EFFECTS OF REM SLEEP DEPRIVATION ON TRIGEMINAL NERVES: INCREASED THERMAL SENSITIVITY

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Allison Elizabeth Overmyer

August 2011

EFFECTS OF REM SLEEP DEPRIVATION ON TRIGEMINAL NERVES:

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ABSTRACT

Sleep is essential for maintaining normal functioning of the nervous system. Importantly, while sleep deprivation increases the level of pain, the pain resulting from injury, trauma, or infection can cause one to become sleep deprived. In such a scenario, a vicious cycle is established and is thought to contribute to chronic pain states as reported in migraine and temporomandibular joint disorder. Sensitization and activation of trigeminal nerves, which provide sensory innervation to much of the head and face and mediate pain and inflammation, are implicated in the underlying pathology of these diseases. I hypothesized that sleep deprivation would cause sensitization of trigeminal neurons. To test my hypothesis I developed and tested a novel rat holding device to measure the effect of rapid eye movement (REM) sleep deprivation on trigeminal nerve sensitivity by measuring changes in response to thermal stimuli. This device is now commercially available and sold as an accessory to the Plantar Test (Ugo Basile). Using my device, I found that REM sleep deprivation lowered the activation threshold of trigeminal nerves to thermal stimuli. Data from my study provide evidence that REM sleep deprivation may contribute to disease progression by lowering the activation threshold of trigeminal nerves to noxious stimuli.

KEYWORDS: trigeminal nerve, nociception, pain, REM sleep, thermal sensitivity

This abstract is approved as to form and content

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Paul L. Durham, Ph.D.
Chairperson, Advisory Committee
Missouri State University

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ACKNOWLEDGEMENTS

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INTRODUCTION

Trigeminal Nerve

The trigeminal nerve, or fifth cranial nerve, is the largest and most complex of twelve cranial nerves and is the great afferent (sensory) nerve of the face, mucous membranes of the head, internal cranial structures, teeth and temporomandibular joints, as well as the efferent (motor) nerve of the first brachial arch (Shankland, 2000a). It originates at the lateral border of the pons through two roots, the sensory and motor root and is comprised of three major divisions: the ophthalmic, the maxillary, and the mandibular.

The ophthalmic or first division (V1) of the trigeminal nerve is the smallest of the three divisions and is purely afferent in function. It supplies sensory branches to the ciliary body, cornea, iris, lacrimal gland, conjunctiva, portions of the mucous membrane of the nasal cavity, sphenoidal sinus, frontal sinus, the skin of the eyebrow, eyelids, forehead, and nose, the tentorium cerebella, dura mater, and the posterior area of the falx cerebri (Shankland, 2001a). The maxillary or second division (V2) of the trigeminal nerve is intermediate in size and is purely afferent in function. It supplies sensory innervations to all structures in and around the maxillary bone and the midfacial region including the skin of the midfacial regions, lower eyelid, side of nose and upper lip, the mucous membrane of the nasopharynx, maxillary sinus, soft palate, palatine tonsil, roof of the mouth, maxillary gingivae, and maxillary teeth (Shankland, 2001b).

The mandibular or third division (V3) of the trigeminal nerve is the largest of the three divisions and is a mixed nerve, meaning it is both afferent and efferent in function.

It is composed of two separate roots, a large sensory root and a smaller motor root (Figure 1). The large sensory root supplies afferent innervation to the teeth and gingivae of the mandible, skin of the temporal region and lower one-third of the face, ear, lower lip, the muscles of the first bronchial arch, the mucous membrane of the anterior two-thirds of the tongue, and floor of the mouth (Shankland, 2001c). The small motor root supplies efferent innervation to the muscles of the first brachial arch which consist of the muscles of mastication, the tensors veli palatini and tympani, the mylohyoid muscle, and anterior belly of the digastrics muscle.

Trigeminal Nerves and Disease

While disease can occur in all areas of the body, diseases associated with the activation of trigeminal nerves are some of the most painful and difficult to treat. Excitation of trigeminal nerves in response to injury or inflammatory stimuli correlates with the region of the head and face based on the innervation pattern of each particular branch. For example, migraine pathology is associated with activation of the V1 branch, while sinus pathology involves primarily the V2 branch, and temporomandibular joint disease(TMJD) pathology with excitation of V3 branch. Migraine pathology is caused by migraine-specific triggers that cause dilation of cranial blood vessels that leads to activation of perivascular trigeminal sensory nerve fibers (Durham, 2008). In response to activation, trigeminal nerves release neurotransmitters that promote peripheral inflammatory responses within the dura and peripheral sensitization of the trigeminal nerve causing associated pain symptoms within the upper facial region. Sinus pathology is caused by upper respiratory infections or allergic rhinitis creating factors which

interfere with ostium patency, mucous protection, or ciliary function causing inflammation, edema, or mucous secretions accumulating in the sinuses (Schor, 1993). These responses sensitize the trigeminal nerve causing pain symptoms within the mid facial region. TMJD pathology is caused by inflammation of the TMJ region and masticatory muscles (Bereiter et al., 2005) leading to activation and sensitization of the trigeminal nerves. TMJD is characterized by impaired jaw function, deviation of deflection, limited range of motion, joint noise, locking, and ultimately pain within the lower facial region (Herb et al., 2006).

Trigeminal Ganglion

The afferent fibers of the trigeminal nerve, except those associated with pressure and stretch receptors and proprioception, have their cell bodies of origin located in the trigeminal ganglion (TG). The nerve cells of the TG are pseudounipolar (Figure 2) and are somatotopically organized from medial to lateral, meaning sensory information in the periphery is preserved in the central nervous system. The peripheral processes of these cells form the ophthalmic, maxillary, and mandibular divisions of the trigeminal nerve while the central processes of the cells form the large sensory root. It is the afferent portion of the trigeminal nerve which provides somatosensory sensations (i.e. mechanorecetion, thermal sensitivity, pressure sensation, nociception, and proprioception) to the entire face (Shankland, 2000b).

Nerve cells located in the TG are of two main morphological types: “large light” (LL) and “small dark” (SD). SD neurons give rise to C fibers (non-mylinated, slow conducting), whereas the fibers of LL neurons are of the A type (mylinated, fast

conducting). SD neurons are concerned with thermo- and mechanoreception, and many of them are nociceptive, while terminals of LL neurons are low threshold mechanoreceptors (Hanani, 2005). SD neurons can be activated by thermal heat stimuli via activation of transient receptor potential cation channel subfamily V member 1 (TRPV 1).

TRPV1 Receptors and Heat

TRPV1 receptors are ligand-gated nonselective cation channels that are considered to be an important integrator of various painful stimuli such as endogenous lipids, capsaicin, heat, and low pH (Cui et al., 2006). These receptors are primarily localized on sensory nerves and initiate and amplify pain and inflammation (Clark et al., 2007). Activation of TRPV1 plays an important role in thermal pain transmission and modulation (Cui et al., 2006) by causing sensitization of nociceptors such that they respond to lower levels of stimulatory molecules or have exaggerated pain responses.

Role of Trigeminal Ganglion in Nociception

To deal effectively with danger, it is imperative for living organisms to know about it and be able to recognize and react to harmful stimuli (Woolf and Ma, 2007). This capability to avoid hazardous situations has been gained during evolution through the development of a specialized apparatus called a nociceptor. Intense stimulation of nociceptors that have high thresholds will elicit a pain sensation that will be processed by the organism's body and result in the organism removing itself from the danger before possible long-term tissue damage has occurred. This is considered acute and protective

under normal conditions and nociceptive types of stimuli can be associated with something being too hot or too cold (Cheng and Ji, 2008).

Stimuli that can cause activation or sensitization of nociceptors are quite diverse and also an individual's response to a particular stimulus is complex. In some cases, further evolution of a nociceptor can result in the development of nociceptor sensitization under injury or diseased conditions, leading to enhanced pain states that the organism cannot avoid (Cheng and Ji, 2008). Stimuli in these circumstances can be defined as one of two pain states, hyperalgesia or allodynia. Hyperalgesia is defined as an increase of pain in response to a painful stimulus (i.e., spraining an ankle causing pain and then doing it again which results in even more pain), while allodynia refers to pain in response to a typically non-painful stimulus (i.e., increased sensitivity such that a typical "warm" stimulus now is perceived as "hot" and thus painful.) For my study, I wanted to investigate whether REM sleep deprivation leads to trigeminal nociceptors sensitization such that a non-painful thermal stimulus provided by an I.R. heat source is now painful.

Sleep and Pain

For our health and well-being, it is imperative to have a good night's sleep to recover, reenergize, and reverse the affects of fatigue. A decrease in the quantity or quality of sleep, which is referred to as sleep deprivation, has significant impacts on public health and is known to trigger irritability, sociability, and dysfunction. It is known that sleep loss results in increased pro-inflammatory agents that are potent pain-inducing and facilitating factors. Thus in this way, a vicious cycle is created since a bad night's sleep enhances pain and pain interferes with a good night's sleep.

What is Sleep?

The state of sleep encompasses almost one third of a person's life and can be defined as a normal reversible state of loss of perception of, and responsiveness to, the external environment (Dodick et al., 2003). While it is generally accepted that a sleeping person does not sense their surroundings nor respond to them to the degree they do when awake, there is not a total lack of sensing and perceiving of external stimuli. For example, intense, discomforting, or especially meaningful stimuli can cause a sudden awakening (Association, 1997; Kryger et al., 2000; Lee-Chiong et al., 2002; Moorcroft, 1993). Disturbances of sleep often mirror disturbances in the physiology of body and mind (Dodick et al., 2003). Therefore, the functions of sleep such as fatigue reversal, biochemical refreshment (i.e., protein synthesis, neurogenesis, metabolic restoration, etc.), immune function, and memory consolidation ensure health and well-being of our systems including our nervous system.

Sleep Cycle

Sleep is regulated under two processes, the circadian rhythm and homeostatic. The circadian rhythm process directs the timing of all 24-hour behavioral, physiological and molecular processes and responds to clues known as zeitgebers (i.e., light, time, and melatonin). The homeostatic process regulates propensity for sleep based on the amount of prior wakefulness and if the resource is previously depleted, the organism will attempt to regain or compensate to ensure that balance is restored. Both processes cooperate with one another and ultimately affect brain wave patterns, which are electrical impulses inside the brain measured in Hertz (Hz) via an electroencephalogram (EEG), to determine

if an organism is in a state of wake or sleep. The state of being awake consists of two wave patterns and the state of being asleep consists of five wave patterns divided into non-Rapid Eye Movement (REM) wave patterns (slow wave (SW) sleep I-IV) and REM wave patterns (REM V).

During the state of being awake, there are beta and alpha waves. Beta waves are irregular, low intensity, and fast frequency (16-25 Hz) that typically occur in an awake, active brain, whereas alpha waves are regular, moderate intensity, and intermediate frequency (8-12 Hz) that typically occur in an awake, but relaxed or drowsy brain. During the state of being asleep, SW sleep I is the transition from alpha waves to theta waves, which are moderate to low intensity and intermediate in frequency (3-1 Hz). SW sleep II is characterized by theta waves. While SW sleep III is less than 50% delta waves, which are intense and low frequency (1/2 to 2-3 HZ), SW sleep IV is more than 50% delta waves. REM V sleep is characterized by sawtooth waves, which are low intensity and mixed frequency (similar to wake wave patterns) (Association, 1997; Kryger et al., 2000; Lee-Chiong et al., 2002; Moorcroft, 1993). It is during REM sleep when the body can commonly have muscular twitches, dreams, muscle atonia, and other physiological body processes. These processes during REM sleep are important for nervous system function in that they cause stimulation for growth, allow repair and maintenance, exercise the brain circuits crucial to survival that are rarely exercised during wakefulness, consolidate/adapt recent memory, and aid learning capabilities (Cohen, 1980). Because of REM sleep's importance for nervous system function, I have chosen to use a model that causes REM sleep deprivation and see how it behaviorally affects an animal's thermal pain threshold.

Sleep Disorders

Sleep disorders are classified into one of three groups: dyssomnias, parasomnias, or medical/psychiatric sleep disorders (Association, 1997). All these disorders interfere in some way with one's ability to get amply quantity or quality of sleep that results in disturbances in the physiology of body and mind. Deep sleep, which encompasses REM sleep among other stages, is known as the restorative part of the sleep cycle for the body so my experimental model therefore mirrors the effects of what individuals endure during REM sleep disorders.

Goals

Prior studies in our laboratory have primarily focused on cellular changes within trigeminal ganglia and spinal trigeminal nucleus in response to inflammatory or noxious stimuli as well as anti-inflammatory treatments. However, behavioral changes have not been investigated. My interests in animal behavior and the increasing interest of behavioral testing in the orofacial field lead me to investigate current behavioral models used to evaluate orofacial pain. I was somewhat surprised to find that there were no standardized models for studying thermal pain. I believe it is important to correlate cellular and molecular changes with behavioral changes because both provide valuable information about the response of the nervous system. Furthermore, behavioral changes are very important factors when looking at REM sleep and nerve homeostasis because typically, it is the behavioral characteristics that are the most pertinent and relevant for understanding human sleep disorders.

I hypothesize that REM sleep deprivation will cause sensitization of trigeminal nerves, thus lowering the thermal pain tolerance threshold. To test my hypothesis, I wanted to evaluate the thermal pain threshold of the facial region of *Rattus norvegicus* (rat) since this organism is traditionally utilized to study cellular and molecular changes. Furthermore, I wanted to develop a reliable means to measure changes in thermal sensitivity because: 1) there has not been a reliable or easily-repeatable method for testing changes in pain threshold behavior in rodents in response to thermal stimuli; 2) there is not a reliable model for testing thermal sensitivity in the facial region of a rodent using the Ugo Basile Plantar Test apparatus; and 3) effects of REM sleep deprivation on the V3 region of the trigeminal nerve have not been extensively studied through cellular and molecular or behavioral means.

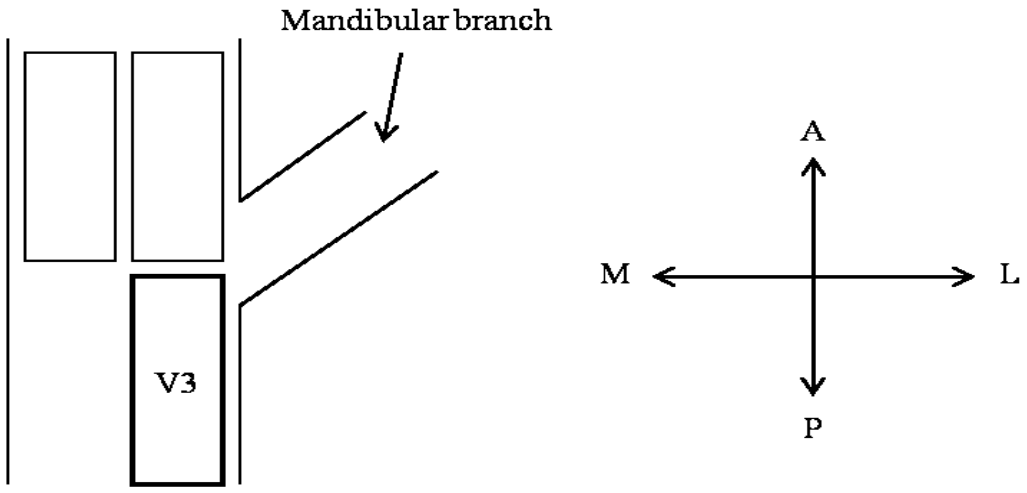

Figure 1. A schematic representation of the V3 region of the right trigeminal ganglion. The V3 region is the largest region of the ganglion and is comprised of both sensory and motor nerves (Abbreviations are as follows: A: anterior, P: posterior, M: medial, L: lateral).

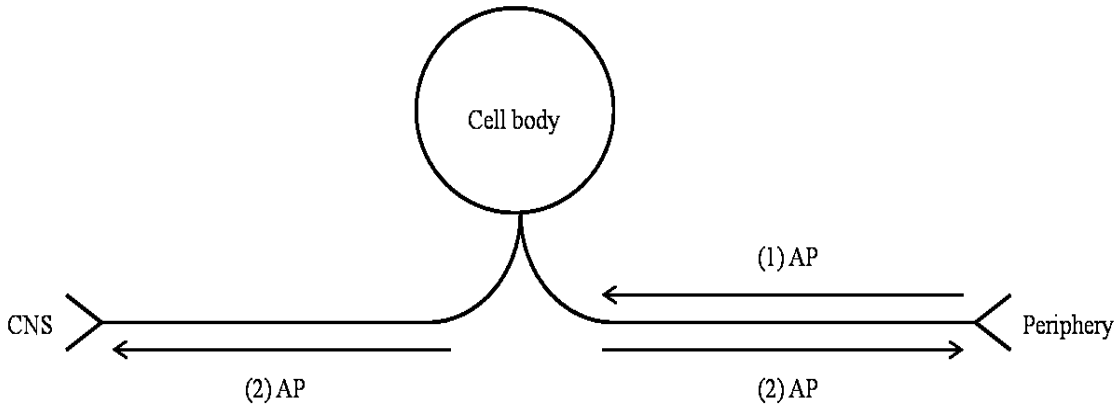

Figure 2. A schematic representation of a trigeminal ganglion neuron. A pseudounipolar neuron with peripheral and central projecting branches is depicted. Activation at the periphery initiates an action potential (AP) to the cell body of the neuron (1) which transmits signals to the central nervous system (CNS) and back to the periphery (2).

METHODS

Animals

Animal studies were approved by the Institutional Animal Care and Use Committee (2008EE approved March 11, 2009) at Missouri State University and were conducted in compliance with all established guidelines in the Animal Welfare Act and National Institutes of Health. A concerted effort was made to reduce the number of animals used and to minimize any suffering. Adult, male or female Sprague-Dawley rats (200-400g) (Charles River Laboratories Inc., Wilmington, MA) were housed in clean, plastic standard rat cages (VWR, West Chester, PA) in an animal holding room or non-standard rat cages in a specialized environmental chamber (Powers Scientific, Inc., Pipersville, PA) on a 12-h light/dark cycle with ambient temperature maintained between 22-24°C and access to food and water *ad libitum*.

True Blue Dye Localization

Female rats (n=3) were anesthetized by injecting 0.3 ml of a mixture of ketamine (45 mg/kg) and xylazine (5 mg/kg) HCl solution intraperitonealy (i.p.). Assessment of proper anesthesia was performed by monitoring the writhing reflex and tail flick reflex. True Blue dye (25 µl; 2 µg/µL in dimethyl sulfoxide, DMSO) was injected into the TMJ capsule, the masseter muscle region, or submandibular muscle region. Injections were performed with a 50 µL Hamilton syringe (Hamilton Company, Reno, NV) and a 26½ G needle (Becton Dickinson, Franklin Lakes, NJ). Five days after injections rats were sacrificed.

TG's were removed from all rats following CO_2 asphyxiation. Ganglia were mounted in Optimal Cutting Temperature compound (OCT) (Sakura Finetek, Torrance, CA) such that the ventral surface was in contact with the slide, quickly frozen, and stored at -20°C. Fourteen-micron longitudinal sections of the entire TG tissue were serially prepared using a cryostat (Microm HM 525, Thermo Scientific, Waltham, MA) set at -20°C and mounted on Superfrost Plus microscope slides (Fischer Scientific, Pittsburg, PA).

Slides containing sectioned ganglia were incubated in 4% paraformaldehyde for 30 minutes and washed three times with phosphate buffered saline (PBS) then covered in Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA). Images were collected using an Olympus DP70 camera mounted on an Olympus BX41 fluorescent microscope and image analysis performed using Olympus Micro-Suite Five image processing software (Olympus, Center Valley, PA). Multiple image alignment (MIA) was utilized to view the entire ganglion by taking eight 40x magnification images and stitching them together to form a single image.

Behavioral Analysis Using Thermal Stimulation (Plantar Test Apparatus)

For the duration of experiments, every morning animals ($n \geq 10$) were individually guided into the holding device and allowed to settle (between 1-5 minutes). Following a short acclimation period to allow the rat to rest its mandible on the glass surface of the device, the Plantar Test apparatus (Ugo Basile, Comerio Varese, Italy) infrared (I.R.) heat source (Figure 3) was directly placed under the submandibular area of the animal and started. Once the animal responded to the stimulus, the I.R. heat source

was removed and the animal was allowed a 30 second resting period before six additional readings were taken. Animals were allowed the 30 second resting period after each reading. When readings were complete, the animal was placed back into its experimental condition and all readings were averaged, the basal (first) reading was subtracted, and daily changes in thermal pain thresholds (in seconds) were recorded.

Converting I.R. Intensity into Degrees Celsius

A thermocouple thermometer (Figure 4) (Fluke, Everett, WA) was placed directly on the glass surface of a Plantar Test apparatus. It was turned on and zero I.R. intensity temperature readings were recorded for 60 seconds (10 second intervals). This procedure was repeated 3 independent times. Next, temperature readings were averaged for each corresponding 10 second interval. Temperature readings were conducted the same for I.R. intensities 10-60 (in increments of 10).

Measuring I.R. Intensity for Consistency

A heat-flux radiometer (Figure 5) (Ugo Basile) was placed directly over the shutter opening of a Plantar Test apparatus. It was turned on and an I.R. intensity energy emittance reading was recorded after waiting 10 seconds; this was repeated for an n=3. Following, energy emittance readings were averaged and a graph was made to show what energy (heat flux (mW/cm^2)) the I.R. intensity emitted. Energy emittance readings were conducted for I.R. intensities 0-99 (in increments of 20).

Designing Prototype of Rodent Holding Device

The Plantar Test apparatus is traditionally used to test thermal threshold responses of an animal's hind paw by placing an animal into a holding box (Figure 6). However, this method does not work well for measuring orofacial thermal threshold responses since the animal does not place its face on the glass surface for a long enough period of time to obtain consistent measurements. Initially, I found that placing an animal into a plastic cone (Figure 7) worked well. However, the animal still had flexibility in its head and paw movements making it impossible to get consistent and accurate readings. Therefore, I decided to try and design a novel holding device that would not only hold the animal similar to current commercial devices, but allow access to the head for consistent thermal threshold readings.

To begin to design a prototype of a device for holding a young adult rat, **c**ardboard was cut and pieced together to form a basic model of a rodent holding device (Figure 8, A). Once the basic design was completed, a male rat was anesthetized by injecting 0.3 ml of a mixture of Ketamine (45 mg/kg) and Xylazine (5 mg/kg) HCL solution intraperitonealy (I.P.) (assessment of proper anesthesia was performed by monitoring the writhing reflex and tail flick reflex) and specific measurements such as: body length, snout length, snout width, body height, etc. were taken. These measurements were then used to modify the general cardboard design and create a new cardboard design that would fit to an animal's body correctly (Figure 8, B). From there, the cardboard design was used as a template to build a plastic prototype (Figure 9) and further modifications were made to optimize the dimension of the device to best fit the contour of the adult rat until a final prototype was completed and successfully used for

holding an animal while allowing oraofacial thermal response readings to be taken (Figures 10-11).

After completion of the final holding device, the lab showed Ugo Basile the design and the company has begun the process of commercializing the device to be sold as an accessory to the Plantar Test apparatus (Figure 12).

Acclimation of Animals in Holding Device

Male rats were guided into a specially built animal holding device and left to sit for 5 minutes. This was conducted over a 4 day period and its purpose was to let the animals become familiar with the device and sit calmly while facial Plantar Test apparatus readings are taken. During the acclimation, if an animal appeared to be too stressed and/or not willing to go into the device, it was pulled from the study.

Cage Models

Control. A control model (Figure 13, A) was made by putting rats in clean, plastic standard rat cages with 1 ½ inches of bedding covering the bottom. These cages allow the animal to lie down and comfortably sleep through all stages of a sleep cycle.

REM Sleep Deprivation Model. To create REM sleep deprivation in my experiments, a REM sleep deprivation model (Figure 13, B) was achieved using a modified version of the flower pot model (Mendelson et al., 1974). Clean, plastic rat cages were modified by installing a 3in. x 3in. x 3in. plastic platform (Office Depot, Inc., Springfield, MO) on one end of the cage. Cages were filled with five liters of warm water and animals were placed on the platform. This platform prevents rats from going

into REM sleep by not providing enough room on the platform when muscle control is lost during REM sleep and allowing the animal to touch the water and wake up.

Environmental Control Model. To rule out water stress as a variable in my experiments, an environmental control model (Figure 13, C) was achieved by modifying the REM sleep deprivation model. Clean, plastic rat cages were modified by installing a 6 in. x 6 in. x 3 in. plastic platform (Office Depot, Inc., Springfield, MO) on one end of the cage. Cages were filled with four liters of warm water and animals were placed on the platform. This platform is large enough for animals to lie down and sleep normally.

Acclimation to Different Cage Models

Male rats ($n \geq 10$) were placed in the environmental chamber for 30 minutes in REM Sleep Deprivation or environmental control cages for a 4 day period to become familiar with the non-standard environment and housing. During the acclimation, if an animal appeared to be too stressed or not able to find the platform and remove itself from the water, it was pulled from the study.

Control, Environmental, REM Sleep Deprivation, and Recovery Procedures

Control. Basal thermal sensitivity readings were taken in the morning starting at 0 hours; subsequently, male rats (n=12) were put in clean standard cages in an animal holding room for 3 days (3 sleep/wake cycles). Everyday food and water were checked and behavioral readings were taken daily.

Environmental Control. Basal thermal responses were recorded in the morning starting at 0 hours; subsequently, acclimated male rats (n=10) were put in clean

environmental control cages at 8:00am in the environmental chamber (Figure 14). The next morning (7am) they were taken out of the cages and allowed to rest in a standard cage for 1 hour, permitting cleaning of cages, checking food, checking water, and allowing time for the animals to calm down before behavioral readings were taken. After the resting period and behavioral readings, rats were again placed in clean environmental control cages and left alone in the environmental chamber. The procedure was done for 3 days (3 sleep/wake cycles).

REM Sleep Deprivation. Basal thermal response values were taken in the morning starting at 0 hours; subsequently, acclimated male rats (n=11) were put in clean sleep deprivation cages at 8:00am in the environmental chamber. The next morning (7 am) they were taken out of the cages and allowed to rest in a standard cage for 1 hour, permitting cleaning of cages, checking food, checking water, and allowing time for the animals to calm down before behavioral readings were taken. After the resting period and behavioral readings, rats were again placed in clean sleep deprivation cages and left alone in the environmental chamber. The procedure was done for 3 days (3 sleep/wake cycles).

Recovery. After the 72 hours, environmental control rats and REM sleep deprivation rats were put in clean standard cages in an animal holding room such that as the control rats. These animals were then allowed 24 hours (1 sleep cycle and 1 wake cycle) of recovery sleep prior to day 1 recovery behavioral readings.

Behavioral Analysis Using Video Recording

Male rats (n=3 for control, environmental control, and REM sleep deprivation) were placed in a rodent incubator at 8:00am and recorded 3 times during the day for 1

hour increments: morning, noon, and early evening for 3 days (3 sleep/wake cycles) (Figure 15). Recorded information was viewed to observe behaviors such as eating, drinking and sleep; sleep behaviors, in particular, were noted and the environmental control and REM sleep deprivation experimental groups were compared to this standard.

Statistics

All thermal testing data were analyzed using an independent samples t-test with SPSS v16.0. Significance was considered at $p < 0.05$. Each condition was repeated in a minimum of 3 independent experiments.

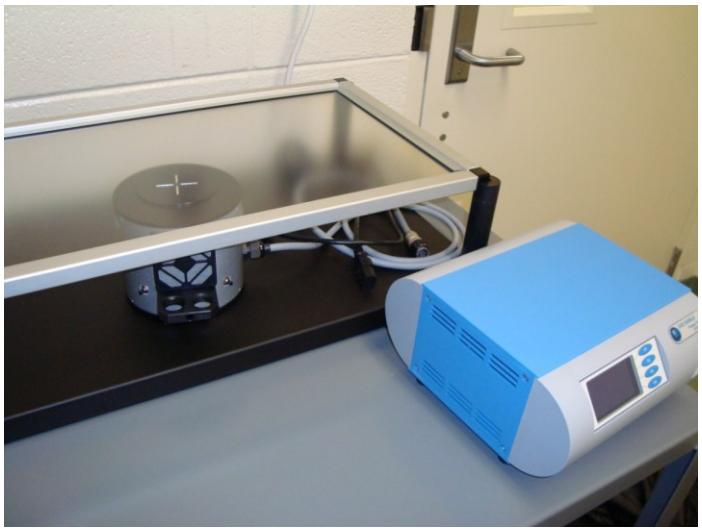

Figure 3. Image of Ugo Basile Plantar Test apparatus. Device used to test thermal thresholds in the submandibular area (V3 region) of experimental animals by using an I.R. heat source to increase skin temperature and then measure the withdraw latency.

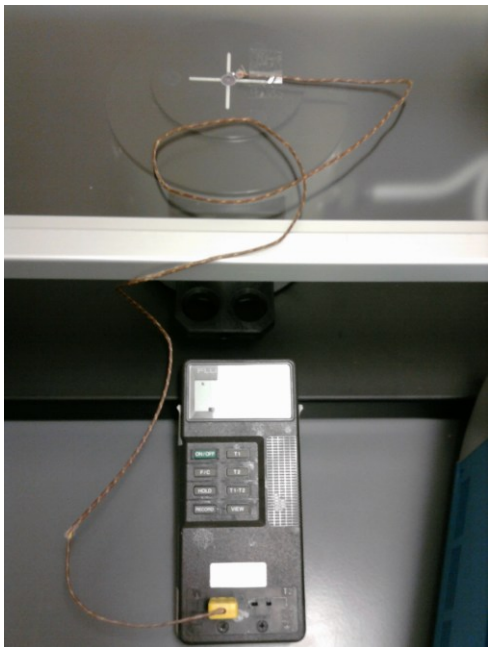

Figure 4. Image of thermocouple thermometer. Device used to measure the temperature at the level of the glass surface that was produced by the I.R. source used in conjunction with the Plantar Test apparatus.

Figure 5. Image of heat-flux I.R. radiometer. Device used to ensure the Plantar Test apparatus emitted the same level of I.R. intensity throughout the study.

Figure 6. Animal placed in a holding box of the Plantar Test apparatus. When the animal becomes quiescent, the I.R. heat source is directed towards the hind paw for a thermal threshold reading.

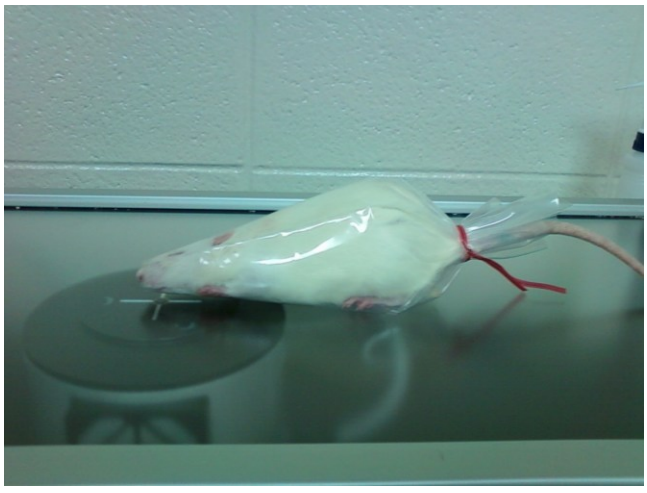

Figure 7. Animal placed in a commercial, plastic restraining cone. The cone was used to position the animal in a way so the heat source hits the submandibular area.

Figure 8. Basic model of rat holding device. Initial holding device design made from cardboard (A) and a modified version based on dimensions of a young, male Sprague Dawley rat (B).

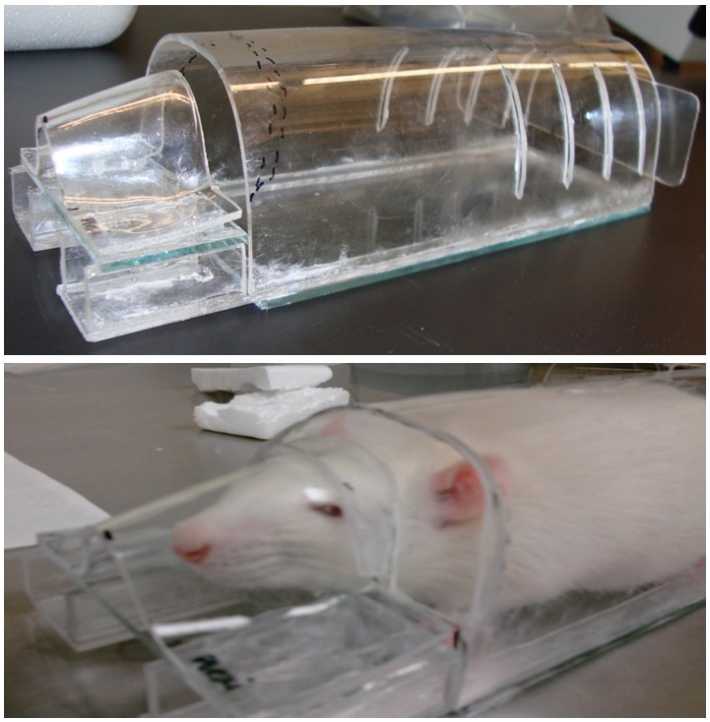

Figure 9. Initial plastic and glass holding device prototype. Modifications were made so the device would contour to head and face of the rat and allow the submandibular area to be placed in contact with the glass for thermal threshold readings.

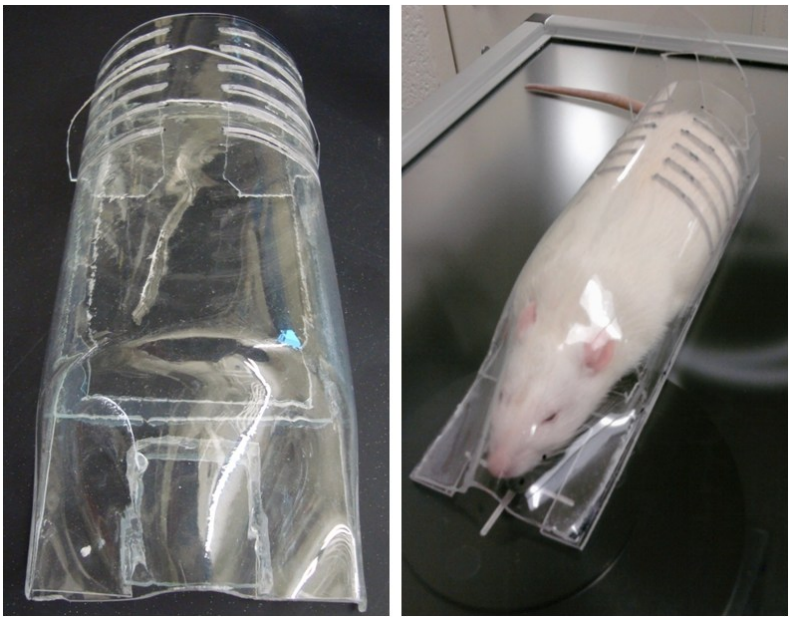

Figure 10. Modified plastic holding device prototype. The form fitted model held the rat well and allowed the submandibular area to be exposed for thermal threshold readings.

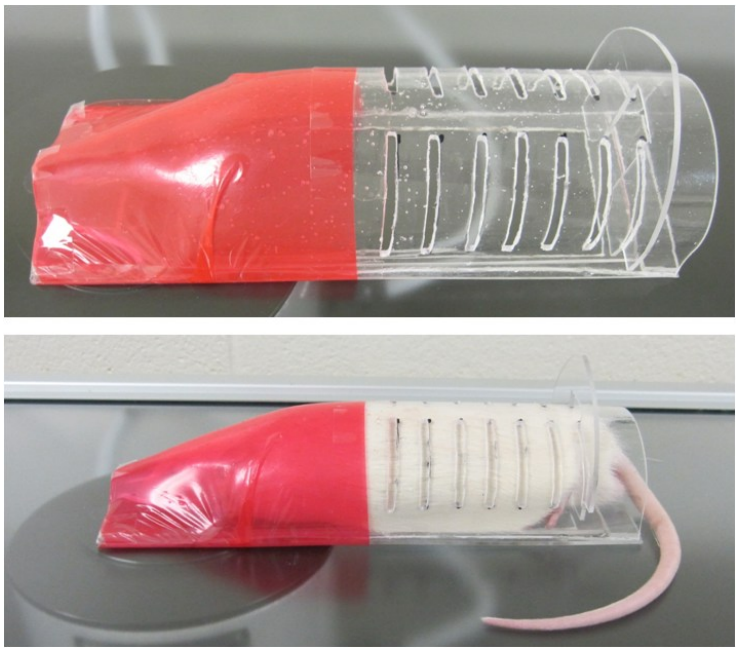

Figure 11. Modified plastic holding device prototype with paraffin. Red paraffin placed over the area where the rat can see blocks the animal's view of surroundings, thus minimizing visual stimuli and shortening the acclimation period and providing a more comfortable environment for the rat.

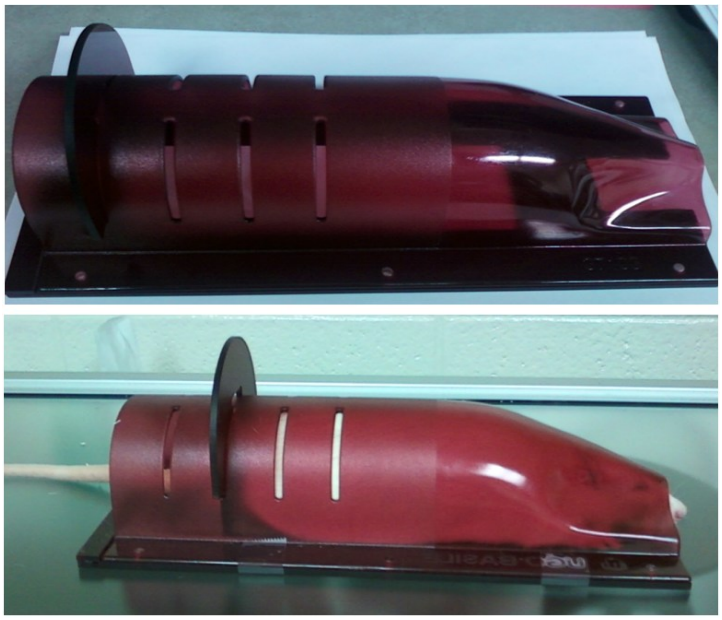

Figure 12. Holding device made professionally by Ugo Basile. The company used my prototype to design and manufacture an animal holding device that is now sold as an accessory for orofacial behavioral tests in conjunction with the Plantar Test apparatus.

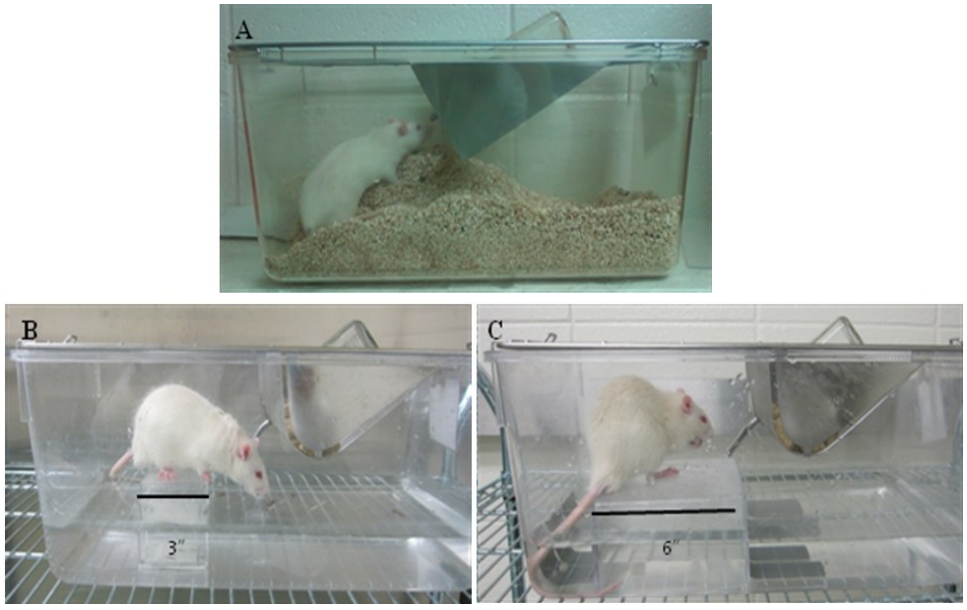

Figure 13. Cages used in experimental procedures. Control cage with aspen bedding (A), REM sleep deprivation cage with a 3 in. x 3 in. x 3 in. platform surrounded by water (B) and an environmental control cage with a 6 in. x 6 in. x 3 in. platform surrounded by water (C).

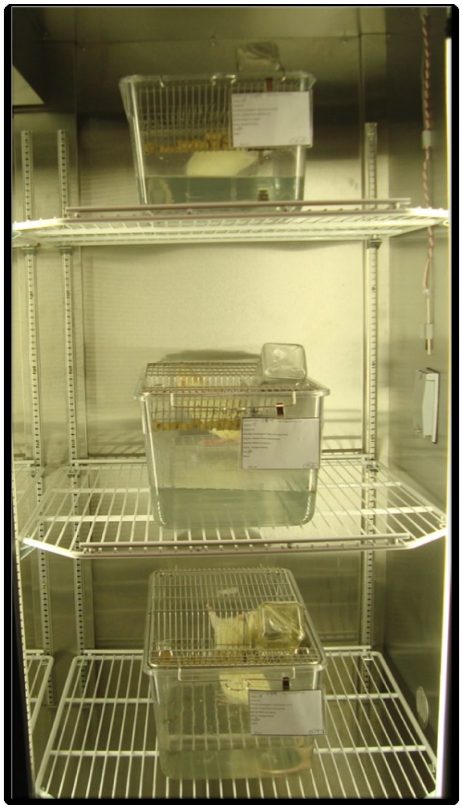

Figure 14. Inside the environmental chamber. A view of the shelves inside the environmental chamber in which experimental cages were placed for 72 hours.

Figure 15. Recording animal behaviors. Cameras were placed inside the environmental chamber and set to record experimental animals during specific times of their sleep cycle (morning, noon, and early evening).

RESULTS

True Blue Dye Localization

Traditionally, our laboratory studies orofacial pain by evaluating cellular and molecular changes in neurons in the V1, V2, or V3 regions of the TG. During experiments, animals are subjected to noxious and/or inflammatory stimuli and then euthanized so TG can be removed and cellular changes in protein and mRNA expression can be investigated. I wanted to focus on behavioral changes in response to thermal (noxious) stimulation of trigeminal nerves using a novel holding device in conjunction with the commercially available Plantar Test apparatus.

Typically, our laboratory studies activation of V3 nerves in response to transient or chronic inflammatory or noxious stimuli injected in the TMJ capsule. Therefore, I wanted to study changes in the thermal sensitivity of V3 nerves in that region of the head and face. However, the Plantar Test apparatus, which uses a heat source placed directly below the animal, was designed to measure thermal withdraw latency in the hind paw that is in direct contact with the glass surface. Thus, I had to find another area on the face that is also innervated by V3 nerves and could be positioned in direct contact with the glass so that upon stimulation, it would elicit the same neuronal changes as if I had tested in the TMJ capsule. Naturally, the area on the face which would be the most accessible for the behavioral test is under the chin in the submandibular region. To determine if this area would indeed cause the same neuronal changes, True Blue, a neuronal labeling dye, was injected into the TMJ capsule, masseter muscle region, and submandibular muscle region to verify by retrograde tracing that these areas are innervated by V3 nerves,

similar to the TMJ capsule. As seen in Figure 16, injections of True Blue in all areas of the face resulted in retrograde transport of the dye into the cell body of neurons located in the V3 region of the ganglion. These results provide evidence that the submandibular region is innervated by V3 nerves and thermal testing in this region of the face should elicit similar responses to those in the area of the TMJ. I could now move forward to standardize a way of using the Plantar Test apparatus that is based on the Hargreave's method of measuring changes in thermoresponsiveness on the submandibular region of my experimental animals.

Converting I.R. Intensity into Degrees Celsius

The Plantar Test apparatus uses an I.R. heat source set to a preferred intensity (0-99) based on how fast you want an animal to respond to the thermal stimulus. Through background research, I learned TRPV1 receptors on neurons are responsible for causing responses to stimuli such as heat; however the heat source must be greater than 43°C. Since I.R. intensities are not represented as a temperature, I wanted to know what those intensities were in comparison to degrees Celsius since this would guide me to the best intensity to use on my experimental animals. After looking at possible devices to help convert the I.R. to an understandable heat, I felt a thermocouple would give the most accurate results. As seen in Figure 17, I created a graph that compares the I.R. intensities from 0-99 (in increments of 10) and how long it takes the Plantar Test apparatus (seconds) to reach a certain temperature (Celsius). Based on my results, I.R. intensities from 70-99 would be predicted to cause thermal responses at a minimum of 6 seconds and a maximum of 10 seconds. However, based on data from my trial runs I consistently

observed most animals responded best between 40-60 I.R. at a minimum of 10 seconds and a maximum of 15 seconds. As a result, I concluded measuring I.R. intensities with a thermocouple and comparing them to degrees Celsius was not a good representation of which intensity to use because I.R. heat absorbance is dependent on multiple variables such as coat color, coat thickness, submandibular distance, etc. After further preliminary testing, I determined 40 I.R. as a good standard intensity to use in my experiments because it not only caused thermal responses in animals, but did so in a temporal manner that allowed accurate readings to be obtained.

Measuring I.R. Intensity for Consistency

The Plantar Test apparatus produces an I.R. heat source through a device which focuses light produced from a halogen bulb through a small shutter. After spending multiple hours learning how to use the apparatus and doing preliminary trials, it appeared the intensities were not eliciting the same responses in animals as they had previously. Before beginning my experiments, our laboratory purchased a heat-flux radiometer. This device is used to measure the emittance from the I.R. source and allows the user to know that the I.R. intensities are the same before each test. Data in Figure 18 shows what energy measurement each I.R. intensity should be if the halogen bulb is working to its full potential. From these measurements, I was able to verify 40 I.R. was consistently maintained at 146 mW/cm^2 throughout my experiments.

Behavioral Analysis Using Thermal Stimulation (Plantar Test Apparatus)

All experimental animals were tested for basal thermal threshold readings before being placed in either control, REM sleep deprivation, or environmental control cages/environments for 72 hours. Every 24 hours the animals were removed from their experimental condition and thermal threshold readings were taken. Following the 72 hour threshold readings, all animals were put in control cages for 24 hours to allow recovery sleep, if applicable, then subsequent 24 hour thermal thresholds were measured. The animals' average change in thermal thresholds for the 72 hour REM sleep deprivation period and the 24 hour recovery sleep period is shown in Figure 19. REM sleep deprived animals at hours 0 and 24 do not show a significant deviation from the control animals' thermal threshold readings. However, at hours 48 and 72 the REM sleep deprived animals exhibited a significantly shorter in withdraw latency when compared to control animals ($p < 0.005$). Following the 24 hour recovery sleep period, the REM sleep deprived threshold readings were similar to levels seen in control animals. For all time points, the environmental control animals and the control animals were not significantly different.

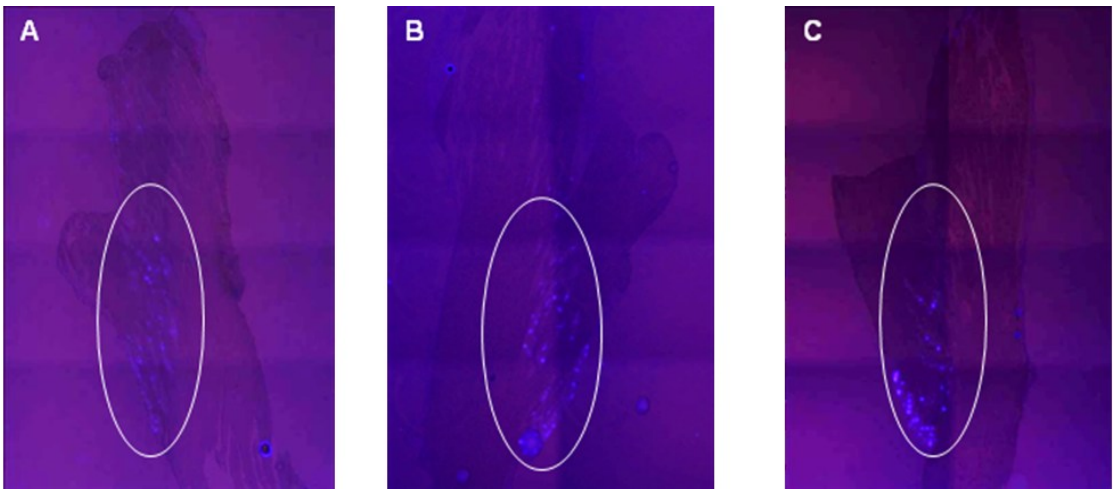

Figure 16. Sections of trigeminal ganglia from animals 5 days after injection of a fluorescent dye in different regions of the face to localize neuronal cell bodies. True Blue, which is taken up by endocytosis in neurons and retrogradely transported to the cell body, was injected into one of three areas in the facial region of the animal: temporomandibular joint (TMJ) capsule (A), masseter muscle (B), or mandibular muscle (C). Circled regions identify the V3 regions within the trigeminal ganglion.

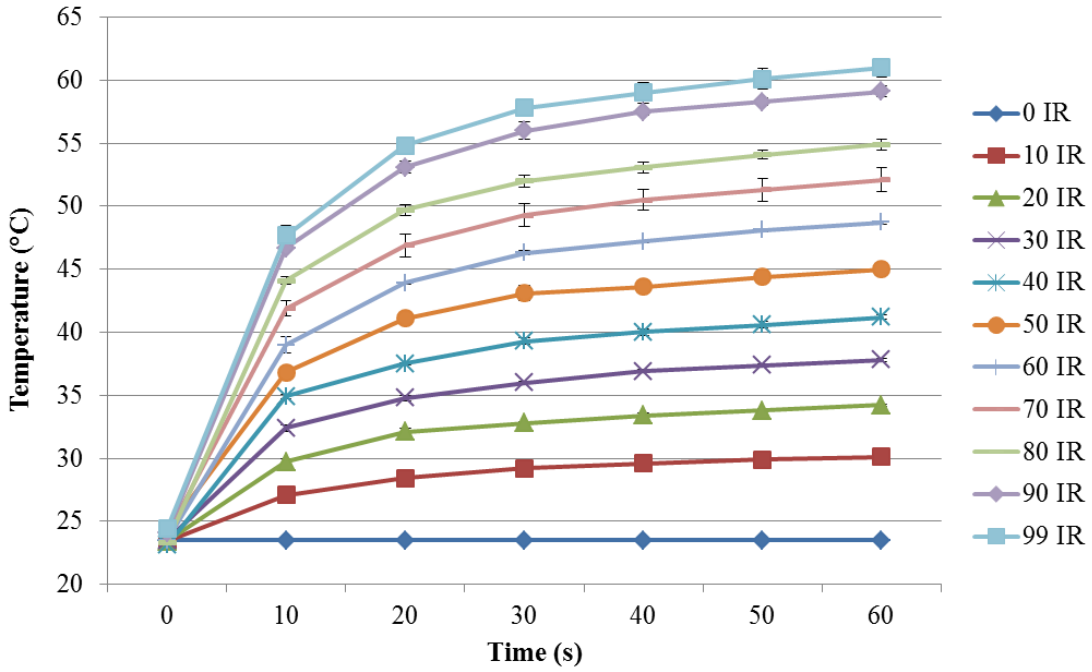

Figure 17. Relationship of I.R. intensity emittance to degrees Celsius. Readings were obtained every 10 seconds using a thermocouple to record changes in temperature readings for Plantar Test apparatus I.R. intensities (0-99 in increments of 10).

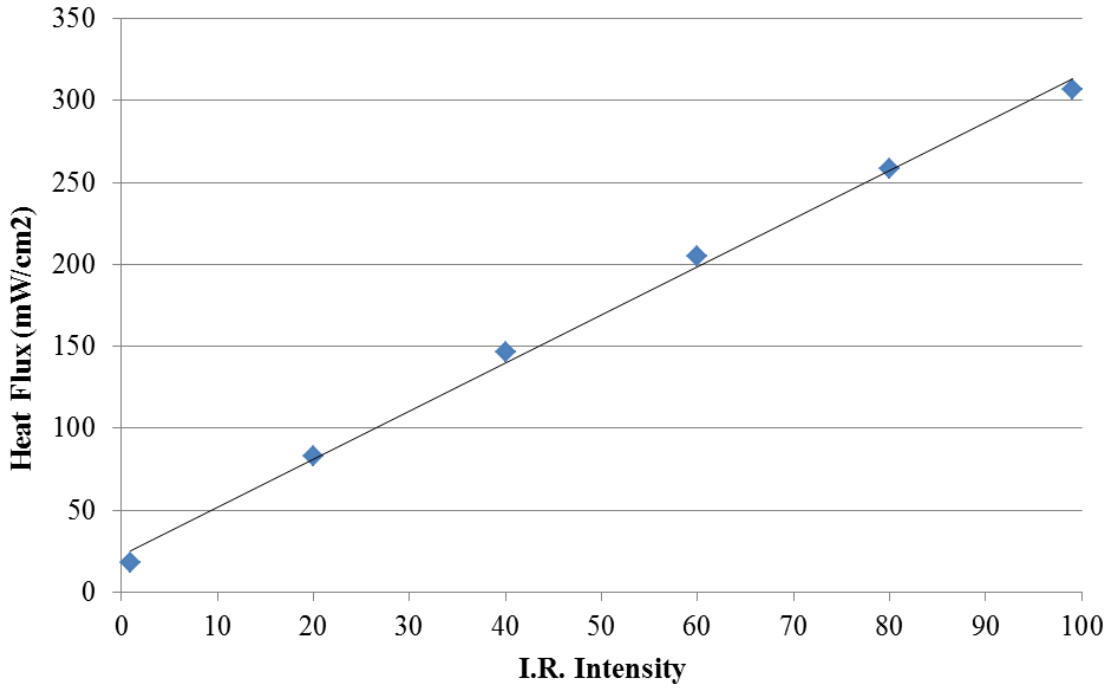

Figure 18. Relationship of I.R. intensity emittance to heat-flux. An I.R. heat-flux radiometer was used to record an energy emittance for Plantar Test apparatus I.R. intensities (0-99 in increments of 20).

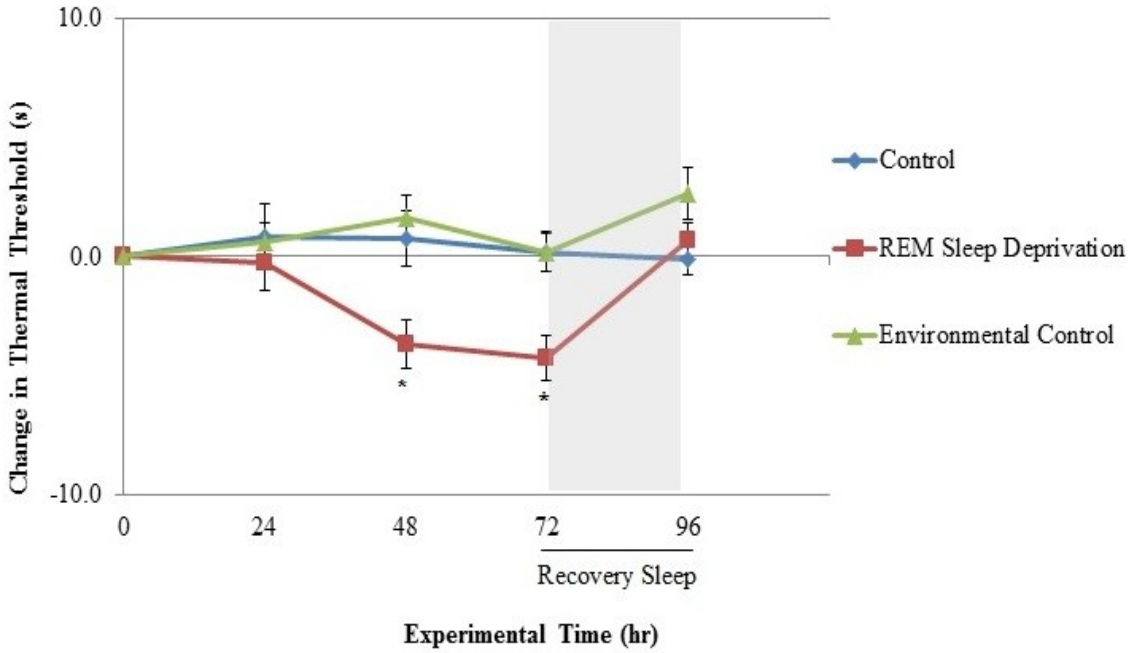

Figure 19. Change in withdraw latency in response to thermal stimuli. Control, REM sleep deprived, and environmental control animals' thermal threshold responses over 96 hours were measured daily. The first 72 hours consisted of REM sleep deprivation, if applicable, and the remaining 24 hours consisted of recovery sleep. * $p < 0.05$.

DISCUSSION

Nociceptor Sensitization

Every organism has a pain threshold of activation as defined by the length of time it takes to feel and respond to pain. Pain is experienced when noxious stimuli (i.e. heat) cause action potential generation in the peripheral nociceptors that leads to excitation of neurons in the central nervous system and release of inflammatory mediators (ATP, protons, nerve growth factor, etc.) and pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, etc.) (Cheng and Ji, 2008). These inflammatory mediators cause phosphorylation of ion channels and activation of signaling pathways in primary neurons, ultimately leading to the sensation of pain. Chronic exposure to noxious stimuli (i.e. diseased conditions) creating prolonged inflammatory responses in the nervous system leads to nociceptor sensitization. This pain hypersensitivity occurs when long-term exposure of inflammatory mediators causes ion channels and signaling pathways to change and increase their expression as well as respond to lower concentrations of inflammatory stimuli. Because of this hypersensitive or primed state, noxious and even non-noxious stimuli will initiate signaling cascades to cause excitation of neurons such that these cells respond in a pathological manner to normal stimuli. For my study, I wanted to see if REM sleep deprivation in animals would promote nociceptor sensitization as measured by a lowering of the nociception threshold to thermal stimuli.

Orofacial Tests to Study Thermal Pain Thresholds

Although diseases can affect any part of the body, those involving the head and face are some of the most painful and difficult to treat. Increased nociceptor sensitivity of trigeminal nerves is implicated in the underlying pathology of orofacial diseases including migraine, sinus, and TMJD pathologies. Orofacial pain in animals can be studied by observing behavioral changes in response to thermal (i.e., heat), chemical (i.e., formalin), or mechanical (i.e., pressure) stimuli. Although there exist research models for testing pain responses in the orofacial area to each of these stimuli, these methods are not widely used because of limitations due to the amount of time that is required to acquire the data and the high variability that increases the number of animals and hence the cost. In addition, another complication one encounters when performing these types of studies is that it is difficult to replicate what typically occurs at the level of the nerves. Typically, tissue injury results in asynchronous activation of peripheral and central neurons but causes synchronous excitation of fibers classically seen in sensory systems (Le Bars et al., 2001). Finally, another major limitation is the fact that these methods are referred to as more of an art than a science since the animals have to be trained and conditioned prior to and during testing. Within the research community, it is common knowledge that not everyone can generate consistent behavioral data especially when using the methods that require one to actually gently restrain the animal during mechanical or thermal testing. Not surprisingly, results from such studies are difficult to repeat even within the same laboratory by different researchers.

Traditionally, orofacial thermal tests have utilized radiant lamps or contact thermodes. Several drawbacks to using radiant lamps include the production of weak

caloric power making the speed of cutaneous heating induction slow and the fact that they emit radiation within the visible and adjacent infrared spectra. Of particular concern is the fact that the emitted radiation is likely to harm surrounding areas such as the eyes during testing. Additional problems are the lack of a focal area and the skin is a poor absorber at this spectra. There are also major problems associated with using thermodes. For example, the effectiveness of thermodes on the skin depends on whether the surface is even and the rate of thermal transfer. The rate of thermal transfer is dependent on the amount of skin contact and the pressure of application, parameters that are difficult to control in awake animals. More recently, infrared diode lasers have been utilized to stimulate trigeminal nerves that innervate facial skin in the rat in studies of thermal sensitivity (Cuellar et al., 2010; Tzabazis et al., 2005). However, in these studies the animal is placed in a stereotactic frame and immobilized. While this method is useful for electrophysiology studies, it is not appropriate for conducting behavioral studies. Unfortunately, a reliable method that requires minimal handling of the animals, short acclimation time, utilizes an I.R. light source, and an automated method for determining time of withdraw latency as currently available for measuring changes in pain thresholds in the hind paw (Plantar Test apparatus) was not available.

In my study, I wanted to modify the Plantar Test apparatus to be able to use the focused I.R. heat source in a way that would allow testing of the pain threshold in response to thermal stimulation of trigeminal nerves. By creating this thermal testing model, orofacial pain can be tested more easily and with less time and money than if using any of the three methods described above. Based on my results, the use of my model may provide a superior method for assessing changes in pain levels caused by

activation of trigeminal nerves, and thus facilitate a better understanding of pain mechanisms associated with diseases of the head and face including migraine and TMJD. Furthermore, creating an animal holding device and standardizing a way to use the Plantar Test apparatus in the orofacial region for thermal testing is important because it not only allows researchers to have a more comprehensible assessment of how orofacial pain effects an individual, but it will also allow researchers to understand possible correlations between cell and molecular changes with behavioral changes.

REM Sleep Deprivation and Pain

In my study, I had to design and test a method for depriving animals of REM sleep. To better control the environment, animals were housed in an environmental chamber that provided a consistent temperature and humidity while minimizing ambient light. The use of the environmental chamber allowed all animals in the REM sleep deprived or environmental control groups to be tested at the same time. Traditionally, the inverted flower pot model has been used to measure cellular and behavioral changes in response to REM sleep deprivation (Machado et al., 2004; Mendelson et al., 1974; Sapin et al., 2009; Shaffery et al., 2002). Using a digital camera mounted inside the chamber, I found that a 3 inch by 3 inch by 3 inch platform was sufficient to allow the animal a place to sleep but awaken upon entering REM sleep and making contact with the water. In contrast, the environmental control cages contained a 6 inch by 6 inch by 3 inch platform that allowed the animals to achieve REM sleep.

A novel finding from my study was that after two nights of REM sleep deprivation, animals had a significant decrease in the withdraw latency time when

compared to control animals. My results provide evidence that following REM sleep deprivation, trigeminal nerves become sensitized such that less stimulus is required to elicit a painful response, evidence of hyperalgesia. However, this difference in thermal sensitivity was not seen in animals returned to normal bedding and allowed to achieve REM sleep during the next 24 hours. Importantly, values for the REM sleep deprived animals were also significantly different from animals housed in the environmental control cages. These results suggest that REM sleep deprivation, rather than stress caused by limited mobility and water, were most responsible for the observed differences in trigeminal sensitivity.

Additional Uses of the Device

Although the main purpose for me designing a holding device was to study behavioral changes in response to thermal activation of trigeminal nerves, use of the device in conjunction with the Plantar Test apparatus could also be used to measure hind paw latency. Thus, the holding device would allow an investigator to study changes in nerves originating from the TG or dorsal root ganglion (DRG), which provides innervation to the hind paw. Based on preliminary studies performed in our laboratory, the standard deviation associated with thermal latency measurements in the hind paw obtained from animals tested in my device versus those from free roaming animals (traditional method) was markedly smaller. Furthermore, using my holding device in this way has the advantage that shorter acclimation times are required (5 minutes/day) compared to the standard free roaming method (30 minutes/day). Another major advantage of using my device is the ability to study both areas simultaneously, which

would greatly aid our understanding of nervous system function in response to noxious or inflammatory stimuli since results could answer questions about how orofacial pain affects the thermal sensitivity of the hindpaw and vice versa.

Another potential application of my holding device is using it in conjunction with Von Frey Filaments to test mechanical allodynia in the head and face of rats. Typically to perform this testing on animals, you must either hold the animal down in a way that is distracting and/or stressful to the animal or you must perform the test while being careful not to cause a withdraw response due to the animal seeing the filament coming towards the facial area making the animal pull away prematurely. Both methods of doing this testing require much habituation time and a skilled technician who has spent many hours performing this method. Hence, the technique is very time-consuming and data generated from such studies are always somewhat subjective since it is difficult to have an animal respond in a repeatable, reliable way. The use of my device would circumvent many of these limitations. In preliminary studies, our laboratory has shown that mechanical allodynia can be measured reliably using my device, which has been modified to allow testing with Von Frey Filaments in the V3 region.

Final Thoughts

Currently, the holding device is designed to comfortably fit and acclimate a rat for thermal testing using the Plantar Test apparatus. Although much research is conducted in rats, there are significantly more studies that utilize mice (especially transgenic and knock-out animals) to study cellular and behavioral changes in response to noxious or inflammatory stimuli. Potentially, my holding device could be modified to facilitate

more behavioral studies in the head and face of mice, which would aid in our understanding of diseases that involves activation and sensitization of trigeminal nerves.

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