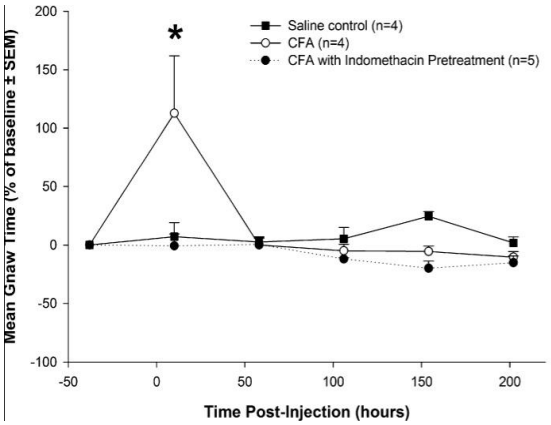

Fig. 5. Effect of masticatory muscle inflammation on gnaw-time. CFA was injected bilaterally into the masseter muscle of mice and their gnaw-time was compared to control saline-injected mice and indomethacin-pretreated mice receiving CFA injection into the masseter muscle. Gnaw-time was significantly increased 10 h post-CFA injection (white circles, n = 4) compared to saline injection (black squares, n = 4) into the masseter muscles (*P < 0.05, Mann–Whitney U test). Indomethacin pretreatment (5 mg/kg intraperitoneal) 30 min. prior to CFA injection (black circles, n = 5) into the masseter muscles completely blocked CFA effects on gnaw-time increase (*P < 0.05, Mann–Whitney U test).

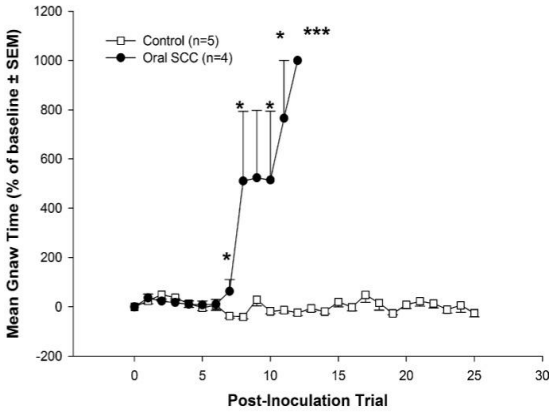

Fig. 7. Effect of oral SCC on gnaw-time. Oral SCC was innoculated into the submental region of mice and their gnaw-time was compared to control culture media-injected mice. Mean gnaw-time was significantly increased in oral SCC-innoculated mice (circles, n = 4) over time compared to mice with control injection (white squares, n = 5) into the submental region ($^{*}P < 0.05$, $^{***}P < 0.001$, Mann–Whitney U test). Mice that reached a 1000% increase in gnaw-time failed to gnaw through the dowel.

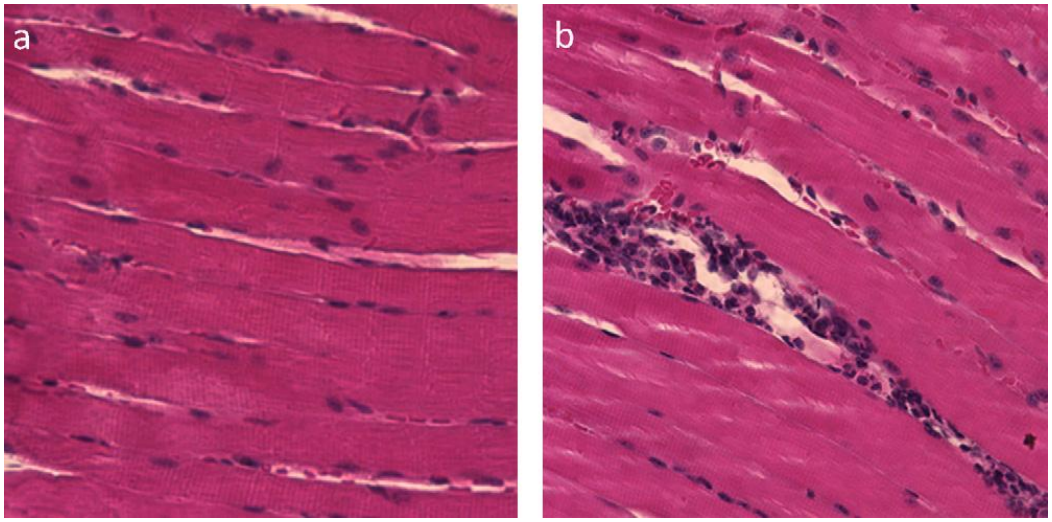

Fig. 6. Histologic evidence of CFA-induced inflammation in the masseter muscle. Histological sections of the masseter muscle were obtained from mice 10 h post-injection of CFA into the left masseter muscle and control saline into the right masseter muscle (H and E stained, n = 4 per side). (a) Normal skeletal muscle architecture of masseter muscle 10 h post-saline injection contrasts markedly with (b) abundant, acute and chronic inflammatory cells infiltrating deep into the muscle fibers. The histology results presented in (a) are representative of 4 control sides and the histology results in (b) are representative of 4 CFA-injected sides.

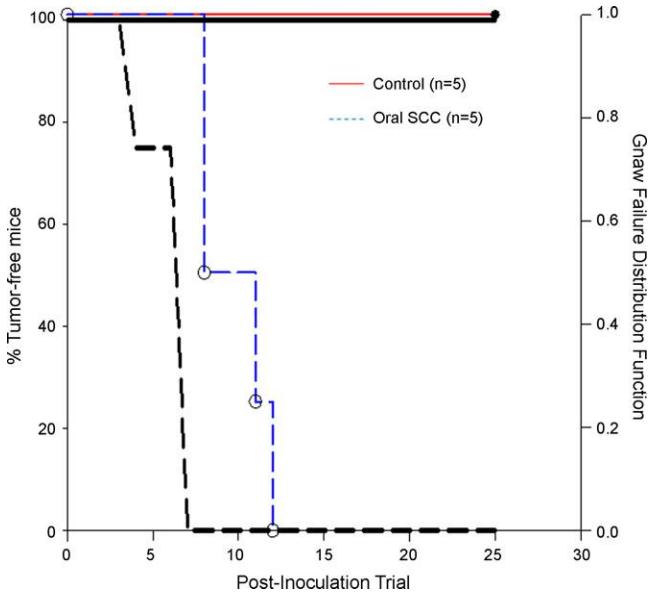

Fig. 8. Plot of tumor onset to gnaw failure in oral SCC-inoculated mice. Left y-axis: tumor onset in mice. Tumor formation was assessed by gross visible appearance in the submental region. Data represent the percentage of tumor-free mice calculated against the total number of mice in their respective control (solid black line, n = 5) and oral SCC-innoculated (dashed black line, n = 4) groups. Right y-axis: gnaw failure function. Data represent the distribution function of gnawing mice calculated against the total number of mice in their respective control (red line, n = 5) and oral SCC-innoculated (dashed blue line, n = 5) groups. The median time to tumor onset is post-inoculation trial 5 whereas the median time to gnaw failure in tumor-bearing mice is post-inoculation trial 8 ($P < 0.01$, Log-Rank Test).

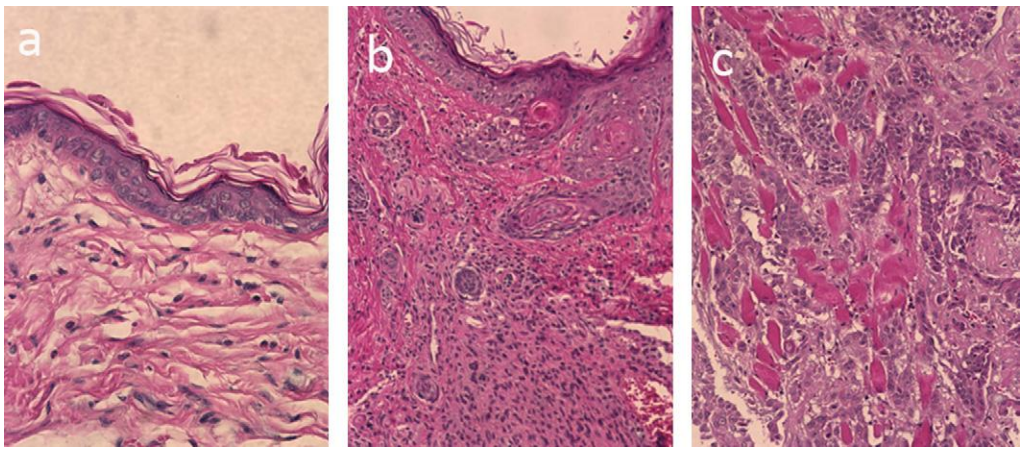

Fig. 9. Histologic evidence of invasive SCC induced by human oral SCC innoculation into the submental region of mice. Histological sections of skin from the submental region were obtained from mice inoculated with SCC (n = 8) and compared to those injected with control culture media (n = 5) in the submental region (H and E stained). (a) Example of normal epidermal/dermal architecture of submental skin 60 days post-culture media injection contrasts markedly with (b) which shows the histologic features of SCC-inoculated mice showing invasion of SCC into dermal layer (30 days post-HSC-3 innoculation) and (c) muscle (60 days post-SCC innoculation). The histology results presented in (a) are representative of 5 control mice and the histology results in (b) and (c) are representative of 8 SCC mice.

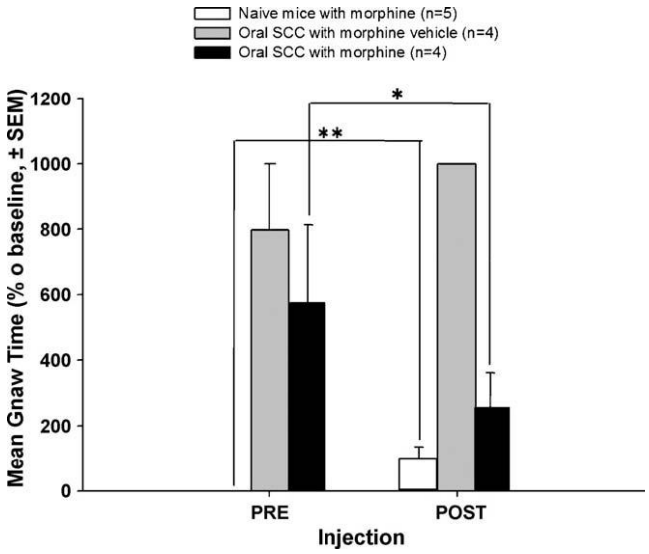

Fig. 10. Morphine attenuates oral SCC-induced increases in gnawing time. Morphine treatment (20 mg/kg intraperitoneal) significantly blocks gnawing time increases in oral SCC-bearing mice (grey bars, n = 4) compared to untreated tumor-bearing mice (black bars, n =4) (*P < 0.05, Mann–Whitney U test). In contrast, naïve mice treated with morphine demonstrate increased gnaw-time (white bar, n =4) (*P < 0.01, Mann–Whitney U test).

4. DISCUSSION

We developed a high throughput, objective, operant assay for orofacial function and validated its ability to detect acute and chronic functional allodynia. The dolognawmeter quantified a behavioral index of nociception in three separate mouse models of orofacial pain (TMJ inflammation, masticatory myositis and head and neck cancer). To confirm that the behavioral dependent measure (gnaw-time) reflects nociception, we restored gnawing function by blocking nociception with the same analgesics used clinically for patients with comparable pathology. Our assay with the dolognawmeter provides a metric for orofacial function and pain that can now be used to investigate molecular mechanisms in the trigeminal system and test analgesics for treating pain in the head and neck.

Our assay exploits a rodent's instinctual response to gnaw through an obstruction in a narrow tube as first described by Ayada et al. (2002). These authors measured gnawing rate by quantifying the mass of plastic gnawed from a hard plastic confinement strip perpetually replaced

on the end of a narrow tube over a fixed period of time; the task is infinite. Our device employs an automated mechanism that records the amount of time required for a mouse to gnaw through an obstruction and actually gain escape. This entails a discrete gnawing task because the mouse always gnaws though the dowel in a similar pattern after training and the device retracts both ends of the dowel from the tube the instant that it is severed. While confined in the dolognawmeter, the animal is unable to turn around but there is no direct restraint as seen in some assays (Amir and Amit, 1978; D'Amour and Smith, 1941; Randall and Selitto, 1957).

Gnawing is a routine, physiologic orofacial function. It is coordinated by the trigeminal somatosensory and motor systems and activates the TMJ, craniomandibular muscles, jaws, incisors, lips, tongue, buccal mucosa, palate and gingiva in a fashion that is similar to the chewing associated with mastication. Functional allodynia originating from pathology in any of these structures is potentially quantifiable with the dolognawmeter. Reflexive orofacial nociceptive assays are less applicable to musculoskeletal nociception generated by function since they often induce an acute, transient painful stimulus in the skin to produce allodynia (Morgan and Gebhart, 2008). In addition most operant orofacial nociceptive assays do not measure orofacial dysfunction resulting from orofacial pathology but rather from noxious cutaneous stimulation (Neubert et al., 2008). In our device, routine voluntary orofacial function involving most of the masticatory complex produces nociception resulting from a clinically relevant pathology such as masseter myositis or oral cancer. An ethical advantage of our device and assay is that the animal controls the stimulus exposure. Thus, this method appropriately follows IASP and NIH guidelines (Zimmermann, 1983).

The dolognawmeter measures functional mechanical allodynia in the rodent orofacial complex. Mechanistically, the source of nociception is likely a combination of chemical and mechanical for all three models studied. The anatomic structures involved in the three models can be inferred

from the site of injury used to create those models. For the TMJ inflammation model the source of nociception is the joint where CFA was injected. The TMJ is a closed space surrounded by dense fibrous connective tissue and extravasation from the joint into surrounding tissues is unlikely. The mechanical allodynia likely occurs during loading of the joint when the mouse is gnawing. Injection of CFA or carrageenan into a joint has been widely used as a model of inflammatory hyperalgesia in articular tissue and produces functional changes consistent with arthritis in patients (Hutchins et al., 2000; Kehl et al., 2000; Tonussi and Ferreira, 1992, 1999). Intramuscular CFA injection produces a valid model for the investigation of muscle hyperalgesia (Harriott et al., 2006). For the masticatory myositis model CFA is injected directly into the masseter muscle. The source of nociception is likely nociceptors within the muscle or the periosteum overlying the mandible. To confirm nociception in the TMJ arthritis and masseter myositis models we completely antagonized the effect of CFA injection on gnawing function by pre-administering a systemic NSAID. We confirmed the infiltrate histologically (i.e. neutrophil infiltrate (Harper et al., 2001)) in both the TMJ and masseter inflammatory models. For the oral cancer model the carcinoma proliferates within the submental space. The submental space is bounded by muscle, periosteum and bone. The source of nociception within the cancer model could be any or all of these structures. We validated the dolognawmeter with these three distinct models of orofacial pathology to demonstrate that the instrument detects orofacial nociception originating from different tissues within the orofacial region. The device can quantify and the investigator can compare dysfunction and thus nociception resulting from different pathologies.

The dolognawmeter possesses a unique combination of experimental and technical advantages. Experimentally, it measures both acute and chronic nociception. We measured nociception hours and days following CFA administration and weeks following SCC inoculation. Assays that quantify stereotyped behaviors such as rubbing and flinching of the head have also been used as an index of acute orofacial nociception; however, these assays are generally subjective, low

throughput and less effective for measuring chronic nociception (Roveroni et al., 2001). Various meal size and interval assays have been proposed to quantify both acute and chronic nociception (Harper et al., 2000, 2001; Kerins et al., 2005; Thut et al., 2007). Feeding assays are potentially confounded by variables that affect appetite including analgesics, systemic disease, time of day, duration of the study, and reward associated with consumption. For example, animals with cancer can weaken, cease eating, and develop cachexia in the absence of nociception. Feeding studies cannot resolve behavioral changes due to orofacial nociception versus nociception originating elsewhere in the body since mice with non-trigeminal nociception demonstrate reduced appetite and feeding rate (Kerins et al., 2005). Appetite is less likely to affect the outcome variable in the dolognawmeter because animals are confined for a relatively brief period in the tube without food. Moreover, the gnawing behavior that we measure does not involve consumption even though it engages most of the orofacial complex used for mastication. The mouse does not consume debris from the dowel since the animal occludes the oral cavity with the tongue, cheeks and lips behind the incisors while gnawing.

High throughput is a technical advantage of the dolognawmeter; the device is fully automated and multiple devices can be run concurrently. This study was undertaken using 30 dolognawmeters. Measurement of the outcome variable is objective and not prone to investigator bias. To accommodate instinctual proclivities of the mouse, a nocturnal prey animal, the experiment takes place in darkness in the absence of the investigator. Initial delay due to habituation, animal loading inconsistencies, or drug side effects is less likely to affect the outcome measure since only the gnaw-times for the second dowels are compared. An animal can delay gnawing on the first dowel until pharmacologic sedation has worn off and/or analgesia has taken effect without influencing the gnaw-time for the second dowel. Thus, the device accommodates pharmacokinetic differences between animals and pre-empts intractable difficulties with pharmacologic sedation, titration and analgesic onset.

A pharmacologic motor effect is less likely to corrupt the outcome variable since the first dowel acts as a prerequisite minimum motor verification task. The dolognawmeter is not prone to false positives when evaluating the efficacy of sedating analgesics since sedation and analgesia produce opposite test outcomes. In most reflexive assays, sedation and analgesia produce a similar test result. For example, in the paw withdrawal assay, sedation and analgesia both increase withdrawal threshold. Without a motor verification task, operant trials such as the mouse facial–thermal–operant assay are likely to produce a false negative when evaluating sedating analgesics since the sedative effect alone can attenuate the operant behavior (Neubert et al., 2008).

We validated measurement of nociception in both cancer and inflammatory models. Using the head and neck cancer pain model we demonstrated oral dysfunction following cancer inoculation. Reduced function secondary to carcinoma-induced nociception was also observed in our previous work demonstrating progressive increase in withdrawal threshold and a decrease in weight bearing in the carcinoma-injected hindpaw mouse model (Schmidt et al., 2007). In both our hindpaw model and the current head and neck cancer model we partially restored function with high dose morphine, the first-line treatment for cancer pain (Schmidt et al., 2007).

The dolognawmeter has potential limitations. Non-nociceptive factors can influence gnawing activity. Systemic illness can weaken an animal and increase gnaw-time since gnawing requires effort. We demonstrated only partial recovery of gnawing function in animals with head and neck cancer. This could be due to a number of variables including subtotal analgesia or systemic illness. Complications concerning systemic disease might be ameliorated with a lower durometer dowel that poses an easier gnawing task. However, gnaw-times are comparable only when using dowels of the same durometer.

Potential behavioral confounders include analgesic side effects such as gnawing stereotypy. Opiates and other dopaminergic drugs induce gnawing stereotypy under specific conditions requiring upright position of the animal or an environment that permits gnawing assisted climbing (Tirelli and Witkin, 1995). The dolognawmeter precludes these conditions and thus prohibits gnawing stereotypy induced by these drugs (Livezey et al., 1995; Tirelli and Witkin, 1995). Morphine administered to naïve mice did not potentiate gnawing activity in the dolognawmeter.

The dolognawmeter quantified an index of nociception to demonstrate functional allodynia in three orofacial conditions. It may also be useful to study painful disorders that are physiologically unique to the orofacial region including toothache, trigeminal neuralgia, and headache. Because the device activates and measures goal-directed behavior, complex diseases such as depression and anxiety could also be studied. We anticipate that future studies will test the validity and identify the shortcomings of the dolognawmeter for these and other diseases.

APPENDIX

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2010.01.012.

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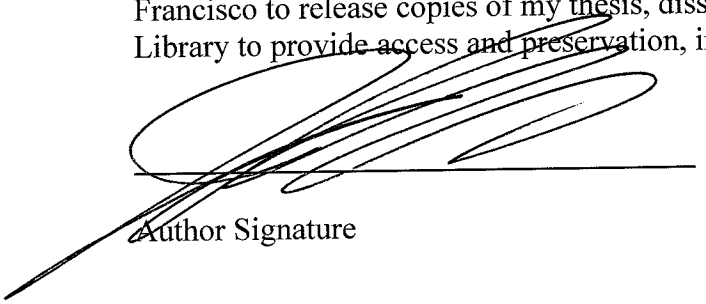

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