

CENTRAL TARGETING OF TRIGEMINAL PRIMARY AFFERENT NERVE TERMINALS
VIA INTRACISTERNAL INJECTION OF RTX AND ITS EFFECT ON PAIN BEHAVIOR

By

MELANIE M. WEXEL

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2008

© 2008 Melanie M. Wexel

To my Mom, for inspiring me to continue education

ACKNOWLEDGMENTS

I thank my committee chair (John Neubert, DDS, PhD) and my committee members (Robert Caudle, PhD and Calegero Dolce, DDS, PhD). I would also like to thank Heather Rossi, Alan Jenkins, Jean Kaufman, and Wendi Malphurs for assisting with the laboratory experiments. Also, I would like to acknowledge the NIH grant #R21 DE16704-01A1 and the Southern Association of Orthodontics grant for financial support.

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	4
LIST OF FIGURES	6
LIST OF ABBREVIATIONS.....	7
ABSTRACT.....	8
CHAPTER	
1 INTRODUCTION	9
Background.....	9
Molecular Targets of Pain	10
Pharmacology	12
Significance	14
Hypothesis and Specific Aims.....	15
2 METHODS	16
Injections.....	16
Pain Induction.....	17
Behavioral Measures	17
Operant Thermal Facial Assessment	17
Mechanical Sensitivity	18
Capsaicin Eye Wipe Sensitivity	18
Immunohistochemistry	18
Statistical Analysis.....	19
3 RESULTS	21
Operant Thermal Facial Assessment	21
Mechanical Sensitivity.....	21
Capsaicin Eye Wipe Sensitivity.....	22
Immunohistochemistry	22
4 DISCUSSION.....	26
5 CONCLUSIONS	31
LIST OF REFERENCES.....	32
BIOGRAPHICAL SKETCH	35

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
3-1. RTX inhibits inflammatory orofacial pain	23
3-2. Mechanical sensitivity assessment	24
3-3. Capsaicin eye wipe response	24
3-4. Effect of intracisternal RTX on TRPV1 expressing cells in the trigeminal ganglia	25
3-5. Effect of intracisternal RTX on TRPV1 staining in the brainstem.....	25
4-1. Central delivery of RTX	30

LIST OF ABBREVIATIONS

ICM	Intracisternal injection into the cisterna magna
TG	Trigeminal Ganglion
RTX	Resiniferatoxin is an agonist of TRPV1. It is an ultrapotent analog of capsaicin that has been identified and used extensively in pain research.
TMD	Temporomandibular disorders. Painful conditions of the temporomandibular joint and muscles of mastication.
TNC	Trigeminal Nucleus Caudalis
TRP	Transient receptor potential. TRP channels have been identified as important modulators of thermal sensations.
TRPV1	Transient receptor potential channel, vanilloid subfamily member 1. Formerly known as the capsaicin receptor or VR1.

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

CENTRAL TARGETING OF TRIGEMINAL PRIMARY AFFERENT NERVE TERMINALS
VIA INTRACISTERNAL INJECTION OF RTX AND ITS EFFECT ON PAIN BEHAVIOR

By

Melanie M. Wexel

May 2008

Chair: John Neubert
Major: Dental Sciences

The TRPV1 selective neurotoxin resiniferatoxin (RTX) was evaluated for use in treating orofacial pain. RTX (250 ng) or vehicle was administered intracisternally in rats to lesion TRPV1 expressing neurons in the nucleus caudalis. Nociception was quantified 7 d following the treatment using an operant facial nociception assay in which the rats place their faces against a thermal stimulus (48°C) while obtaining a reward. Stimulus and reward contacts were monitored. The central lesions were verified by immunohistochemistry. RTX produced an analgesic effect to inflammatory pain as indicated by significantly greater ($P < 0.05$) reward licking events (1490 ± 360) and reward pain ratio (14.9 ± 2.5) when compared to vehicle-treated rats (Licks: 396 ± 86 ; Pain Ratio: 0.9 ± 0.1). RTX eliminated TRPV1 labeling in the nucleus caudalis but not in the trigeminal ganglion. These data indicate that RTX lesions may be used to selectively treat orofacial pain.

CHAPTER 1 INTRODUCTION

Background

Uncontrolled pain is a global problem of epidemic proportions. The cost of chronic pain is estimated to be about \$80 billion per year, with as much as 40% associated with orofacial pain (Israel and Scrivani 2000). Orofacial pain disorders including temporomandibular disorders (TMD), trigeminal neuralgia, headaches (e.g., migraine, tension-type), and myofascial pain compose a significant proportion of this widespread phenomenon. Although these disorders affect an estimated 20% of the U.S. population, there is a significant discrepancy in the amount of research focusing on the facial region and trigeminal system (Lipton et al. 1993). Further research and understanding of the trigeminal pain pathway mechanisms are needed for the development of novel facial pain therapies.

Opioids are currently the main form of treatment for moderate to severe pain. Unfortunately, these drugs have dose-limiting central side effects including sedation and respiratory depression. These side effects combined with their unpredictable response can vastly undermine the quality of life for patients being treated for chronic pain conditions. Since few effective analgesic drugs have emerged in the last 100 years, the search for better pain treatments remains a priority.

With the discovery of the capsaicin receptor (TRPV1) there is promise of uncovering the exact molecular mechanisms involved in pain transduction. Capsaicin is the main pungent ingredient of “hot” chili peppers that causes the activation of many nociceptive neurons (Szallasi and Blumberg 1990). TRPV1 is well known to have a role in pain modulation, both as an activator and inhibitor of transduction. However, the exact mechanisms that are involved with TRPV1-mediated pain inhibition are largely unknown. For example, the role of desensitization

versus neuronal death following treatment with vanilloids to produce pain relief is currently being debated. Understanding these mechanisms is crucial for further development of innovative molecular pain management therapies.

Agonists of TRPV1 including resiniferatoxin (RTX), an ultrapotent analog of capsaicin, have been identified and used extensively in pain research. RTX specifically deletes TRPV1 receptors and therefore can be used to investigate the role of TRPV1 on the elements of pain biology. This research not only aims to investigate the therapeutic capabilities of RTX but also to use RTX as a molecular neurosurgical tool to further understand pain mechanisms with the removal of the TRPV1 receptor.

Molecular Targets of Pain

Heat and vanilloid agents such as capsaicin evoke similar “burning” sensations, which led to the discovery that they have a common molecular pathway. It has been postulated for many years that nerve endings detect temperature and physical changes by ion channels. Recently, a number of transient receptor potential (TRP) channels have been identified as important modulators of thermal sensations. There are six TRP channels that have been cloned and characterized as thermoreceptors. This family has been shown to mediate extracellular calcium influx in response to low intracellular levels (Clapham 1996). Thermoreceptors can be activated by heat (35-45°C) or cold (17-35°C) and thermonociceptors are activated at noxious temperatures below 15°C and above 43°C (Caterina et al. 1997; LaMotte and Campbell 1978; Tominaga et al. 1998). These extreme temperatures can burn or freeze the skin and produce high frequency firing of A δ and C nociceptive fibers (Caterina et al. 1997; McKemy et al. 2002; Peier et al. 2002). TRPV1 is the mammalian heat thermonociceptor that we will focus on in this study.,

Hot chili peppers were believed to evoke burning pain through capsaicin-induced ion currents in sensory neurons. Investigations in the late 1980s led to the conclusion that the

capsaicin receptor was a non-specific cation channel with limited selectivity for calcium (Wood et al. 1988). The first evidence to support the hypothesis that ion channels were responsible for the burning response mechanism was the identification of heat gated ion channels in certain primary afferent neurons (Cesare and McNaughton 1996). In 1997, the transient receptor potential channel, vanilloid subfamily member 1 (TRPV1) receptor, formerly known as VR1, was cloned and characterized as a major molecular component for detecting both chemical and thermal pain (Caterina et al. 1997).

Gene expression for TRPV1 has been found to occur predominantly in small diameter cells of the unmyelinated c-fibers of trigeminal and dorsal root sensory ganglia (Caterina et al. 1997). TRPV1 is also expressed all along the primary afferent neurons from the peripheral distribution in the skin and deep tissues of the face to the presynaptic terminals (Caterina et al. 1997; Denda et al. 2001). TRPV1 is a ligand gated channel that is selective for cations with a very high relative permeability to divalent cations, including calcium. Although, TRP family receptors also respond to many other stimuli (e.g. temperature, protons, lipids, phorbols, phosphorylation, and pressure), TRPV1 is the only channel activated by capsaicin/vanilloids (Gunthorpe et al. 2002).

TRPV1 can be thought of as an integrator of noxious stimuli. Agonists (capsaicin and RTX) and potentiators (protons) act by reducing the temperature threshold for activation (Gunthorpe et al. 2002; Szallasi and Blumberg 1996; Tominaga et al. 1998). The mechanism of action of TRPV1 is largely unknown, but it appears that binding or direct thermal activation of the TRPV1 receptor causes a conformational change that allows the influx of cations into the cell. This influx creates an action potential that begins the cascade of nerve transmission that ultimately results in central perception of pain as described above (Caterina et al. 1997).

Pharmacology

TRPV1 receptors have a number of agonists including capsaicin, the main pungent ingredient of “hot” chili peppers and RTX, an ultrapotent analog of capsaicin derived from the euphorbia rubber plant (Szallasi and Blumberg 1990). Capsaicin and RTX can both activate and inhibit the signaling from nociceptive neurons. Inhibition of nociception by capsaicin is primarily produced by desensitization of the neuron, while RTX can specifically delete TRPV1 expressing neurons (Caterina et al. 1997; Caudle et al. 2003; Karai et al. 2004).

When capsaicin binds to TRPV1, activation of the channel allows the influx of sodium which results in depolarization and subsequent pain. Then, the influx of calcium leads to acute desensitization (Caterina et al. 1997; Szabo et al. 1999; Szallasi and Blumberg 1996). RTX is very potent for binding the TRPV1 receptor (Acs et al. 1996) and in contrast to capsaicin, the high affinity binding of RTX keeps the channel open. This allows for excess calcium influx in conjunction with release of intracellular stores, and subsequent cytotoxicity (Caterina et al. 1997; Szallasi et al. 1999). The ability of RTX to selectively eliminate cells expressing the TRPV1 receptor allows for its’ use both as a therapeutic prospect, and a tool to study the role of vanilloid receptors in pain modulation.

In vivo studies have investigated various methods of targeting the dorsal root ganglia and trigeminal nerve both peripherally and centrally with RTX. In 1999, Szabo et al. compared the effects of RTX injected epidurally and subcutaneously in rats. Epidural administration was found to be selective for the spinal cord region, while the subcutaneous approach gave a generalized analgesic effect. The epidural approach was found to produce profound, long-lasting segmental analgesia to C-fiber mediated pain as judged by temperature withdrawal latency (Szabo et al. 1999). The elimination of peripheral nerve endings of the hindpaw showed a long term, reversible attenuation of nociceptive transmission based on increased thermal hindpaw

withdrawal latency (Neubert et al. 2003). Kissin et al. completely prevented pain hypersensitivity caused by the carrageenan inflammatory process and plantar incisions by prophylactically applying RTX percutaneously to the sciatic and saphenous nerves (Kissin et al. 2002; Kissin et al. 2005b). This group also showed a decrease in carrageenan induced edema and pain with RTX injections in the knee joint (Kissin et al. 2005a). In 2004, Karai et al. injected RTX into hindpaws of rats and the lumbar cerebrospinal fluid of dogs and was able to demonstrate efficacy in both species. Of significance, the pain response was blocked while keeping the sensations of touch, proprioception, motor function and mechanosensitive nociception (Karai et al. 2004).

There is precedence to using RTX within the trigeminal system. Karai et al. applied RTX into the trigeminal ganglia (TG) via a stereotaxic approach through the brain, while Neubert et al. accessed the trigeminal ganglia via the infraorbital foramen (Karai et al. 2004; Neubert et al. 2005a). Both studies demonstrated that RTX can specifically delete TRPV1 within the orofacial region, as evidenced by a loss of capsaicin eye wipe response. In this behavioral assay, a 0.1% solution of capsaicin is applied to the cornea of the eye and the number of eye wipes with the paws is counted for 1 minute. Capsaicin normally elicits extreme burning pain when applied in this fashion, producing over 50 wipes/min on average. Animals treated with RTX in the trigeminal ganglia have a complete elimination of this response. Immunohistochemistry data also revealed that RTX blocked inflammation induced spinal c-fos induction, a marker for nociceptive activity induced within the second order neurons (Neubert et al. 2003).

Reduction of orofacial pain responses have also been achieved by using specific toxins via an intracisternal injection approach of substance P conjugated to saporin (SP-SAP) (Simons et al. 2002). While this drug specifically targeted the superficial medullary neurons expressing the

neurokinin 1 receptor (NK-1), this intracisternal approach may also be used to target the central terminals of trigeminal primary afferent fibers. Surprisingly, this region of the brainstem is relatively unprotected and is easily accessible via a percutaneous approach through the atlanto-occipital membrane overlying the cisterna magna. The cisterna magna is an ideal site to target TRPV1 in the brainstem to evaluate the effects of RTX on orofacial pain. Although there is evidence that application of RTX to the peripheral nerve terminals produces a loss of TRPV1 expressing cells in the trigeminal ganglia; the effects on TRPV1 cells in the trigeminal ganglia with a intracisternal injections are unknown (Neubert et al. 2003). This is one of the outcomes that we investigated.

One of the limiting factors for studying pain in the face relates to the limited number of valid behavioral outcome measures. Assessment of mechanical sensitivity using von Frey filaments to elicit a head withdrawal response is the typical orofacial pain outcome measure. However, this approach has numerous limitations, including that the animals must be restrained. Our group has devised an alternative to typical reflex and unlearned behavioral measures by using an operant thermal assessment system (Neubert et al. 2005a). Briefly, this system measures orofacial pain behavior by providing positive rewards for tolerance of thermal nociceptive stimulation. A food fasted animal is given the choice of placing its face on a hot (e.g., 48°C) thermode in order to obtain a reward of sweetened condensed milk. (Neubert et al. 2005a). This method readily lends itself to testing of trigeminally-mediated pain and was one of the primary outcome measures of this study. This system is described in detail in the materials and methods.

Significance

Currently, the most commonly used drugs and treatment approaches vastly undermine the patients' quality of life and many times remain inadequate. There is a need for greater

understanding of the trigeminal pathways of pain in order to develop novel pain management techniques. This study hopes to investigate pain mechanisms within the trigeminal system that will also evaluate new therapeutic approaches to treating chronic pain.

Hypothesis and Specific Aims

We hypothesized that deletion of trigeminal TRPV1 expressing primary afferent pain fibers via targeting of the central terminals will significantly affect pain in the orofacial region. We tested this hypothesis with a number of specific aims. Aim 1 was to evaluate the effects of central RTX-treatment on orofacial pain by behaviorally characterizing thermal and mechanical sensitivity following central administration of RTX or vehicle and induction of orofacial inflammation. Aim 2 was to histologically characterize the changes in the TRPV1 expressing cells in the trigeminal ganglion and evaluate the relative amount of TRPV1+ fibers in the nucleus caudalis following central administration of RTX or vehicle.

CHAPTER 2 METHODS

Male Sprague Dawley rats (200-300g, $N = 30$) were lightly anesthetized (isoflurane, 1-2.5%, inhalation) 1 day prior to testing, and the face was shaved using clippers, followed by application of depilatory cream. Excess cream was removed with a moistened paper towel to minimize skin irritation. Rats were food fasted (12-15 hrs) prior to each testing session but were provided with standard food chow immediately following each session and on non-testing days. Animals were brought into the behavioral procedure room 1 hr prior to testing at the same time each day and allowed to acclimate to the temperature and ambient noise of the room. Water and food was made available *ad libitum* when animals were not in a testing session. Animal weight was recorded weekly. Animal testing procedures and general handling complied with the ethical guidelines and standards established by the Institutional Animal Care & Use Committee at the University of Florida, and all procedures complied with the Guide for Care and Use of Laboratory Animals (Council 1996).

Injections

We targeted TRPV1 receptors on the central terminal of the trigeminal primary afferent neurons by intracisternal injection into the cisterna magna. Animals were anesthetized (2.5% isoflurane, USP, inhalation) and the posterior skull region overlying the cisterna magna was disinfected with betadine. A 27 gauge, ½ inch needle attached to a 0.3ml plastic syringe was then directed so as to touch the occiput. Note that contacting the bony surface provides distinct tactile feedback. The tip was sequentially moved caudally until the needle punctured the dura overlying the cisterna magna. Once the needle was in place, aspiration was performed to check for cerebrospinal fluid, and then RTX (250 ng) or vehicle (0.25% Tween 80 in phosphate buffered saline, 0.05% ascorbic acid) was delivered.

Pain Induction

Carrageenan, an inflammatory agent derived from seaweed, was used to induce inflammation and subsequent heat sensitivity (Ng and Ong 2001). A volume of 200 μ l of a 40 mg/ml, 8mg total solution was administered by subcutaneous injection into the face 1 week following intracisternal RTX or vehicle injections. Animals were tested 3 hrs post-carrageenan injection at 48°C.

Behavioral Measures

The animals were trained for two weeks prior to injections to allow them to acclimate to the reward-aversion testing boxes. Baseline thermal (37°C, 48°C), mechanical (Von Frey), and cap eye wipe responses were recorded on all rats prior to injections.

Operant Thermal Facial Assessment

Orofacial thermal sensitivity was assessed using a reward-conflict operant paradigm, as described previously (Neubert et al. 2005a). Briefly, unrestrained animals were placed into reward-aversion testing boxes. The animals were given a choice to endure a painful thermal stimulus (48°C + inflammation) in order to receive a reward consisting of sweetened condensed milk solution. Sensors were placed on both the reward bottle and stimulus thermode and data was recorded automatically as a “licking” or “facial contact” event whenever the animal contacted either part. The threshold for detection of facial contacts and licking contacts was set at 1.0 V and an event was registered when the signal went above threshold and ended when the signal drops below threshold. For orofacial thermal sensitivity, six outcome measures were evaluated: (1) reward intake; (2) total number of licking events; (3) total number facial contacts; (4) cumulative facial contact duration; (5) ratio of reward/facial contacts; (6) duration per contact for the facial contacts. Data analyses were completed using custom-written routines (generously

provided by Dr. Charles Widmer, University of Florida) in LabView Express (National Instruments Corporation) and Excel (Microsoft).

Mechanical Sensitivity

Mechanical sensitivity was evaluated as a reflexive pain outcome measure. An electronic aesthesiometer (IITC Inc., Woodland Hills, CA) was used to assess the threshold of painful response to mechanical stimulation. The rigid tip attached to the probe was pressed against the skin overlying the superficial masseter until an aversive response was displayed and the probe was removed. An aversive response included withdrawal of the head, vocalization, twitching of the back, struggling against restraint, or any combination thereof. The sensor displayed the force (g) needed to achieve this aversive response and this value was recorded by the investigator. Three measurements were taken at each test site, alternating between the left and right sides.

Capsaicin Eye Wipe Sensitivity

The capsaicin eye wipe response was assessed before and 1 hour after the cisterna magna injections of RTX and vehicle. A capsaicin solution (0.1%, intraocular, 50 μ l) was placed directly into the cornea and the number of eye wipes was counted for one minute (Karai et al. 2004). In preliminary studies, we found that RTX delivered intracisternally completely eliminated the capsaicin eye wipe response. Therefore, we used this assay to verify that the RTX injections were successful. RTX injected rats that did not experience elimination of the capsaicin eye wipe response were re-injected.

Immunohistochemistry

We investigated the expression of TRPV1 in the trigeminal ganglion (TG) and the trigeminal nucleus caudalis (TNC) following RTX or vehicle treatment in the cisterna magna. After animals were euthanized, right and left trigeminal ganglia and brainstem tissues were

immediately harvested and placed into a 10% formalin solution and post-fixed overnight. Samples were paraffin embedded, sectioned (10 μ m) and mounted on Fisher Plus slides. Following deparaffinization and epitope unmasking with Target Retrieval Solution (S1700, Dako, Carpinteria, CA, 60C overnight), sections were blocked with 10% normal goat serum (S-1000, Vector Laboratories, Inc., Burlingame, CA) followed by a peroxidase quenching (Dako Peroxidase Blocking Reagent, S2001) and incubated overnight at 4°C with the TRPV1 primary antibody (1:10,000, rabbit anti-VR1, Affinity Bioreagents). Antibody detection was performed using the Vectastain Elite ABC Goat anti-Rabbit IgG and Peroxidase Substrate Kits (SK-4700 and SK-4100, Vector Laboratories, Inc., Burlingame, CA) and visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Lab, SK-4100). Control specimens for assessment of non-specific binding were processed in parallel, with the omission of the primary antibody. Histological sections were visualized under light microscopy.

A blinded observer chose two sections from different levels of the TG and TNC for each group (treated vs. non-treated) and the number of TRPV1 immunoreactive and non-immunoreactive cells within a specific, standardized area were counted by visual inspection (100x magnification); the ratio of TRPV1 to the total number of small to medium sized cells was calculated. For brainstem sections, the presence of positive staining was graded by an observer blinded to the animal treatments using a 3 point scale: 0=none; 1=light staining; 2=heavy staining. Ten sections at random for each treatment were scored by visual inspection (10x objective).

Statistical Analysis

Data normality was assessed (Kolmogorov-Smirnov with Lilliefors Significance test) and the appropriate statistical analyses were completed to determine whether the effects of

temperature were significant. An ANOVA was used to evaluate the effects of treatment (none, inflammation/RTX, inflammation/vehicle) on mechanical sensitivity and operant thermal facial assessment outcomes at 48°C (SPSS Inc). An ANOVA was also used for the capsaicin eye wipe testing response to evaluate differences between the treatment groups and baseline. When significant differences were found, post-hoc comparisons were made using the Tukey HSD, using a probability level of 0.05.

In the trigeminal ganglia, the ratio of TRPV1 to the total number of cells in the two treatment groups (RTX, vehicle) was compared using an unpaired t-test. The Kruskal-Wallis test was used for brainstem histological comparisons. A significance level of $P < 0.05$ was used in all instances.

CHAPTER 3 RESULTS

Operant Thermal Facial Assessment

Previous studies by Neubert et al demonstrated significant temperature effects on the operant behavior of untreated animals (Neubert et al. 2006; Neubert et al. 2005b). In these studies, a number of outcomes were significantly decreased at temperatures $\geq 45.5^{\circ}\text{C}$, including reward licking events, reward/attempts and the facial duration/contact ratios. Additionally, carrageenan-induced inflammation produced a significant decrease on these outcome measures (Neubert et al. 2005b). Based on these studies, we chose to use the carrageenan-inflammation model and test animals at stimulus temperatures $\geq 45.5^{\circ}\text{C}$. Following intracisternal injections, vehicle animals that were tested at 48°C showed no significant difference from 48°C baseline values (data not shown). Note that RTX animals were not tested at 48°C prior to carrageenan treatment because in preliminary studies these animals displayed insensitivity at this noxious stimulus temperature and therefore risked severe burning of their faces.

There was a significant difference between RTX and vehicle groups for all operant thermal facial outcome measures following inflammation with carrageenan, when tested at 48°C (Figure 3-1). Five outcome measures were significantly higher in the RTX animals: intake, licking contact events, duration, ratio licks/contacts, and ratio facial duration/contacts. Facial contact events were significantly lower for RTX animals. Overall, these data indicate an analgesic effect following intracisternal RTX treatment.

Mechanical Sensitivity

At baseline, there was no significant difference in mechanical sensitivity between the two groups. After intracisternal treatment, the RTX/carrageenan group demonstrated a significantly higher threshold ($P < 0.05$) as compared to baseline and vehicle/carrageenan groups (Figure 3-2).

The vehicle treated animals had a significantly lower threshold ($P < 0.05$) following carrageenan inflammation, as compared to baseline values. These results indicate that carrageenan produced mechanical hyperalgesia that was blocked by intracisternal RTX treatment.

Capsaicin Eye Wipe Sensitivity

The capsaicin eye wipe response was completely eliminated in RTX animals and remained intact in vehicle injected animals. There was no significant difference between the eye wipe response of baseline and vehicle animals following capsaicin application. (Figure 3-3)

Immunohistochemistry

There were no significant differences found in the ratio of TRPV1-expressing cells in the trigeminal ganglia for RTX-treated animals compared to vehicle-treated (Figure 3-4). There were significant differences found in the level of TRPV1 staining of the trigeminal nucleus caudalis between the two treatment groups (Figure 3-5). The RTX group showed a complete elimination of TRPV1-positive staining in the nucleus caudalis. All RTX-treated animal sections (N=15) were scored zero for no staining, while all sections (N=15) from vehicle-treated animals were scored two for heavy staining. Collectively these data indicate that the RTX intracisternal treatment was specific to lesioning of the central terminals of the TRPV1-expressing neurons, but did not lesion the entire neuron.

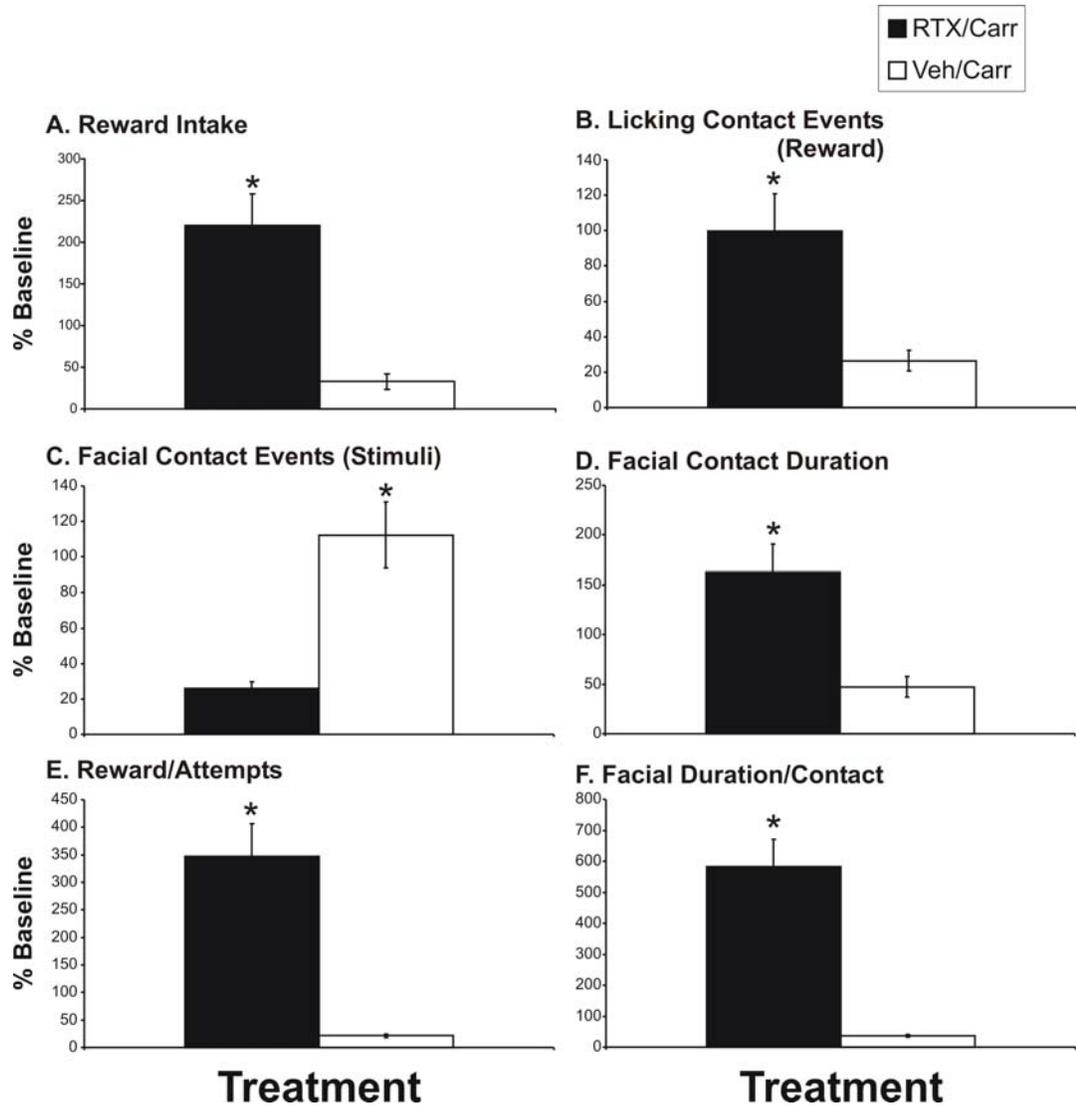


Figure 3-1. RTX inhibits inflammatory orofacial pain. There was a significant difference ($*P < 0.05$) in all outcomes between RTX and vehicle animals. These measures were taken 3h following induction of orofacial inflammation. Note that all behavioral outcome measures are expressed as a percent of 48°C baseline values.

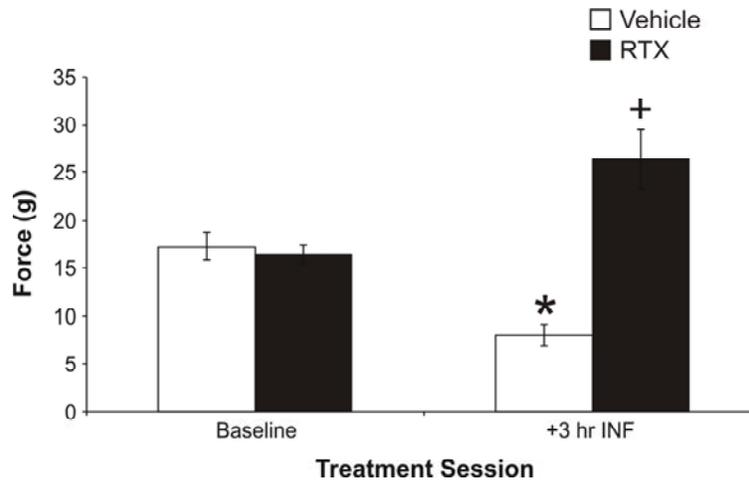


Figure 3-2. Mechanical sensitivity assessment 3h after inflammation using Von Frey filaments. RTX significantly increased the threshold compared to both vehicle and baseline ($+P<0.05$). Vehicle treated animals displayed a significantly lower threshold ($*P<0.05$) as compared to baseline.

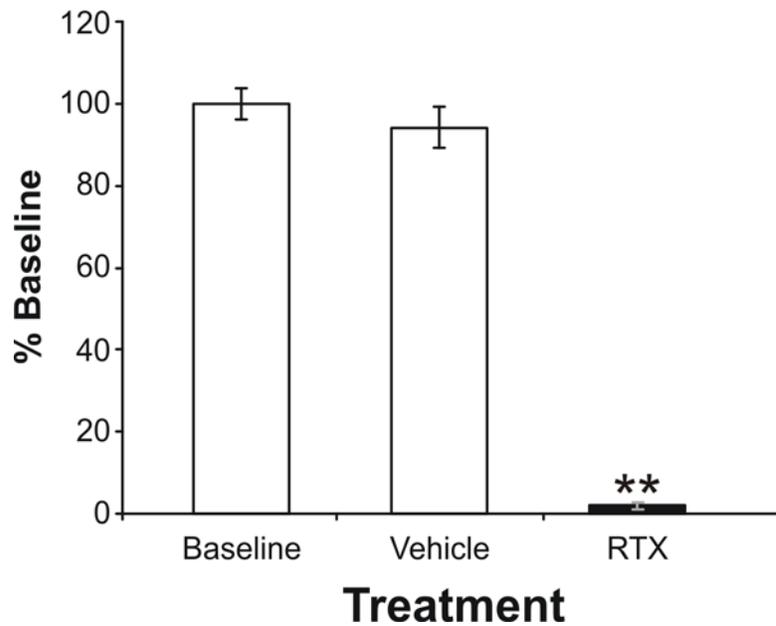


Figure 3-3. Capsaicin eye wipe response. RTX significantly eliminated the number of eye wipes following application of 0.1% capsaicin to the eye ($P<0.05$).

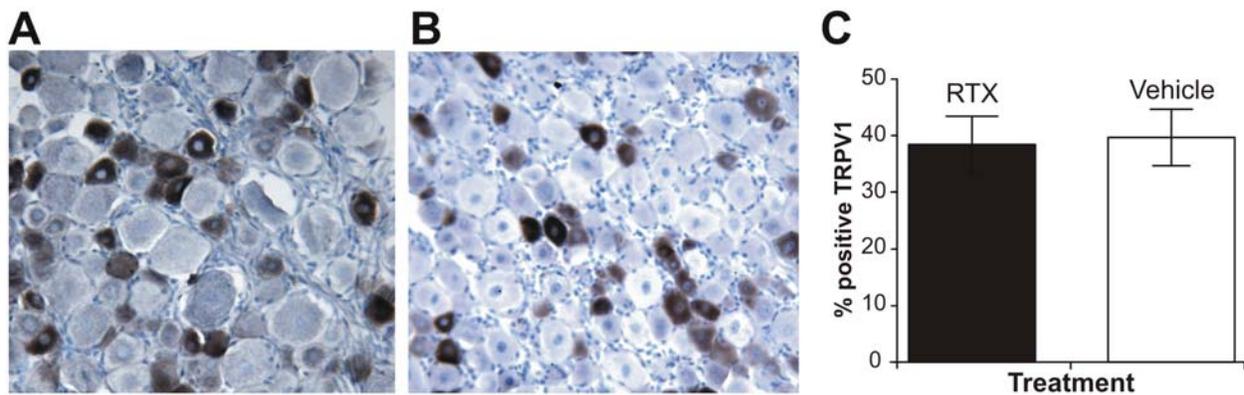


Figure 3-4. Effect of Intracisternal RTX on TRPV1 expressing cells in the trigeminal ganglia. Intracisternal (ICM) injection of RTX does not significantly reduce the number of TRPV1+ neurons within the trigeminal ganglia. Immunohistochemical analysis demonstrated that RTX (A) and vehicle (B) treated animals had a similar proportion (C) of TRPV1+ cells 2 weeks following intracisternal treatment with either RTX or vehicle.

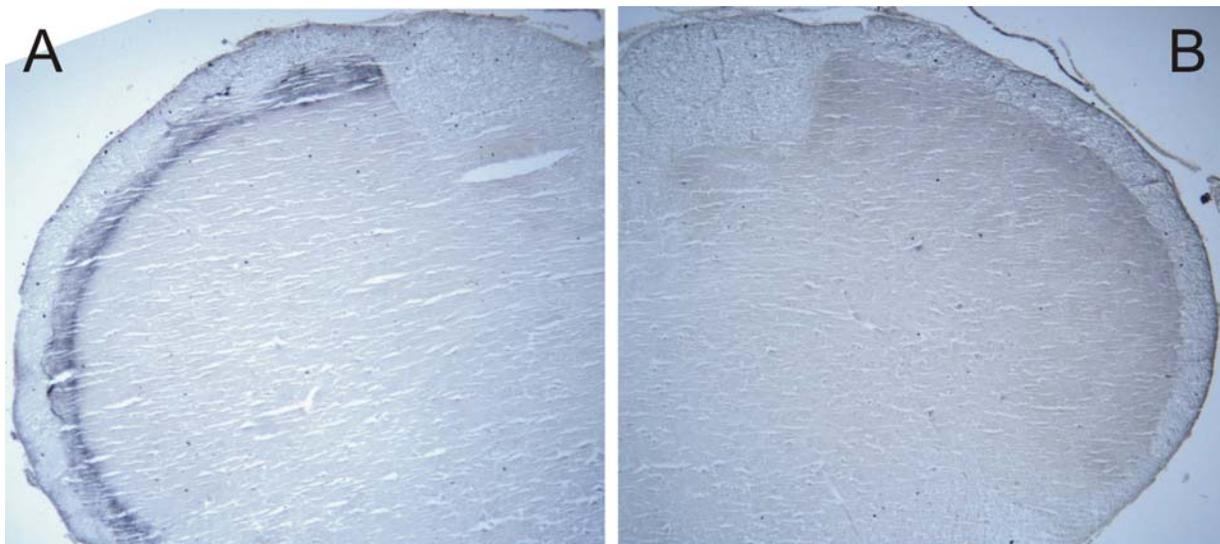


Figure 3-5. Effect of intracisternal RTX on TRPV1 staining in the brainstem. This is a representative histological section of TRPV1+ staining in the brainstem following either intracisternal (ICM) injection of vehicle (A) or RTX (B, 250ng, 10 μ l). Note that there was complete elimination of TRPV1 staining in the brainstem for the RTX-treated animal.

CHAPTER 4 DISCUSSION

To evaluate the effects of a therapy in the orofacial region of animals, one must consider the use of validated behavioral outcome measures. We used a variety of tests that included reflex, unlearned, and operant measures to evaluate both mechanical and sensory aspects of pain. A novel operant thermal test assay was used to examine changes in thermal sensitivity after intracisternal targeting of TRPV1 receptors in the brainstem using RTX and subsequent inflammation in the face. Vehicle/carrageenan animals tested at 48°C demonstrated behavior indicative of hyperalgesia whereas the behavior of RTX/carrageenan animals indicated analgesia.

All six of the behavioral outcomes were significantly different for the RTX-treated animals as compared to the vehicle-treated animals. For the RTX group, five of the outcomes significantly increased (intake, licking contact events, duration, ratio licks/contacts, ratio facial duration/contacts), which we interpret as an analgesic response. Additionally, the decrease in facial contact events also indicates analgesia because the animal was able to keep its face on the thermode for a longer duration, therefore requiring fewer contacts to access the reward. For vehicle-treated animals, there was a significant decrease in intake, licking contact events, duration, licks/contacts ratio, facial duration/contacts ratio, as compared to baseline testing at 48°C. This is typical of a hyperalgesic response after carrageenan inflammation and has been documented previously (Neubert et al. 2005b). However, there was no significant difference between baseline and vehicle facial contact events (*data not shown*). This is not completely surprising, because the number of facial contact events can increase due to the limited duration the animal can tolerate its face on the thermode. Since there is reduced duration with elevated contact attempts the duration/contact ratio was very low in vehicle rats, confirming hyperalgesia.

We assessed mechanical sensitivity using Von Frey filaments, with a head withdrawal being the endpoint to stimulation. This was completed to evaluate whether RTX is primarily affecting thermo-sensing versus mechano-sensing neurons. We found that the RTX group had a significantly ($P < 0.05$) higher threshold than both vehicle and baseline, while the vehicle group demonstrated mechanical hyperalgesia with a significantly ($P < 0.05$) lower threshold than RTX and baseline. This finding suggests that some mechanical nerve transmission is also being affected by the intracisternal RTX injections. This is interesting given our prior studies (Neubert et al. 2003; Neubert et al. 2008a; Neubert et al. 2005a), in which RTX was given subcutaneously in the foot, percutaneously in the trigeminal ganglion, and perineurally in the sciatic nerve, demonstrating little effect of RTX on mechanical sensitivity. This difference in mechanical sensitivity between peripherally and centrally administered RTX would suggest that the site of drug application may have different effects on mechanical responses.

We completed a histological survey of TRPV1 in various points in the trigeminal sensory pathway, including at the cell body level in the trigeminal ganglion and at the brainstem level where the primary afferent neurons project centrally into the nucleus caudalis. In previous studies, perineural application of RTX around the infraorbital nerve ($p < 0.05$) demonstrated a loss of TRPV1-expressing cells in the TG and orofacial pain responses are eliminated (Neubert et al. 2008b). Initially, we hypothesized that the deletion of TRPV1 from the presynaptic terminal of TRPV1 expressing neurons would also lead to deletion of their cell bodies within the TG following intracisternal RTX application. On the contrary, this study showed that centrally delivered RTX causes specific elimination of TRPV1-positive staining in the nucleus caudalis but no differences in the ratio of TG TRPV1 expressing cells following RTX treatment, suggesting that the central terminal is being cleaved off in a receptor specific fashion (Figure 4-

1). This has interesting implications clinically, as central administration of RTX may have the advantage over peripheral delivery systems. This is due to its specific targeting of the pre-synaptic TRPV1 fibers with no damage to the TG TRPV1 cells or the peripheral fibers. The peripheral elimination of TG TRPV1 cells and infraorbital nerve fibers may come with a price as the cell body may also perform other tasks and functions. For example, different growth factors (e.g., nerve growth factor) and neuropeptides (e.g., substance P) are colocalized in cells expressing TRPV1 and elimination of these factors may have implications on healing.

Although ~20% of the U.S. population is affected by a facial pain disorder, there still remains many questions regarding mechanisms of trigeminal pain processing. Current treatment modalities for chronic orofacial pain disorders can include the use of pharmaceutical agents such as the opioid receptor agonists (e.g., morphine) that also cause CNS and respiratory depression. The goal of this project was to explore the use of alternative analgesic agents that lack these untoward side effects. Targeting the TRPV1 receptor with the agonist RTX in rats has led us one step closer to understanding molecular mechanisms of trigeminal pain transduction in humans. This knowledge will provide a foundation for development of novel therapeutic approaches that specifically target the trigeminal pain pathway without inducing sedative side effects and diminished the quality of life for those dealing with chronic facial pain.

Further investigation is necessary to evaluate the negative side effects that may occur due to removal of this receptor. For example, with loss of sensation associated with elimination of the TRPV1 receptor, the response to painful stimuli as a protective mechanism against injury in these patients may be compromised. Also, TRPV1 may have other roles of which we are not aware that could complicate the therapy. For these reasons, RTX therapy should initially be

reserved to improve the quality of life for patients with terminal illnesses causing chronic facial pain.

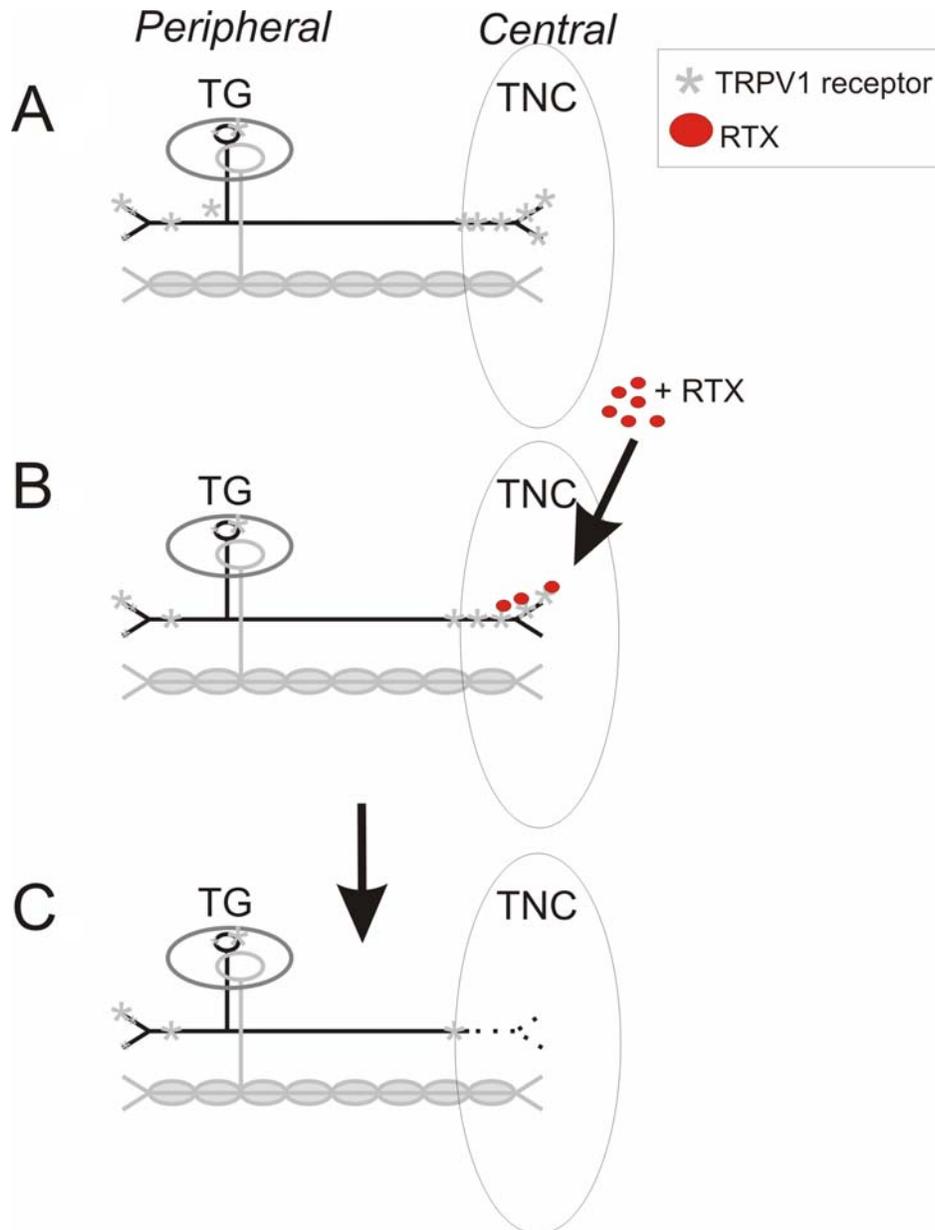


Figure 4-1. Central delivery of RTX A. Baseline animal with peripheral and central TRPV1 receptors intact B. Delivery of RTX intracisternally C. Specific deletion of TRPV1 only on the presynaptic fibers of the TNC with no effect on the TG cell bodies.

CHAPTER 5 CONCLUSIONS

The use of RTX has confirmed the role of TRPV1 in trigeminal pain processing. This molecular understanding of the trigeminal pain mechanism is essential for the development of novel pain management techniques. In studies completed in the lab including the body of work in this project, both peripheral and central administration of RTX in the trigeminal region eliminated pain behavior in animals. We conclude that intracisternal RTX treatment may be an effective means for specific pain control in the orofacial region.

LIST OF REFERENCES

- Acs, G., Palkovits, M. and Blumberg, P.M., Specific binding of [3H]resiniferatoxin by human and rat preoptic area, locus ceruleus, medial hypothalamus, reticular formation and ventral thalamus membrane preparations, *Life Sci*, 59 (1996) 1899-908.
- Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway, *Nature*, 389 (1997) 816-24.
- Caudle, R.M., Karai, L., Mena, N., Cooper, B.Y., Mannes, A.J., Perez, F.M., Iadarola, M.J. and Olah, Z., Resiniferatoxin-induced loss of plasma membrane in vanilloid receptor expressing cells, *Neurotoxicology*, 24 (2003) 895-908.
- Cesare, P. and McNaughton, P., A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin, *Proc Natl Acad Sci U S A*, 93 (1996) 15435-9.
- Clapham, D.E., TRP is cracked but is CRAC TRP?, *Neuron*, 16 (1996) 1069-72.
- Council, N., In: *Guide for the Care and Use of Laboratory Animals.*, National Academy Press, Washington, DC, 1996.
- Denda, M., Fuziwara, S., Inoue, K., Denda, S., Akamatsu, H., Tomitaka, A. and Matsunaga, K., Immunoreactivity of VR1 on epidermal keratinocyte of human skin, *Biochem Biophys Res Commun*, 285 (2001) 1250-2.
- Gunthorpe, M.J., Benham, C.D., Randall, A. and Davis, J.B., The diversity in the vanilloid (TRPV) receptor family of ion channels, *Trends Pharmacol Sci*, 23 (2002) 183-91.
- Israel, H.A. and Scrivani, S.J., The interdisciplinary approach to oral, facial and head pain, *J Am Dent Assoc*, 131 (2000) 919-26.
- Karai, L., Brown, D.C., Mannes, A.J., Connelly, S.T., Brown, J., Gandal, M., Wellisch, O.M., Neubert, J.K., Olah, Z. and Iadarola, M.J., Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control, *J Clin Invest*, 113 (2004) 1344-52.
- Kissin, E.Y., Freitas, C.F. and Kissin, I., The effects of intraarticular resiniferatoxin in experimental knee-joint arthritis, *Anesth Analg*, 101 (2005a) 1433-9.
- Kissin, I., Bright, C.A. and Bradley, E.L., Jr., Selective and long-lasting neural blockade with resiniferatoxin prevents inflammatory pain hypersensitivity, *Anesth Analg*, 94 (2002) 1253-8, table of contents.
- Kissin, I., Davison, N. and Bradley, E.L., Jr., Perineural resiniferatoxin prevents hyperalgesia in a rat model of postoperative pain, *Anesth Analg*, 100 (2005b) 774-80.

- LaMotte, R.H. and Campbell, J.N., Comparison of responses of warm and nociceptive C-fiber afferents in monkey with human judgments of thermal pain, *J Neurophysiol*, 41 (1978) 509-28.
- Lipton, J.A., Ship, J.A. and Larach-Robinson, D., Estimated prevalence and distribution of reported orofacial pain in the United States, *J Am Dent Assoc*, 124 (1993) 115-21.
- McKemy, D.D., Neuhausser, W.M. and Julius, D., Identification of a cold receptor reveals a general role for TRP channels in thermosensation, *Nature*, 416 (2002) 52-8.
- Neubert, J.K., Karai, L., Jun, J.H., Kim, H.S., Olah, Z. and Iadarola, M.J., Peripherally induced resiniferatoxin analgesia, *Pain*, 104 (2003) 219-28.
- Neubert, J.K., Mannes, A.J., Karai, L.J., Jenkins, A.C., Zawatski, L., Abu-Asab, M. and Iadarola, M.J., Perineural resiniferatoxin selectively inhibits inflammatory hyperalgesia, *Mol Pain*, 4 (2008a) 3.
- Neubert, J.K., Mannes, A.J., Keller, J., Wexel, M., Iadarola, M.J. and Caudle, R.M., Peripheral targeting of the trigeminal ganglion via the infraorbital foramen as a therapeutic strategy, *Brain Res Brain Res Protoc*, 15 (2005a) 119-26.
- Neubert, J.K., Rossi, H.L., Malphurs, W., Vierck, C.J., Jr. and Caudle, R.M., Differentiation between capsaicin-induced allodynia and hyperalgesia using a thermal operant assay, *Behav Brain Res*, 170 (2006) 308-15.
- Neubert, J.K., Rossi, H.L., Mannes, A.J., Jenkins, A.C., Wexel, M. and Iadarola, M.J., Central targeting of trigeminal TRPV1-expressing neurons for control of orofacial pain, In preparation (2008b).
- Neubert, J.K., Widmer, C.G., Malphurs, W., Rossi, H.L., Vierck, C.J., Jr. and Caudle, R.M., Use of a novel thermal operant behavioral assay for characterization of orofacial pain sensitivity, *Pain*, 116 (2005b) 386-95.
- Ng, C.H. and Ong, W.Y., Increased expression of gamma-aminobutyric acid transporters GAT-1 and GAT-3 in the spinal trigeminal nucleus after facial carrageenan injections, *Pain*, 92 (2001) 29-40.
- Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S. and Patapoutian, A., A TRP channel that senses cold stimuli and menthol, *Cell*, 108 (2002) 705-15.
- Simons, C.T., Gogineni, A.G., Iodi Carstens, M. and Carstens, E., Reduced aversion to oral capsaicin following neurotoxic destruction of superficial medullary neurons expressing NK-1 receptors, *Brain Res*, 945 (2002) 139-43.

- Szabo, T., Olah, Z., Iadarola, M.J. and Blumberg, P.M., Epidural resiniferatoxin induced prolonged regional analgesia to pain, *Brain Res*, 840 (1999) 92-8.
- Szallasi, A. and Blumberg, P.M., Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes, *Brain Res*, 524 (1990) 106-11.
- Szallasi, A. and Blumberg, P.M., Vanilloid receptors: new insights enhance potential as a therapeutic target, *Pain*, 68 (1996) 195-208.
- Szallasi, A., Blumberg, P.M., Annicelli, L.L., Krause, J.E. and Cortright, D.N., The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons, *Mol Pharmacol*, 56 (1999) 581-7.
- Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I. and Julius, D., The cloned capsaicin receptor integrates multiple pain-producing stimuli, *Neuron*, 21 (1998) 531-43.
- Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J. and Bevan, S., Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture, *J Neurosci*, 8 (1988) 3208-20.

BIOGRAPHICAL SKETCH

Melanie Wexel received her Bachelor of Science at James Madison University, in Harrisonburg, Virginia in 2000. In 2004, she completed her Doctor of Dental Surgery degree at Virginia Commonwealth University School of Dentistry, in Richmond, Virginia. In 2005, she received a certificate of continuing education after a 1-year fellowship in orthodontics at the University of Florida. She began her orthodontic residency in 2005 at University of Florida College of Dentistry. She is expected to graduate with a certificate of orthodontics and a Master of Science in May 2008. She plans to practice orthodontics in central Virginia.