CHARACTERIZATION OF FACIAL PAIN USING AN OPERANT BEHAVIORAL TESTING PARADIGM AND EVALUATING THE ROLE OF TRANSIENT RECEPTOR POTENTIAL CHANNELS IN FACIAL PAIN

By

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To my biological family and my Florida family, who have kept me going, Mr. Booker, my first biology teacher, and the rats.

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Pain normally serves an adaptive purpose, but injury and prolonged inflammation can lead to changes that produce chronic pain. In order to better understand and treat chronic pain, scientific study must use methods that model and assess pain in a manner relevant to the human condition. Operant assessments of pain accomplish this by providing the animal with a conflict between a desired state and potential pain. In these studies, an operant method was used to establish the ability of rats to obtain a milk reward while stimulating their faces in the presence of a single stimulus or two stimuli. Similarly a person with chronic pain may forgo rewarding activities to avoid pain. This method was then used to assess the effects of transient receptor potential (TRP) channel agonists and of chronic constriction injury (CCI) on successful task completion. The TRP channel melastatin 8 is activated by cold, and another TRP ankyrin 1 may also be activated by noxious cold. Both of these receptors are co-expressed with TRP vanilloid 1, activated by noxious heat. Thus, any of these channels may affect the perception of cold *in vivo*. Without TRP channel manipulation or injury, successful task completion declines sharply with increasing heat, but only slightly with increasing cold. Menthol, a TRPM8 agonist, and icilin, a TRPM8/TRPA1 agonist, led to a decline successful task completion to 10 degrees and

enhanced cold avoidance respectively, suggesting that cold allodynia was induced. Activation and lesion of TRPV1 hindered and enhanced successful task completion with painful cold respectively, supporting a role for TRPV1-expressing afferents in cold nociception. CCI is a common model of chronic neuropathic pain. CCI-treated rats exhibited impaired success with 10, 37 degrees, and rough stimulation, with differing duration and temporal patterns. Although aversive behaviors were observed, success was not significantly affected with 48 degree stimulation in these animals. Changes in TRPV1 expression and intensity of TRPM8 expression were also observed following CCI, supporting current evidence that these channels contribute to thermal allodynia accompanying neuropathic injury. These findings provide a foundation for clinically relevant means of evaluating pain mechanisms and analgesia.

CHAPTER 1
INTRODUCTION

Clinical Significance

Pain normally serves as a protective function by alerting us to the presence of an external, noxious stimulus or by bringing some imbalance in our internal state to our attention (i.e. aches experienced during infection). However, disease and injury can lead to changes in the nervous system that produce and maintain pathological, chronic pain. Chronic pain can be highly distressing and excruciating, with either no external cause, or it can be triggered by normally pleasant sensation (e.g. the gentle touch of a loved one). Treatment to mitigate these effects can be expensive and associated with severe side effects that may be as intolerable as the pain. Over time, some medications may lose their effectiveness. These aspects of chronic pain have motivated researchers to understand how these features develop so that we may improve treatment techniques. In order to better address the needs of the patient, basic research must use models and methods of pain measurement that more accurately reflect the problems we are trying to overcome.

Chronic pain in the orofacial region is experienced by 20 to 25% of the United States, including patients suffering from a broad spectrum of disorders such as trigeminal neuralgia, temoporomandibular joint (TMJ) disorders, and headaches (e.g., migraine), as well as pain associated with trauma to the face and mouth. Despite the clinical prevalence of orofacial pain, basic research has primarily used the rodent hindpaw and sciatic nerve as the standard model for neuropathic pain, with few studies focusing on pain involving the face or trigeminal nerve. It is assumed that the processing of cutaneous sensations is equivalent in the sciatic and trigeminal pathways, but differences in receptor expression and function have been noted (Kayser et al., 2002; Kobayashi et al., 2005) that could have a measurable effect on the development and

treatment of chronic pain states in these areas. Also, conditions such as chronic headache and trigeminal neuralgia are unique to the trigeminal system. To provide the best assessment and treatment of chronic pain within the orofacial region, basic science must include a specific assessment of trigeminal sensory processing.

Additionally, assessment of pain in experimental animals has relied heavily on reflexive withdrawal of the hindlimbs from either a hot or punctate mechanical stimulus. The withdrawal reflex represents the most immediate and basic response to a damaging stimulus, often preceding conscious perception of pain. This may not be the most appropriate method for evaluating cold sensitivity in general, or neuropathic pain specifically. Whereas heat can be immediately damaging with brief exposure, prolonged exposure to cold is required before injury occurs, thus the drive for reflexive withdrawal from localized cold stimulation is likely not as pronounced as for damaging heat. With respect to neuropathic pain, the capacity of this chronic pain to prevent a patient from enjoying and functioning in daily life is what drives the patient to seek treatment. Thus, in the laboratory, assays that measure the capacity of pain to hinder normal function or rewarding task would more accurately reflect the patient experience. Operant assays can accomplish this goal.

Benefits of Incorporating Operant Assessments of Pain into Scientific Research

Behavioral evaluation of pain has relied primarily on withdrawal thresholds (Hargreaves et al., 1988; Imamura et al., 1997), which are spinally-mediated or unlearned behaviors such as isolated grooming (Deseure and Adriaensen, 2002; Deseure and Adriaensen, 2004), which are brainstem-mediated (Mauderli et al., 2000). While such measures have been important for laying the foundations of our understanding of pain, they can be evoked in decerebrate animals (Woolf, 1984) and thus do not directly evaluate higher order cortical processing of pain. It has become increasingly evident that chronic, maladaptive pain states are not simply the result of changes at

the peripheral and spinal level of nociception, but are maintained by changes at the cortical level (de Leeuw et al., 2005; Seifert and Maihofner, 2008). Therefore, the evaluation of pain and analgesia in experimental animals should incorporate methods that directly evaluate cortical processing by requiring that the animal make a decision about its environment.

Operant pain assays rely on an animals' natural avoidance of unpleasant or noxious stimuli. Unlike many reflex-driven tests, the animal is allowed to move freely in the testing box. This eliminates stress associated with restraint (Mauderli et al., 2000), or anticipation of painful stimulus that the animal can see approaching, which is particularly problematic for testing the face. Restraint stress has been shown to enhance nociceptive responses (Gameiro et al., 2005; Gameiro et al., 2006) and increase operant escape from painful heat (King et al., 2003; King et al., 2007). Stress can therefore enhance pain even if that is not the intent of the experimenter.

In addition to fully evaluating all levels of nociceptive processing and reducing potential stress, operant methods also remove some of the bias that can arise in the execution and evaluation of reflexive withdrawal or unlearned behaviors as responses to pain. The influence of experimenter bias in basic scientific research has been commented on (Eisenach and Lindner, 2004) and demonstrated through statistical analysis of studies conducted between laboratories (Crabbe et al., 1999) and of experienced individuals within a single laboratory (Mogil et al., 2006). Even when careful effort is made to blind the experimenter to treatment group, reflex and unlearned responses require that the assessing scientist make a judgment regarding what responses should be considered "nociceptive". Such judgments vary depending on experience and personal interpretation. Operant methods for assessing pain remove the experimenter from the application of the pain stimulus and often involve a permanent record of the behavioral response, which can be re-examined as desired.

Due to these limitations, we have developed an operant assay for evaluating facial pain. This method assesses the animal's ability and willingness to place its face in contact with a stimulus to obtain reward. The more pain the animal experiences, the less successful it should be at performing this task, which has been validated (Neubert et al., 2005). This behavioral assessment provides a means of examining the role of various molecular mediators in thermal processing *in vivo*, as well as changes that occur following neuropathic injury.

Role of Transient Receptor Potential Channels in Thermal Processing

Vanilloid Transient Receptor Potential Channels Feel the Heat

In the last decade, several members of the transient receptor potential (TRP) receptor family have been identified as molecular mediators of thermal stimuli (see Table 1-1), which has considerably enhanced our understanding of peripheral sensory processing. The most thoroughly studied of these, and the first to be characterized was TRP vanilloid 1, a critical molecule for encoding noxious heat (Caterina et al., 1997; Caterina et al., 1999; Caterina et al., 2000). TRPV1 also mediates hyperalgesia following inflammation (Caterina et al., 2000; Neubert et al., 2008; Wexel, 2008), incisional pain (Pogatzki-Zahn et al., 2005), and contributes to neuropathic pain (Walker et al., 2003; Levine and Alessandri-Haber, 2007). Additional members of the vanilloid family, TRPV2 (Caterina et al., 1999), TRPV3 (Xu et al., 2002), and TRPV4 (Guler et al., 2002), are also important for mediating responses to painful heat and warmth respectively.

Discrepancies noted between the *in vitro* activation range of TRPV1 (>42°C) and the impairment observed in TRPV1 knock-out mice (>49°C) (Caterina et al., 2000; Davis et al., 2000) led to further speculation and investigation regarding the encoding of heat sensation and pain. It has been suggested that a small number of TRPV1 negative nociceptors may be needed to produce a withdrawal response (Julius and Basbaum, 2001) and recently demonstrated that TRPV1 provides the primary heat signaling via lamina V, but only part of the input to lamina I

(Eckert et al., 2006). Additional investigation indicates that a subset of heat responsive nociceptors rely on TRPV1/2 independent mechanisms (Woodbury et al., 2004). The encoding of heat therefore involves a number of molecular mediators, including the four vanilloid TRP channels and potentially others. The expression patterns of these molecules, as well as the convergent inputs they provide to different levels of the dorsal horn encode the nuanced experience of warmth and painful heat.

Transient Receptor Potential Channels Make Cold Hurt

The molecular mediators of cold have been a hot topic in pain and sensory processing for the last six years, fueled by the discovery and characterization of two putative cold receptors in the TRP channel super family: TRP melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1). TRPM8 is activated by cooling compounds, such as menthol, and by temperatures at and below 25°C (McKemy et al., 2002; Peier et al., 2002). TRPM8 was never disputed a molecular mediator for innocuous cold perception. However, the channel is expressed in cells with both nociceptive and non-nociceptive characteristics (Xing et al., 2006; Dhaka et al., 2007; Takashima et al., 2007; Dhaka et al., 2008). Initial studies led some to suggest that TRPM8 served primarily in innocuous cold perception and that some other molecule or molecules encoded painful cold stimulation (McKemy, 2005), but subsequent work has cemented a role for TRPM8 in cold pain (Xing et al., 2006; Colburn et al., 2007; Dhaka et al., 2007; Xing et al., 2007; Dhaka et al., 2008).

Another receptor characterized a year later appeared to match the expectations for a molecular transducer of painful cold. TRPA1 (formerly ANKTM1), was shown to be activated by cooling, with a lower threshold in the nociceptive range (<18°C) (Story et al., 2003), in menthol insensitive (TRPM8 negative) neurons (Bandell et al., 2004). TRPA1 is expressed exclusively in cells with nociceptive characteristics that also express TRPV1 (Kobayashi et al.,

2005). It is also activated by a number of pungent compounds that produced burning or pricking sensations, such as icilin, mustard oil, and cinnamon aldehyde (Bandell et al., 2004; Garcia-Anoveros and Nagata, 2007). Others failed to replicate activation of TRPA1 *in vitro* by cold (Jordt et al., 2004; Nagata et al., 2005). This discrepancy has been attributed to the observation that TRPA1 activity is dependent on intracellular calcium (Doerner et al., 2007) and that cooling in heterologous expression systems can increase local calcium and coincidentally activate the TRPA1 (Zurborg et al., 2007). However, direct cooling activation of TRPA1 was demonstrated at physiological resting membrane potential using a calcium free, inside-out patch clamp (Sawada et al., 2007), providing further *in vitro* evidence that TRPA1 is activated directly by painful cold.

Encoding Thermal Sensations is a Group Effort

Studies of knock-out mice conclusively demonstrated that the lack of TRPM8 profoundly impaired irritation induced by acetone, withdrawal from intensely cold stimuli, discrimination between cool stimuli, cold avoidance, and icilin-induced shaking (Colburn et al., 2007; Dhaka et al., 2007), indicating that TRPM8 is required for both cold perception and nociception. In contrast, impairments in cold responses were not observed in TRPA1 knock-out mice by one laboratory (Bautista et al., 2006), and only to a small degree in a modest sample of females knock-out mice by another laboratory (Kwan et al., 2006).

However, the findings in TRPA1 knock-out mice do not necessarily rule out a role for this receptor in encoding certain aspects of painful cold. TRPM8 and other cold activated molecules likely maintain the normal cold perception in TRPA1 knock-out mice. There is evidence to suggest that TRPA1 acts as a mechanoreceptor (Kwan et al., 2006; Cahusac and Noyce, 2007; Kindt et al., 2007). It is possible that TRPA1 mediates the pricking or tingling sensations that accompany dramatic cooling, whether by direct activation from cooling or by coincidence

detection of another channel that mediate activation via increased local calcium concentration. The burning aspects of cold pain are likely mediated by fibers that co-expression of TRPM8 and TRPV1 (Okazawa et al., 2004; Kobayashi et al., 2005; Xing et al., 2006; Dhaka et al., 2007). There are also other cold nociceptors not expressing either TRPA1 or TRPM8 (Babes et al., 2002; Madrid et al., 2006; Munns et al., 2007) that contribute to the encoding of cold pain. Thus, like heat, there are a myriad of molecular players with differing patterns of expression and connectivity that are responsible to for the various nuances of cold perception.

Using Operant Testing to Evaluate the Mechanisms of Normal and Neuropathic Pain

Operant behaviors provide an additional and clinically relevant means of evaluation thermal and mechanical sensitivity, particularly cold sensitivity. This behavioral testing method in combination with pharmacological manipulation of TRP channels has provided us with additional insights regarding the processing of cold stimuli. Finally, we demonstrated that neuropathic pain can be measured using this method, and that it can provide insights regarding the role of peripheral, cognitive, and affective factors in pain-related decision making. The results reported here support recent work related to the mechanisms of cold nociception and sensation. These findings also lay a foundation for future applications of operant pain assessment to study the mechanisms and treatment of pathological pain.

Table 1-1. Summary of activation range, agonists, and antagonists for thermally activated Transient Receptor Potential (TRP) channels.

TRP channel	Activating stimuli	Agonists	Antagonists
TRPV1 (VR1)	42-52°C, acidity	Capscaicin, RTX, ethanol (Levine and Alessandri-Haber, 2007), camphor (Xu et al., 2005), 2-APB (Colton and Zhu, 2007), anandamide (Ross, 2003)	I-RTX (Jhaveri et al., 2005), capsazepine, BCTC (Behrendt et al., 2004), AMG0347 (Steiner et al., 2007)
TRPV2 (VLR1)	>52°C	2-APB (Colton and Zhu, 2007), cannabidiol (Qin et al., 2008)	Gadolinium , Lanthanum (Leffler et al., 2007)
TRPV3	>33°C	2-APB (Colton and Zhu, 2007), camphor (Moqrich et al., 2005), carvacrol, eugenol, thymol (Levine and Alessandri-Haber, 2007)	
TRPV4	27 - 42°C, hypotonicity, acidity, mechanical	4*PDD (Strotmann et al., 2003)	
TRPM8 (CMR1)	<25°C	Menthol, icilin (2μM) (McKemy et al., 2002)	Capsazepine, BCTC (Behrendt et al., 2004)
TRPA1 (ANKTM1)	<18°C (debated), mechanical	Bradykinin, mustard oil, icilin (50 μM), cinnamaldehyde, menthol (<100μM) (Garcia-Anoveros and Nagata, 2007)	Menthol (>100 μM) (Karashima et al., 2007)

BCTC = 4-(3-chloro-pyridin-2-yl)-piperazine-1-carboxylic acid (4-tert-butyl-phenyl)-amide, 2-APB = 2-aminoethoxydiphenyl borate, 4*PDD = 4,*-phorbol 12,13-didecanoate

CHAPTER 2
CHARACTERIZATION OF OPERANT RESPONSES TO THERMAL AND MECHANICAL FACIAL STIMULATION WITHOUT PAIN INDUCTION

Operant assessments of cutaneous sensation and pain have been employed in recent years with respect to hindpaw stimulation. These assessments present thermal stimuli individually (Mauderli et al., 2000; Vierck et al., 2004; Vierck et al., 2005; Vierck et al., 2008), in pairs presented simultaneously (Lee et al., 2005; Vierck et al., 2005; Jabakhanji et al., 2006; Walczak and Beaulieu, 2006; Vierck et al., 2008), or as a thermal gradient (Lee et al., 2005). However, none have reported an operant method for evaluating facial pain, regardless of stimulus type. Also, no operant methods currently exist to evaluate mechanical sensitivity in any part of the body. In this chapter, the operant method for evaluating facial pain is described, including modifications made to evaluate mechanical sensitivity. This method evaluates a rodent's ability to obtain a milk reward while in contact with a stimulus. The rodent may be presented with one stimulus, or may choose between two stimuli to obtain the reward. The stimulus could be any temperature within a range from -4 to 52°C or one of two textured mechanical stimuli. We used the single stimulus task to characterize operant responses of male rats to individual thermal and mechanical stimuli in the absence of pain manipulation. We also used the preference task to assess affective aspects of pain processing and determine what effect previous experience might have on preference. These findings provide the basis for evaluating the effects of TRP channel modulation (Chapter 3) and neuropathic injury (Chapter 4).

Operant Methods for Evaluating Facial Pain

Animals

Male hairless Sprague-Dawley rats (Charles River, Raleigh, NC, 5-8 weeks old) were used for all experiments described in this and the following chapters, unless otherwise specified. Three to five rats were housed in large cages with enrichment (as described in (Rossi and

Neubert, 2008). A standard 12-hour light/dark cycle was maintained and rats were allowed access to food and water ad libitum when not being tested. Weights were recorded every week to monitor general health. 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 (Institute of Laboratory Animal Resources, 1996).

Single Stimulus Task

Facial testing was conducted using a reward-conflict paradigm as described previously (Neubert et al., 2005). Briefly, rats were trained to drink sweetened condensed milk while making facial contact with a single thermode and lick-tube (Object 2-1). During the training period (approximately 2 weeks) baseline intake was recorded and box distance from the sipper tube was gradually increased. Rats were considered ready for stimulus testing once they were able to drink an average of 10 grams of milk with their faces in correct contact with the thermodes, set at 37°C. Following training, rats were either tested at a range of thermal stimuli to establish a stimulus response pattern, or they were tested at a particular stimulus to establish a baseline of behavior before the induction of a pain state, drug administration, or a combination of the two. To maximize thermal stimulus contact, the facial testing region for each animal was depilitated under light isofluorane anesthesia (inhalation, 2.5 %) once a week or as needed. Rats were also fasted overnight for 13-15 hrs to motivate performance, but no more than three times per week. All stimulus testing was conducted within an ambient temperature of 20 to 26°C.

For every 20 or 30-minute testing session, milk intake was measured and raw data recorded using Windaq software and hardware. This raw data was transformed into a numerical form using a custom-written subroutine in Labview (courtesy of Dr. Charles Widmer). The numerical data was used to calculate the number of licks and the number of stimulus contacts,

from which the success ratio is derived. The success ratio is the number of licks (successful attempts) divided by the number of stimulus contacts (total attempts).

Stimulus Preference Task

The thermal preference of the rats was recorded as previously described (Rossi et al., 2006). Rats were trained in the single task condition, as described above, and initially placed in the thermal preference apparatus with both thermodes set at 37°C to allow them to become accustomed to this new task. A second such session was recorded to ensure rats did not demonstrate a side preference. Rats were able to move freely from one side of the compartment to the other and explore both thermodes at will. Unless otherwise specified, the start side was not controlled by the experimenter, but was recorded as part of the offline data analysis. When tested repeatedly with a combination of stimuli, the hot and cold (or soft and rough) sides of the testing chamber were alternated to prevent learned aversion or preference for one side of the box.

Following data acquisition, raw data files for each side of the thermal preference box were examined together to determine the starting side and the number of switches made between sides. The raw data was transformed to a numerical form and licks, stimulus contacts, duration, and the success ratios for each side of the preference apparatus were determined as in the single stimulus task. Additionally, because the rats have a third option of abstaining from task completion to avoid either stimulus, the time spent unstimulated was determined by subtracting the total stimulated time from the total testing time (1200s). Licks, stimulus contacts, and duration could be expressed either as raw data or as a percentage of the total.

Assessment of Mechanical Sensitivity

We adapted our existing thermal testing apparatus by placing a Velcro-covered plastic sleeve over the thermodes to serve as a rough or soft mechanical stimulus (Figure 2-1). The number of licks made in contact with the mechanical stimulus were recorded and calculated in

the same manner as the thermal testing analysis. However, contact with the mechanical stimulus cannot be directly measured because the sleeve blocks the completion of the electrical circuit needed to register facial contact. Therefore, stimulus contacts were determined indirectly by compressing the raw licking data and counting the number of licking bouts (Figure 2-2). Slight differences were occasionally noted between the appearance of the compressed windaq file and the visual output in Labview (Figure 2-2), so for the sake of consistency all bouts were counted from the compressed windaq file. Our ratio for assessing successful task completion was then calculated by dividing licks (successful attempts) by licking bouts (approximate total contacts or attempts). For comparison of these mechanical stimuli with smooth metal, this analysis method was also applied to licking data obtained with 37°C –smooth metal stimulus.

Mechanical preference was also evaluated by covering the thermodes on each side of the preference box with rough and soft stimuli. For each stimulus, outcomes were calculated as described above. Side switching was also determined in off-line analysis, but may underestimate absolute switching because mechanical stimulus contacts without licks could not be recorded. Unstimulated duration was also calculated by subtracting the sum of the licking durations on both stimuli from the total testing time (1200s). In some sessions the start side was controlled by the experimenter. In these instances the rat would be placed in the box on one side with the middle barrier in place. The barrier was removed once the rat made contact with the stimulus and after this point the rat was allowed to explore the preference apparatus freely.

Statistical Analysis

All statistical analyses described herein were performed using SPSS (SPSS, inc. v.14 or 16). One-way analysis of variance (ANOVA) was used to compare the effects of individually presented thermal and mechanical stimuli on operant behavioral outcome measures. Because the individual thermal data was represented by a large sample size consisting of multiple cohorts

tested repeatedly at different times, data was submitted to a box plot analysis, and outliers were removed on an outcome by outcome basis, where an outlier is defined as any datum greater or less than one and a half times the interquartile range. Of the 1024 data points, 2, 9, and 8% were identified as outliers and removed from licks, stimulus contacts and success ratio respectively.

For both thermal and mechanical preference data, paired t-tests were used to determine the difference between the percentage of licks on each stimulus and repeated measures ANOVA was used to determine significant differences in the percentage of time spent on either stimulus or unstimulated. One-way ANOVAs were used to compare licks and time spent on either stimulus or off the thermode across stimulus pairs. For thermal preference data only, success ratios for the single stimulus condition versus pairing with another stimulus were also evaluated using one way ANOVA. To evaluate the effect of previous experience on time with either stimulus or unstimulated, repeated measures ANOVA was used. Post-hoc comparisons were made using Tukey's test for all one way ANOVAs, and the least squared differences test (LSD) for repeated measures ANOVAs. Statistical significant was set to $p<0.05$ for all analyses.

Operant Behavioral Profile under Normal Conditions

Operant Responses to Thermal Stimuli

Individual stimuli

Rats were tested with a range of thermal stimuli from noxious cold (-4°C) to noxious heat (52°C). There was a significant effect of temperature on licks ($F_{9, 1006} = 50.666$), stimulus contacts ($F_{9, 933} = 73.864$), and success ratios ($F_{9, 955} = 104.301$, $p<0.001$ for all outcomes; Figure 2-3). Cold and cool stimuli produce a modest reduction in licks and success ratios, relative to neutral (37°C) or warm (42°C) stimulation. Cold stimuli did not significantly increase stimulus contacts. In contrast, hot stimuli produce a sharp decline in licks and success ratio as temperature increases, which is accompanied by an increase in stimulus contacts at the most

noxious hot temperatures (48 and 52°C). These data indicate that successful task completion is hindered more by noxious heat than noxious cold. With noxious heat, rats must make frequent contacts with the stimulus in order to obtain the milk reward, while this strategy is not necessary with cold stimulation. However, the fact that there is a reduction in successful task completion in the presence of cold stimulation indicates that cold is more aversive than neutral (37°C) or warm (42°C) stimuli.

Thermal preference

Although individuals may exhibit a side preference when exposed to a pair of neutral temperatures, this preference is not consistent across testing sessions and when all rats outcomes are averaged for each stimulus no side bias is observed, as previously reported (Rossi et al 2006). With few exceptions, individual rats exhibit a temperature preference each time they are exposed to hot and cold pair of stimuli (Table 2-1). We tested a range of cold stimuli paired either with 45 or 48°C. Most individuals preferred 45°C regardless of the cold stimulus paired with it. In contrast, when non-noxious cold stimuli (10, 18, and 24°C) are paired with 48°C, most rats prefer the cold stimuli. When 48°C is paired with -4°C, noxious cold, most rats prefer 48°C.

The rats' individual preferences are predictive of the mean group licking preference and time distribution for each stimulus combination. For all pairs including 45°C as the hot stimulus, 45°C is strongly preferred, as indicated by percentage of testing time and percentage of total licks (Figure 2-4 A, B). There was no significant difference in the percentage of time or licks spent on the 45°C stimulus or the cold stimulus when compared across stimulus pairs. However, rats did spend slightly more time at 24°C than at 18 or 10°C and as a consequence unstimulated time is significantly smaller when 24°C is the cold option.

As indicated by individual preferences, non-noxious cold stimuli (10, 18, 24°C) are preferred when paired with noxious 48°C. In contrast, when 48°C is paired with a noxious cold

stimulus (-4°C), 48°C is preferred (Figure 2-4 C, D). For all pairs, there was no significant difference in the percentage of time spent on the hot side (10-15%), regardless of whether or not the cold stimulus was noxious (Figure 2-4 C). In contrast, percentage of time and licks on the cold stimulus, are significantly greater for non-noxious temperatures than -4°C (Figure 2-4 C, D). Conversely, the percentage of licks on 48°C is significantly lower when 48°C is paired with non-noxious cold stimuli (Figure 2-4 D). As was the case for 45°C, the percentage of unstimulated time is modulated by the intensity of the cold stimulus, with the lowest unstimlated time spent with the least intense stimulus (24°C) and the greatest unstimulated time spent with the coldest stimulus (-4°C) (Figure 2-4 D).

Taken together, these data indicate that the percentage of time on the hot stimulus is consistent across stimulus pairs. In the case of 48°C, this is even true when the preference switches from cold to hot (10, 18, 24°C versus -4°C). These data also indicate that the percentage of time spent on any stimulus also decreases with increasing intensity. As a consequence, the percentage of time spent unstimulated is the greatest when both stimuli are very intense (-4 and 48°C) and the lowest when both stimuli are the least intense (24 and 45°C).

Modulation of successful task completion for each stimulus in the thermal preference task

Success at 45°C was not significantly changed by being paired with any of the cold stimuli, although it was slightly increased (Figure 2-5A). In contrast, success at all of the cold stimuli paired with 45°C was decreased relative to when the stimuli are presented alone (Figure 2-5B). Interestingly, the success ratios for 10, 18, and 24°C are significantly different from each other in the presence of 45°C, but not when these cold stimuli are presented individually, which we have demonstrated previously regarding individual cold stimuli (Rossi et al., 2006).

In contrast, success at 48°C is modulated by cold in an intensity dependent fashion. Success at 48°C is significantly decreased when paired with -4 or 10°C, as compared when 48°C

is presented alone, and no different when paired with 18 or 24°C (Figure 2-5C). Conversely, success at -4 and 10°C are lower with 48°C than when presented individually (Figure 2-5D). Success at 18 and 24°C are greater with 48°C than alone, but only significantly so for 18°C (Figure 2-5D). As with 45°C, an effect of cold stimulus intensity is more apparent when paired with 48°C than when presented alone.

Taken together, these data indicate that pairing stimuli can modulate successful task completion at either stimulus as compared to when that stimulus is presented alone. While the effects observed at 45°C can be explained by the strong preference for 45°C, the effects at 48°C suggest that there is an interaction between the two stimuli independent of preference that can modulate successful task completion at each stimulus. It is possible that stimuli 10°C and below cross sensitize with noxious heat, resulting in less success than when stimuli are presented individually.

Using the thermal preference task to condition aversion or preference

In addition to characterizing thermal preference, time distribution, and the effect of stimulus interactions on successful task completion, we also sought to determine if prior experience in the thermal testing apparatus could affect thermode preference the following day. Rats (n =10) were tested first at 37 and 37°C, exhibiting no side preference and spending about 60% of the testing time unstimulated (Figure 2-6A). They were then tested at 42 and 10°C, exhibiting a strong preference for the left, 42°C thermode and spending about 45% of testing time unstimulated. This preference was not the result of a bias in the starting side (Table 2-2). Twenty four hours later, the rats were tested at 10 and 10°C. Rather than exhibiting no group preference, as would be expected with two equal stimuli, rats spent significantly more time on the left thermode than the right (Figure 2-6A). The time spent on the right thermode with 10 and 10°C was also significantly lower than the time spent on the right thermode at 37 and 37°C,

while there were no significant differences between time on the left thermode, or unstimulated time for either same stimulus pair. The rats also exhibited a bias for starting on the left thermode (Table 2-2). Taken together, these findings suggest that the left-thermode preference (right thermode avoidance) of the previous day was sufficient to produce a conditioned preference when one should not have occurred.

We were also able to use previous experience to obscure subsequent preference. Rats were first tested at 52 and 18°C, which induced a preference for 18°C. Again, a bias in start side was not observed (Table 2-3). We generally observe that simply switching the hot and cold stimuli the following day does not have a significant effect on thermal preference. However, changing one of the stimuli by a few degrees could have a significant impact on behavior. Therefore, the next day the hot and cold stimuli were switched, the cold stimulus was maintained at 18°C, while the hot stimulus was decreased to 48°C. Rather than exhibiting a preference for 18°C as indicated above, the preference was abolished. After being exposed to noxious heat on the left thermode, fewer rats started on the left side. The next day the hot and cold stimuli were switched and 18°C was paired with 48°C. Rather than exhibiting a preference for 18°C as indicated above, the preference was abolished. Rats also spent significantly less time on the left thermode when it was set to 18°C as compared both to the 18°C right side thermode of the previous day, and the 18°C left side thermode two days later (Figure 2-6B). Unstimulated time was also significantly greater when preference for 18°C was abolished than when it was present. Fewer rats also started on the left side (Table 2-3). As reported above, there was no significant difference in time spent on the 48°C stimulus when preference was abolished versus present. There was also no significant difference in time spent on 18°C when it was paired with 52°C as compared to when it was paired with 48°C and preference was intact. Taken together, these data

indicate that previous experience can condition avoidance of one thermode that leads to avoidance of that side and thus an apparent lack of preference.

Operant Responses to Mechanical Stimuli

Individual stimuli

Rats were tested with soft and rough mechanical stimulation, as well as a neutral thermal stimulation (37ºC) to compare the responses to the mechanical stimuli with that of a smooth metal thermode. There were no effects of mechanical stimulation on licks ($F_{2,97} = 2.109$, $p = 0.127$) or bouts (an indirect measure of stimulus contacts; $F_{2,97} = 2.453$, $p = 0.091$; Figure 2-7 A,B). However, there was a significant effect of mechanical stimulation on licks per bout (an indicator of successful task completion; $F_{2,97} = 8.275$ $p<0.001$; Fig.2-7 C). The greatest success occurred with the soft stimulus and no significant difference in success was observed between smooth metal and the rough stimulus (Figure 2-7 C). Taken together, these data indicate that rats find the soft mechanical stimulus the least aversive or most pleasant, even when compared to smooth metal.

Mechanical preference

Preference for mechanical stimulation in naïve rats was dependent on the starting stimulus. The majority of rats starting voluntarily on the soft stimulus exhibited a strong preference for this stimulus. In contrast, when rats began with the rough stimulus, no particular preference was favored by the group (Table 2-4). When a soft preference was exhibited, rats that began on the rough stimulus spent more licks and time on the rough stimulus as compared to those that started on the soft stimulus, while licks and time on the soft stimulus were not affected by start side (Figure 2-8). As a consequence, total licks were slightly higher and unstimulated duration was lower when rats started with the rough stimulus than with the soft stimulus (Figure 2-8). These findings indicate that starting side can influence mechanical preference. When soft

is the first stimulus encountered there is little drive to sample the rough stimulus. When the rough stimulus is the first encountered, it may be mildly aversive, motivating exploration of the other stimulus. However, it is not so aversive that the soft preference is consistently maintained across multiple testing sessions. The decreased unstimulated duration and the increase in licks observed when rough is the starting stimulus may suggest that the rats sample both sides more frequently to arrive at the soft preference. In support of this idea, rats starting with soft stimulation had an average of one successful switch, while when rough stimulation is first they average three successful switches.

Discussion of Normal Operant Responses

In this chapter, we demonstrated that operant task completion is strongly inhibited with increasing heat intensity, but only somewhat hindered by increasing cold intensity. However, when thermal stimuli of equivalent intensity are paired, the aversiveness of cold stimuli is apparent. Noxious cold and hot stimuli are also able mutually hinder successful task completion, suggesting that these stimuli activate a common nociceptor population and pain pathway. A novel, operant methodwas also used to assess mechanical sensitivity. Like operant responses to cold stimuli, successful task completion is not strongly hindered by rough stimulation in normal rats. Unlike the strong preferences exhibited for thermal stimulus pairs, mechanical preference testing revealed only a modest preference for soft stimulation, which depended on the starting stimulus.

Single Stimulus Response

We have previously shown that increasingly intense hot stimuli produce a steep decline in successful task completion when stimuli are presented individually (Neubert et al., 2005). In contrast, increasingly intense cold stimuli only produce a slight decline in successful task completion, where significance was only observed at -4°C (Rossi et al., 2006). The thermal

stimulus response shown here reflects these findings and provides a summary of normal responses taken from multiple cohorts of male hairless Sprague Dawley rats spanning a three year period.

These findings are similar to another operant assay that evaluates pain based on how long rats spend on an illuminated (i.e. normally aversive) thermoneutral platform in order to escape thermal stimulation of the paws (Mauderli et al., 2000; Vierck et al., 2004). These authors also observed that escape duration increases steeply with increasing heat, but the slope of responses with increasing cold is nearly flat (Mauderli et al., 2000; Vierck et al., 2004). These findings are in contrast with a reflexive withdrawal study, which reported steep decline in withdrawal latency from 5 to -5°C stimulation delivered by a peltier-cooled floor when a 100s cut off was applied to define nociception (Allchorne et al., 2005). In contrast to these findings a more recent study, which applied a safety cut off of 180s, found that naïve rats responded with latencies >180s for all cold stimuli tested (20-0°C) (Tanimoto-Mori et al., 2008). The results of the latter study are in agreement with operant findings described here and by Vierck, Mauderli, and colleagues (Mauderli et al., 2000; Vierck et al., 2004).

Discrepancies between the two withdrawal studies could be related to the different cut off times. Additionally, they could be related to differences in testing order, which was not specified by either author, although they did indicate that different stimuli were tested on different days. We have previously demonstrated that the testing of hot stimuli in the operant facial assay is not order dependent (Neubert et al., 2005), however initial experiments indicated that success with painful cold (2°C) was artificially low if this was the first stimulus the rats were exposed to after training. Thus it is possible that responses to cold stimulation could be more susceptible to order effects than heat responses.

With respect to the translational relevance of cold pain assessment in experimental animals, it may be especially important to include paradigms that assess responses to multiple cold exposures rather than a single stimulating event. Operant paradigms follow the former stimulation pattern and reflex assays the latter. Descriptions of painful sensations produced by cold stimulation are more variable than those describing painful heat, which is likely related to differences in the populations of cold receptors being activated. Human subjects report that repeated cold stimulation of glabrous skin produces a deep radiating ache that increases in proportion to stimulus intensity (Mauderli et al., 2003), whereas contact within the noxious range can produce a cold "burning" sensation (Morin and Bushnell, 1998) likely the consequence of deep c fibers that respond to both noxious cold and heat (Simone and Kajander, 1996). The "deep radiating ache" could be a composite of activity of non-nociceptive cold receptors in superficial skin and deep nociceptors near vessels (Mauderli et al., 2003). Behaviorally distinguishing different populations of cold nociceptors, particularly the a delta population, as can be done with laser stimulation for heat related responses (Tzabazis et al., 2005; Tran et al., 2008) could provide an important dimension to understanding the development of cold allodynia characteristic of neuropathic pain.

Thermal Preference and Unstimulated Time

Our findings with respect to single stimuli imply that while cold stimuli may not be as immediately painful as heat, they may still be aversive. To more directly address the influence of affect on thermal processing, thermal preference between disparate hot and cold stimuli was assessed. The thermal preference findings indicate that where intensity is equivalent, warm or hot stimuli are preferred. By pairing cold stimuli with 45°C, a hot stimulus of equivalent intensity, the aversive nature of these stimuli are revealed by the strong heat preference. This has also been shown in a hindpaw assay, where mice were able to move freely between a neutral

(31°C) floor and a cooled floor (Walczak and Beaulieu, 2006). The rats' preference for heat when both stimuli are equal in intensity has also been demonstrated in humans. Cold pressor pain is rated more unpleasant than contact heat, although both have the potential to be painful (Rainville et al., 1992). In a recent study a 41°C stimulus was rated more pleasant than a 12°C stimulus, although neither stimulus was indicated as painful and rated with similar intensity by the subjects (Rolls et al., 2008).

In contrast to most two-choice preference assays that stimulate the paws, this facial preference assay is actually a three choice task that provides the option of forgoing stimulation entirely to avoid contact with painful or aversive stimuli. This measurement is analogous to behavior produced by a single-stimulus shuttle box assay, where increasingly noxious stimulation to the hindpaws results in increasing time spent on a thermoneutral escape platform (Vierck et al., 2004). In general, rats spent 40% or more of testing time unstimulated, but we observed that the percentage of unstimulated time could be modulated by the aversiveness of the stimuli presented. Thus, unstimulated time and thermal preference likely reflect the affective and cognitive components of pain-related decision making in experimental animals. Evaluation of unstimulated time in addition to thermal preference is important because a treatment could potentially have no effect on thermal preference, but could increase or decrease unstimulated time via equivalent pro- or anti-nociceptive action on both stimuli respectively.

Interaction Between Stimuli

In a multi-stimulus assay, it is important to consider the potential for each stimulus to recruit cell populations that can enhance or block transduction of the other stimulus, presumably enhancing or blocking pain. In this assay we can assess how successful rats are at obtaining milk reward in contact with either stimulus and compare this with success for each stimulus when it is presented alone. We observed that success ratios could be modulated by pairing hot and cold

stimuli. When non-noxious cold and 45°C were paired, an insignificant increase in success at 45°C was observed, as well as robust decrease in success at cold. These changes in success are likely due primarily to the strong 45°C preference exhibited for these stimulus pairs, although the slight elevation of success at 45°C when paired suggests that the cold stimuli might weakly block heat pain at this intensity.

In contrast, the effect of pairing on success at noxious heat (48°C) depended on the intensity of the cold stimulus, but not necessarily thermal preference. In the presence of low intensity cold (18 or 24°C), successful task completion was not changed for 48°C, but was increased for 18 and 24°C, following a pattern predicted by thermal preference for the cold stimuli. However, in the presence of high intensity cold (10 and -4°C), success at 48°C was reduced, despite the fact that 48°C is preferred when paired with -4°C. The opposite was also true; success was reduced for 10 and -4°C in the presence of 48°C. These findings suggest that intense cold activates substrates that can facilitate heat pain, and vice verse.

This cold-induced facilitation of heat pain could be due to the activation of peripheral nociceptors that respond to both cold and heat, or the convergent, facillatory input of distinct cold and heat-responsive nociceptors onto second order neurons in the dorsal horn of the trigeminal nucleus caudalis. The latter possibility is more likely, as TRPM8 and TRPV1, molecular mediators of cold and heat respectively, are minimally co-expressed in naïve trigeminal ganglia (Kobayashi et al., 2005). TRPA1 has also been proposed to respond to nociceptive cold stimulation, but this is debated. TRPA1 and TRPV1 are highly co-expressed and agonists of this channel have been shown to weakly enhance cold pain (Albin et al., 2008). Although TRPM8 and TRPV1 co-expression is reported to be very low in naïve trigeminal ganglia (Kobayashi et al., 2005), this does not necessarily mean that such a population could not

have a significant influence on thermal processing in the context of alternating or mixed hot and cold stimulation. It is also possible that the cold and hot nociceptors have convergent inputs within the trigeminal spinal nucleus. Lingual application of the TRPM8 agonist menthol has been shown to cross-sensitize subjects to the irritation produced capsaicin, a TRPV1 agonist (Cliff and Green, 1996). Additionally, local field potentials in the trigeminal nucleus caudalis respond to mixed thermal stimuli and agonists of TRPM8, TRPA1, and TRPV1 (Zanotto et al., 2007; Zanotto et al., 2008). Assessing changes in success in our thermal preference task could provide a means of studying the interaction between cold and hot nociceptors *in vivo*.

Conditioned Response in the Thermal Preference Assay

Thus far, we have established that this thermal preference assay can assess intensity related properties of pain by measurement of stimulus duration and interactions between cold and hot nociceptors by examining success changes. This assay also can also assess affect related properties of pain by the thermal preference and time spent unstimulated. Another common paradigm used to assess pain affect is conditioned place aversion (CPA), where an inflammatory pain state becomes associated with one compartment in a two or three chambered box and avoided by the animal in future trials (Vaccarino et al., 1992; Sufka, 1994). We found that we could use the stimulus pairs to condition a preference where one should not exist, or abolish a preference that should exist, in a manner similar to conditioned place aversion. However, while CPA generally necessitates separate sensory testing using reflex withdrawal to evaluate intensity-related properties of pain (Colpaert et al., 2006; van der Kam et al., 2008), the facial thermal preference assay has the potential to assess both intensity and affect aspects of pain simultaneously.

Operant Assessment of Mechanical Sensitivity

The first operant assessment of sensitivity to tactile stimuli is described here. While no operant assessment has been devised to assess mechanical sensitivity in the hindpaw, a recent study has assessed withdrawal latencies from a textured cork floor, which is comparable to the task defined here (Tanimoto-Mori et al., 2008). Limitations of this assessment include the inability to directly measures contacts with the mechanical stimulus and the lack of variety in the mechanical stimuli we are able to provide currently. The approximation of stimulus contact by counting bouts could introduce experimenter bias and, as mentioned, it cannot assess unrewarded contacts unless some other method is included. Photobeams between the stimulus bars could be used to more directly measure stimulus contacts and duration. Additionally, the current set of stimuli likely only produces innocuous, mildly aversive sensations, which is supported by our preference finding and the influence of starting stimulus on preference. This assay could benefit from inclusion of additional stimuli, with a consistent gradation from innocuous to painful sensation. We are exploring this possibility using varying grits of sandpaper to broaden the assessment potential of this assay.

Despite these issues, a clear difference in successful task completion was observed for the three individual stimuli evaluated. Surprisingly, operant responses to smooth metal and rough velcro did not significantly differ, while clearly success was greatest for the soft stimulus. This indicates that the soft stimulus is more pleasant than either rough velcro or smooth metal, even when the metal is maintained at an innocuous temperature (see single thermal stimulus response). Mechanical preference testing also supports the idea that soft is more pleasant than rough, but the fact that preference could be influenced by starting stimulus also indicates that rough is only mildly aversive.

Conclusion

In this chapter, normal operant responses to thermal stimuli reveal differences between cold and hot stimulation, which are further emphasized in the thermal preference task. The thermal preference assay can assess both intensity and affective aspects of facial pain simultaneously. Additionally we describe a means of assessing mechanical allodynia in an operant manner. These methods of assessment provide a clinically relevant means for examining mechanisms of pain because they can assess both the direct response to the stimulus and the avoidance of the stimulus. This is relevant to the patient experience because an individual may feel the need to forgo rewarding experiences to avoid pain or pain may begin to outweigh the reward. Thus, this chapter lays the foundation for evaluating the role of TRP channels in pain processing.

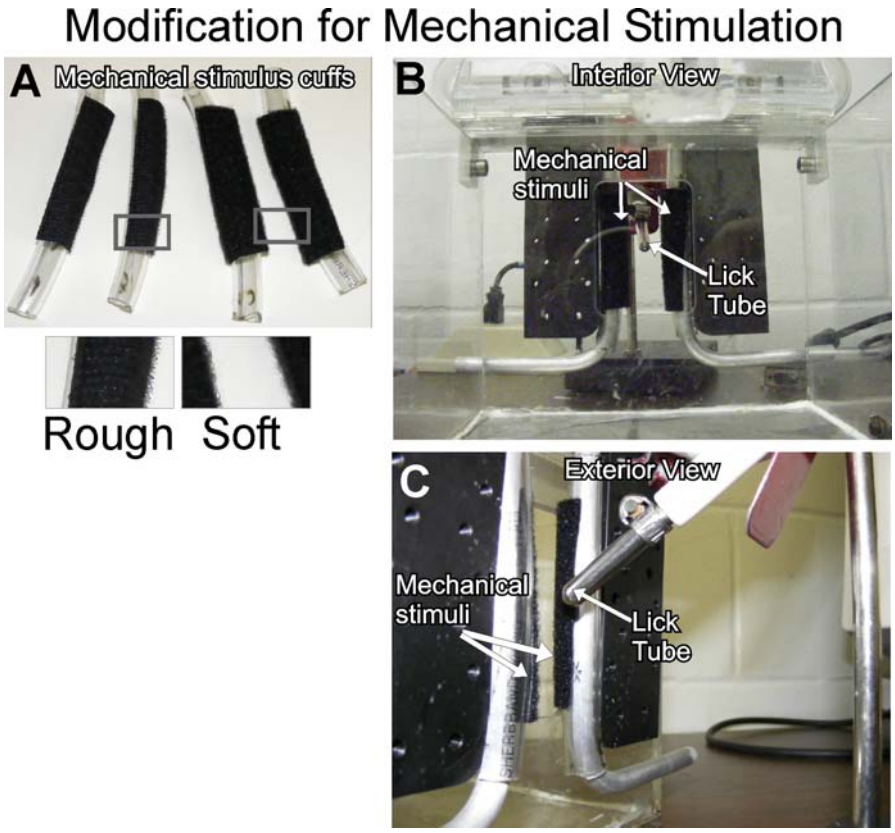


Figure 2-1. Modification made to the operant testing apparatus to assess mechanical sensitivity. A) Mechanical stimulus cuffs were made from plastic tubing (Fisher, wall thickness 1/16 mm) 11.4cm long, cut open on one side, with a 6.4cm length of rough or soft velcro (see magnified view for texture) attached to the region accessible to the rat from the inside of the box. B) View from the inside of the box, with mechanical stimulus cuffs on the thermode. The animal must brush its face against this stimulus in order to access the lick tube just outside the opening. Additionally, the movements made by the animal to lick and adjust head position provide brushing or scratching contacts while the animal is in contact with the soft or rough stimulus respectively. C) Side exterior view of the reward access.

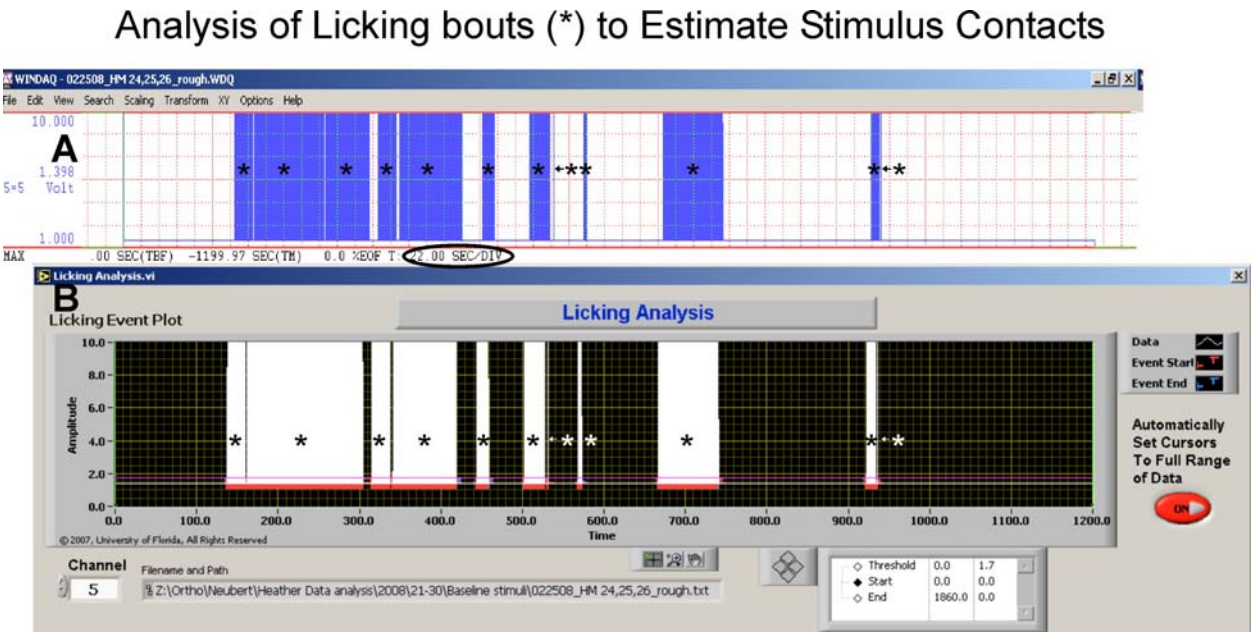
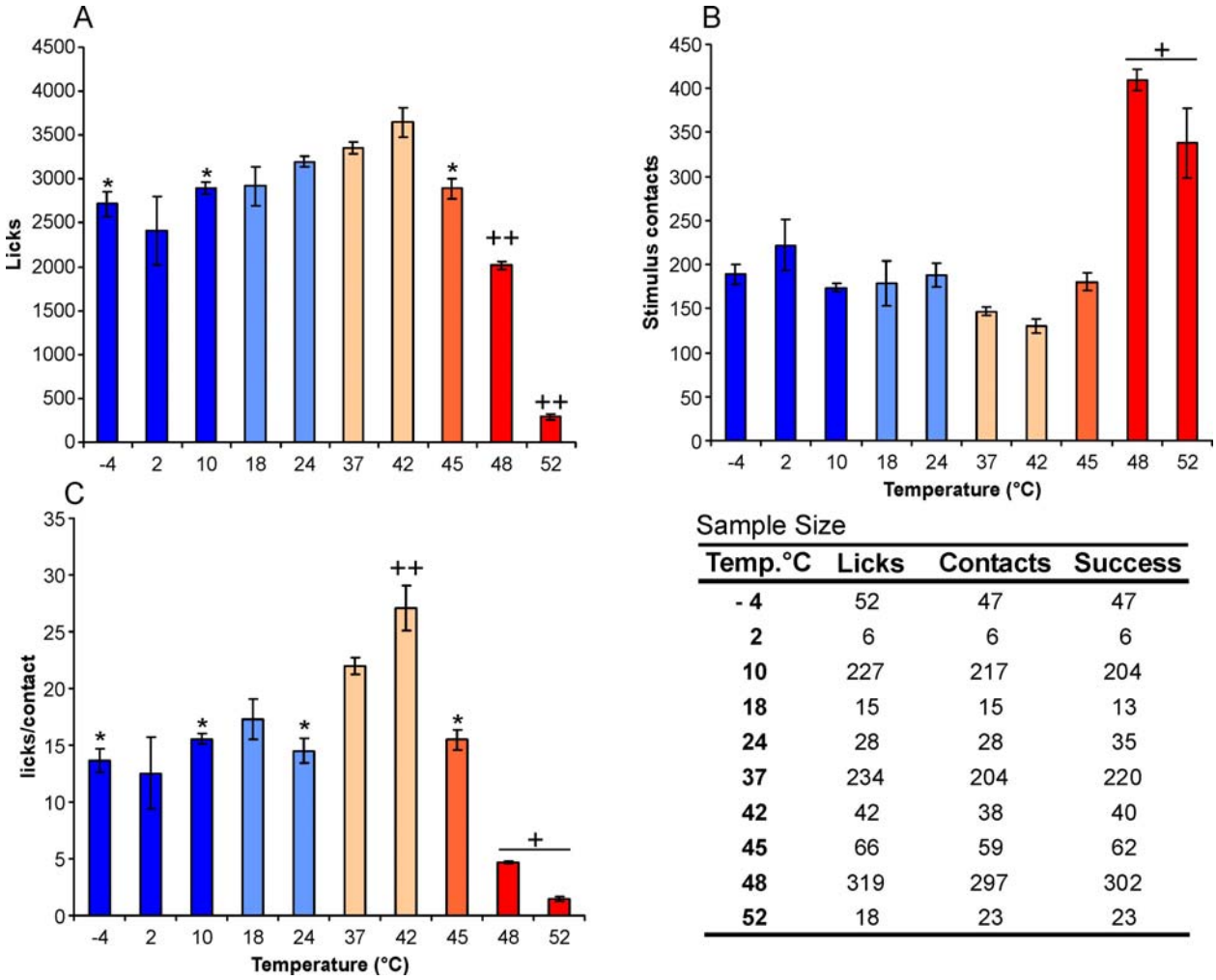


Figure 2-2. Example of licking bout determination based on compressed outputs of raw data in Windaq or Labview. A) Licking bouts for Channel 5 are indicated by astricies, and the compression rate is circled (22 seconds/division; where a division is the width of one red box). There are twelve bouts of licking visible in this output. B) The same output viewed in Labview, which indicates eleven bouts rather than twelve. Please note, the original images were captured and copied into Corel draw, which has reduced the image quality. This has made it difficult to view two of the brief bouts that were visible originally by viewing the raw data directly.



Sample Size

Temp.°C	Licks	Contacts	Success
- 4	52	47	47
2	6	6	6
10	227	217	204
18	15	15	13
24	28	28	35
37	234	204	220
42	42	38	40
45	66	59	62
48	319	297	302
52	18	23	23

Figure 2-3. Effect of single thermal stimuli on operant task completion. A) Licks, B) stimulus contacts, C) and licks/contact (success ratio) were determined when rats were tested with thermal stimuli ranging from very cold (-4°C) to very hot (52°C). Dark blue = cold, light blue = cool, tan = warm, orange = moderate heat, red = noxious heat. Table indicates the number of rats for each stimulus and outcome measure. * indicates a significant difference from 37 and 42°C, + indicates significant difference from all stimuli cooler than the temperature indicated, and ++ indicates significant difference from all other stimuli. Significance is set at $p < 0.05$, and are determined by one-way ANOVA and post-hoc Tukey's test. All data are mean ± SEM.

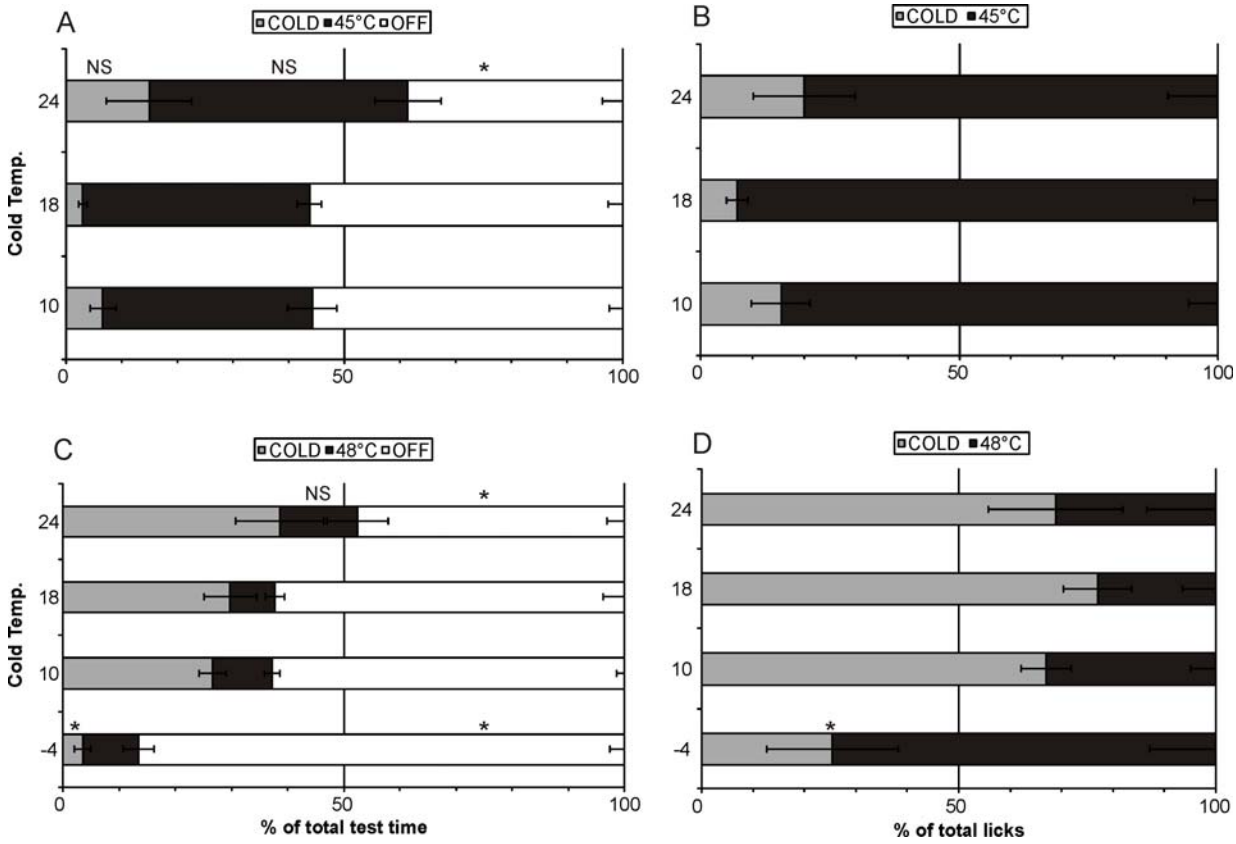


Figure 2-4. Distribution of time spent on the cold, hot, or off the thermode and percentage of licks spent at either thermode when cold stimuli were paired with 45 or 48°C. A) More time is spent on the 45°C stimulus when paired with a 10, 18, or 24°C cold stimulus. Significantly less time is spent unstimulated when the cold stimulus is 24°C. B) The majority of licks also occur in contact with the 45°C stimulus. C) More time is spent on the non-noxious cold stimulus (10, 18, 24°C) when paired with 48°C. In contrast, more time is spent at 48°C when the cold stimulus is noxious (-4°C). The lowest percentage of time of the thermodes occurs when the cold stimulus is 24°C and the most when it is -4°C. D) More licks occur at innocuous cold than at 48°C, and the opposite is true when -4 and 48°C are paired. * indicates $p<0.05$ across different pairs, determined by ANOVA with Tukey's test post-hoc. All data are mean ± SEM.

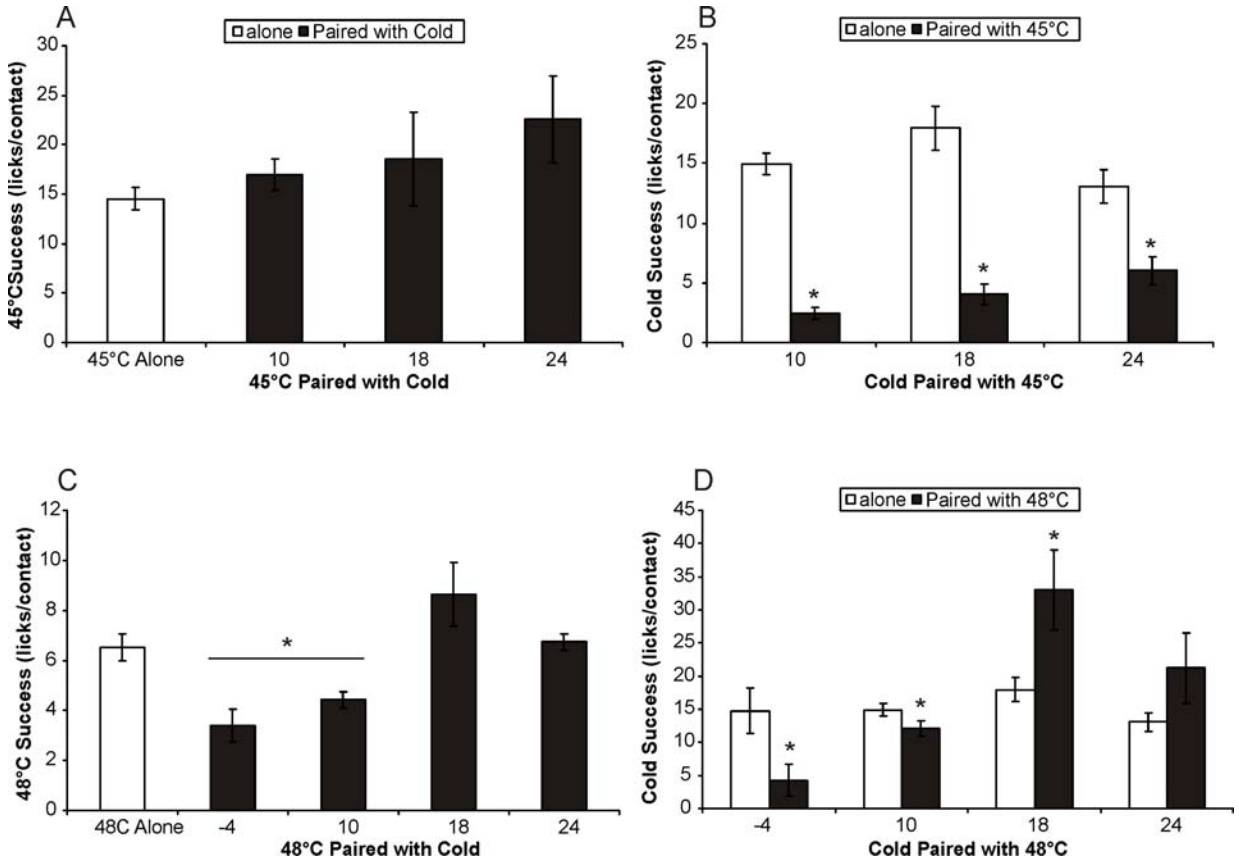


Figure 2-5 Successful task completion when stimuli are paired or presented alone**.** A) Success at 45°C is not significantly increased by pairing with non-noxious cold stimuli as compared to when it is presented alone (n = 29). B) Success at the cold stimuli (10, 18, 24°C) is significantly decreased when paired with 45°C. (10°C n = 41, 18°C n = 12, 24°C n = 14). C) Success at 48°C is significantly reduced when paired with -4 and 10°C, but not significantly different when paired with 18 or 24°C as compared to when it is presented alone (n = 28). D) Success is significantly decreased at -4 and 10°C when paired with 48°C, significantly increased at 18°C, and not significantly increased at 24°C. * indiciates $p<0.05$ for success alone versus paired, determined by ANOVA and Tukey's test post-hoc (except -4°C, which was determined by paired t-test). All data are mean ± SEM.

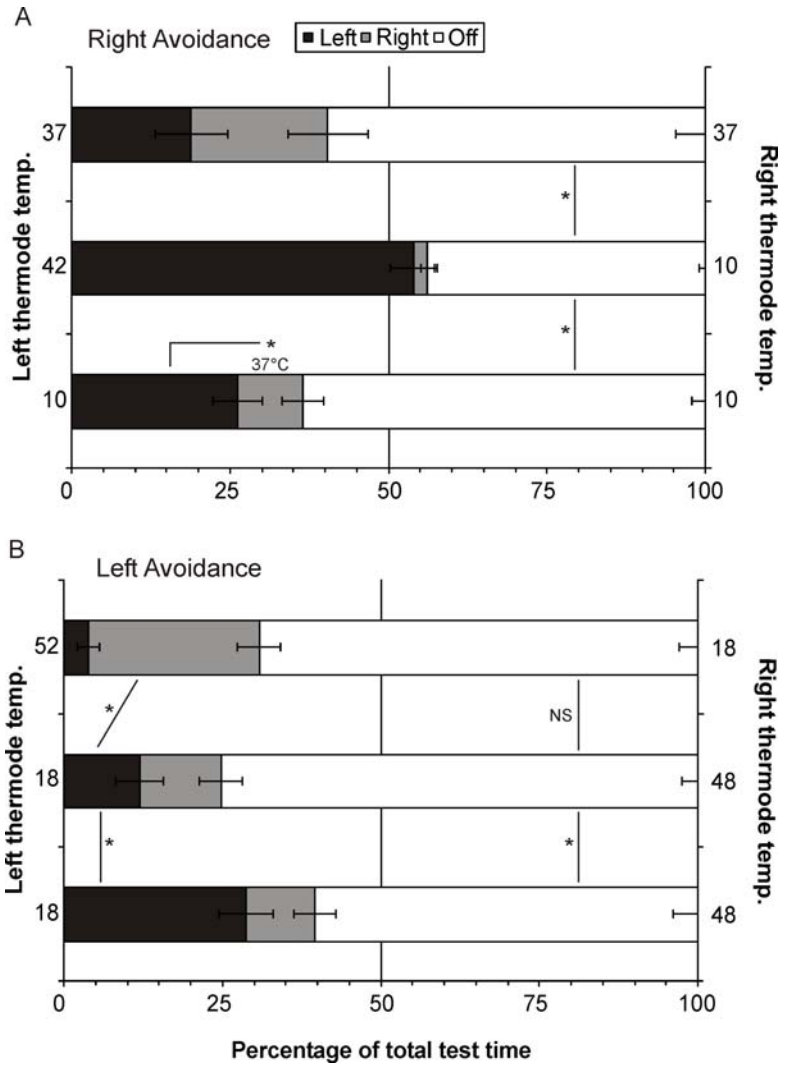


Figure 2-6. Preference can be conditioned or abolished by stimulus exposure 24 hours prior. A) Two equivalent stimuli normally do not produce a preference, as indicated by 37 and 37°C shown on the top. However, when preceded by a preference inducing combination (42 and 10°C), more time is spent on the left thermode (previously preferred) than the right (previously avoided). The time spent on the right 10°C thermode is also significantly different from the right thermode set at 37°C. B) Previous experience can also hinder the presentation of a stimulus driven preference. In this case, a strong preference for the right thermode (and avoidance of the left thermode) was induced with a combination of 52 and 18°C. The following day, rats were tested at a combination of 18 and 48°C and did not show a preference. The time spent on the left side 18°C thermode, which was previously avoided, was significantly lower than the time spent at 18°C the previous day or two days later when the 18 and 48°C stimulus combination was repeated. * indicates $p < 0.05$, with repeated measures ANOVA and post-hoc LSD.

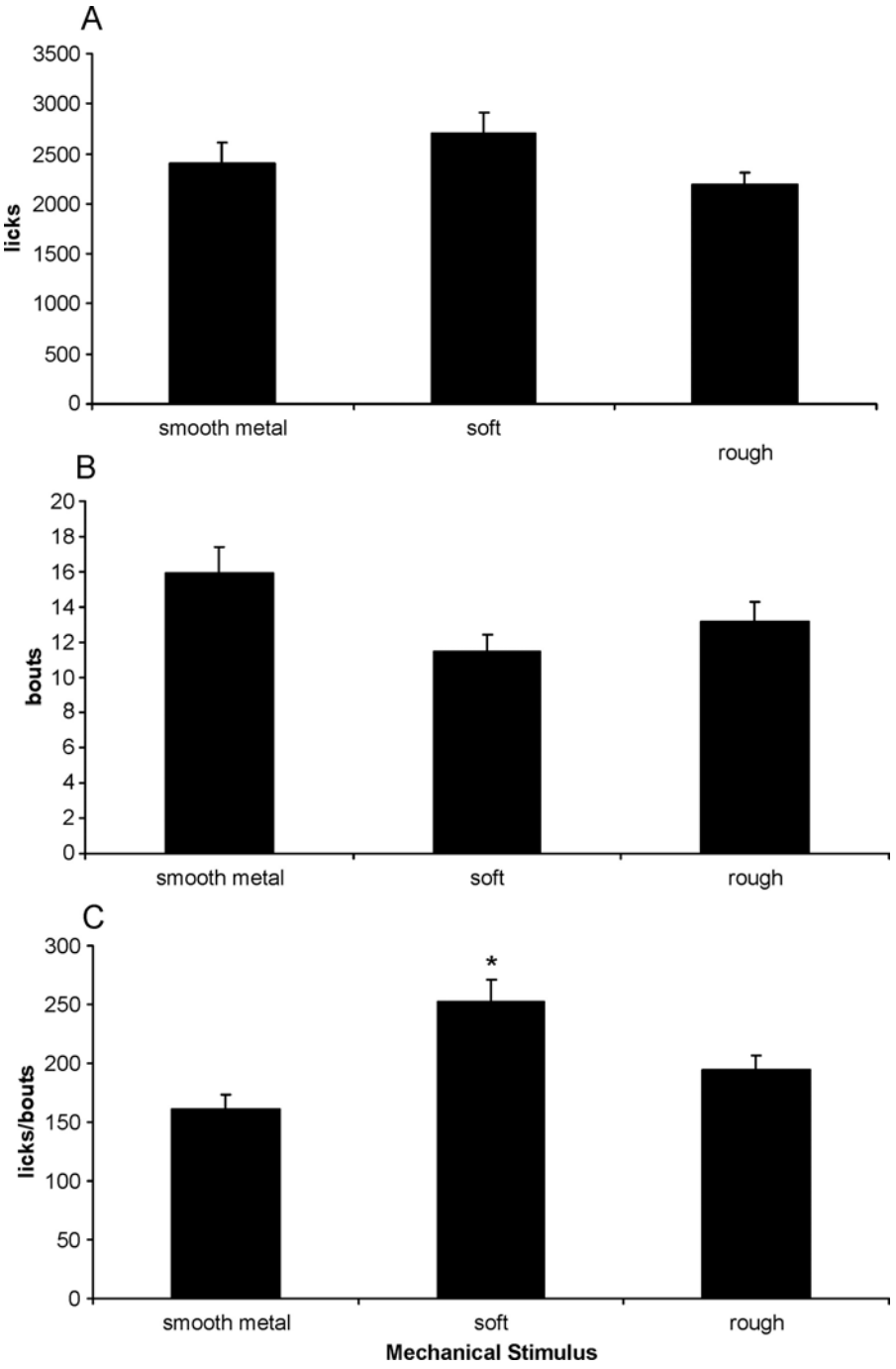


Figure 2-7. Effect of smooth, soft, or rough mechanical stimulation on operant task completion. A) Licks, B) bouts of licking (an indirect measure of stimulus contacts), C) and licks per bout (a success measure) were determined when rats were tested with a smooth metal stimulus (37C, n = 30), a soft stimulus (n = 20), and a rough stimulus (n = 48). There were no significant effects of stimulus type on licks or bouts, but licks/bout were significantly greater with soft stimulation than either smooth or rough. * indicates $p < 0.05$, as determined by one-way ANOVA and post-hoc Tukey's test. All data are mean ± SEM.

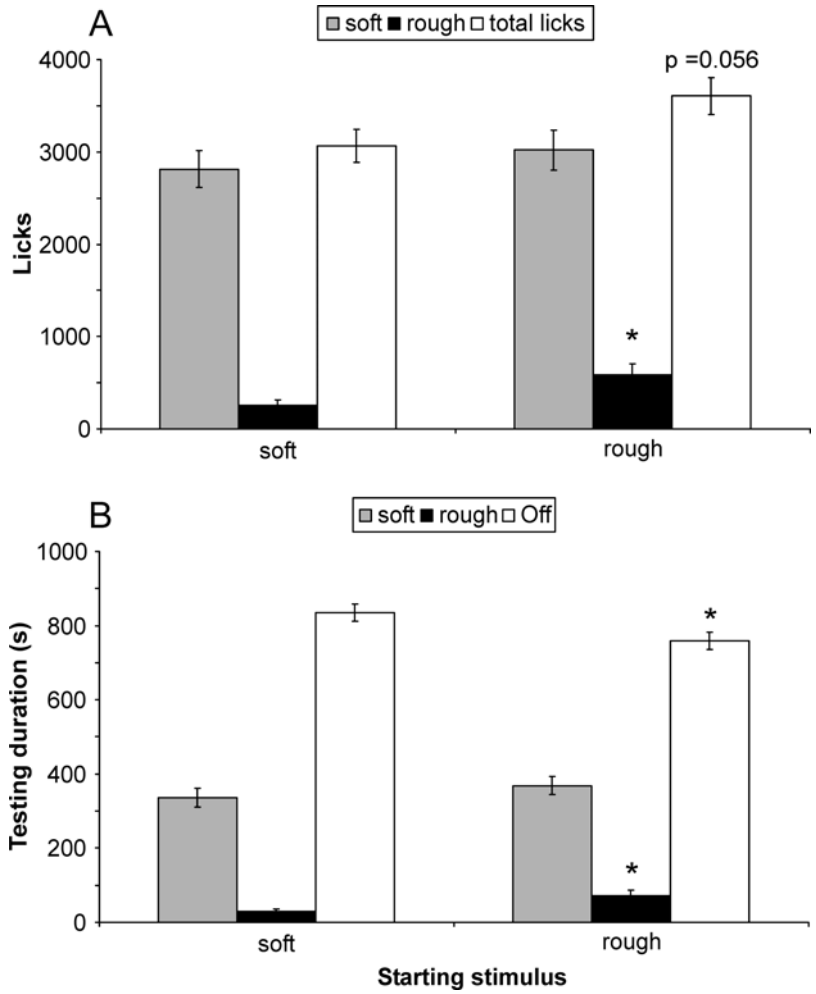


Figure 2-8 Effect of starting side on licks and duration spent with the soft and rough stimuli, or on total licks and unstimulated time when soft is preferred. A) Licks with soft stimulation were not different, while licks with rough stimulation were greater when rough was the starting stimulus. Difference in total licks between the two starting conditions did not reach statistical significance. B) Time spent in contact with soft stimulation was also not effected by starting stimulus, while time spent on the rough stimulus was greater when this was the starting stimulus and unstimulated time was significantly reduced. * indicates significant difference between soft or rough as the starting stimulus (determined by unpaired t-test). $p < 0.05$ All data are mean ± SEM.

Table 2-1. Number of rats exhibiting a cold, hot, or no preference at the stimulus combinations tested (bold numbers indicate the group preference).

Temperature Pair (°C)	Cold Preference	Hot Preference	No Preference	Total n
-4 & 48	1	**5**	1	7
10 & 48	**37**	15	2	54
18 & 48	**8**	1	0	9
24 & 48	**7**	2	0	9
10 & 45	2	**12**	0	14
18 & 45	0	**9**	0	9
24 & 45	2	**10**	0	12
37 & 37	11	14	2	27

(For 37&37°C, the count in the "cold preference" column reflects number of rats with a left thermode preference).

Table 2-2. Influence of previous experience on frequency of start side and preference with 10 and 10°C stimuli 24 hours later.

	Preference Induced	
First Day	Left 42°C	Right 10°C
n rats started on this side	4	6
n rats preferred this side	10	0
Second Day	Left 10°C	Right 10°C*
n rats started on this side	8	2
n rats preferred this side	8	2
Total n		10

* Please note that the two rats who started on the right exhibited a left-side preference and the two rats that preferred the right started on the left.

Table 2-3. Influence of previous experience on frequency of start side and thermal preference 24 hours with 18 and 48°C stimuli 24 hours later.

	Preference Abolished	
First Day	Left 52°C	Right 18°C
n rats started on this side	5	4
n rats preferred this side	1	8
Second Day	Left 18°C	Right 48°C
n rats started on this side	3	6
n rats preferred this side	4	5
Total n		9

Table 2-4. Effect of start side on the number of rats preferring soft, rough, or neither mechanical stimulus.

Starting Stimulus	Soft Preference	Rough Preference	No Preference	Total Rats
Soft	25	3	2	30
rough	14	16	10	40

Note: rough start includes both experimenter controlled and voluntary occurrences; no differences were noted in preference pattern between involuntary or voluntary rough start.

Object 2-1. Video clip of rats performing the single stimulus operant task.

CHAPTER 3
PHARMACOLOGICAL MANIPULATION OF OPERANT BEHAVIOR USING TRANSIENT RECEPTOR POTENTIAL CHANNEL AGONISTS

Findings within the last two decades have uncovered six transient receptor potential channels that are involved in the encoding of thermal stimuli. Among these, TRPV1, TRPM8, and TRPA1 play an important role in normal and pathological pain perception. Doubt regarding the role of TRPM8 in cold nociception has been recently laid to rest with the profound lack of cold-mediated responses exhibited by two independently developed strains of TRPM8 knock-out mice (Colburn et al., 2007; Dhaka et al., 2007). However, conflicting results still fail to resolve what role if any TRPA1 may play in cold nociception. Also, both TRPM8 and TRPA1 exhibit overlapping expression patterns with TRPV1 (Kobayashi et al., 2005; Xing et al., 2006). Thus molecular receptors for cold and heat may interact with each other with respect to different stimuli by their expression within a single primary afferent neuron. These expression patterns define subpopulations of noci- and thermoceptors that may exhibit overlapping connectivity with second order neurons. Thus, co-expression of TRP channels and connectivity of TRP-expressing primary afferents likely contribute to the complex and nuanced experience of thermal, chemical, and painful stimuli.

In this chapter, we examine the effects of various TRP channel agonists on operant responses to cold stimuli, thermal preference, and unlearned cold-mediated behaviors. We examine the capacity of the different TRP channel agonists to modulate operant responses to cold stimuli. We used the TRPM8 agonist menthol, the TRPM8/TRPA1 agonist icilin, and the TRPV1 agonists capsaicin and resiniferatoxin (RTX). RTX is an ultrapotent TRPV1 agonist, which binds to the channel and causes a massive influx of cations, including calcium. This results in apoptotic cell death when RTX is applied peripherally or excitotoxic degradation of the primary afferent terminal when applied centrally (Clapham, 1996; Wexel, 2008). Thus, this

agonist can be used to specifically ablate afferents that express TRPV1, coincidentally removing channels that are co-expressed with TRPV1. We demonstrate in this chapter that cold pain can be manipulated both by the cooling agonists menthol and icilin, as well as the burning agonist capsaicin. We also demonstrate that selective ablation of TRPV1-expressing profiles using the ultrapotent agonist RTX can significantly impair pain specific responses, while leaving cold avoidance intact.

Methods

Animals

Most of the animals were housed as described in chapter 2, including six female hairless rats that were used to examine the effects of menthol on cold sensitivity (Rossi et al., 2006). The effect of capsaicin on operant responses to 10°C and the effects of peripheral RTX on operant responses to cold were examined with haired male Sprague Dawley rats (Jackson Laboratories, 250-300g) conventionally housed in pairs. Sprague Dawley rats with normal fur were shaved and depilitated under anesthesia (isoflurane, 2.5% with oxygen) two to three time per week.

Preparation and Administration of TRP Channel Agonists

Menthol (10%) was dissolved in a vehicle of 1.6% ethanol in Tween80 and phosphate buffered saline (PBS) and administered subcutaneously (s.c., 150 µl) in each cheek. Icilin was dissolved in dimethyl-sulfoxide (DMSO) to a volume of 100µl for intraperitoneal (i.p.) injections or 10µl for intracisternal (i.c.m.) injections. A commercially available capsaicin cream was used for topical application of capsaicin (0.035%, Capzasin P; Chattem, INC; Chattanooga, TN). Capsaicin was applied bilaterally to the cheeks, left on for 5 minutes, removed with water, and the skin was dried with a cotton ball. Resiniferitoxin (RTX, 250 ng, LC Laboratories, Woburn, MA) was dissolved in a vehicle of 0.25% Tween 80 in PBS, 0.05% ascorbic acid to a volume of 50 µl for infraorbital nerve applications, or a volume of 10 µl for intracisternal injections.

Topical capsaicin application, s.c. injections and i.p. injections were performed on awake, restrained animals.

Operant behavioral testing was conducted immediately following capsaicin removal, 15 minutes following menthol or vehicle administration, 45 minutes following icilin or vehicle administration, and twenty four hours to two weeks following RTX or vehicle administration. The effects of topical capsaicin, menthol, and icilin are not permanent; therefore these drugs could be administered more than once in individual animals. However, because capsaicin can desensitize heat responses with repeated application, a period of at least one week was allowed to pass before administering capsaicin a second time. A cross-over design was used to administer menthol and vehicle. To avoid sensitization or desensitization from these compounds, as well as sensitivity from multiple injections, a recovery period of two days minimum passed between treatments (one week between i.c.m. injections). The effects of RTX are long lasting; therefore comparisons between RTX and vehicle treatment are inter-individual.

Infraorbital and Intracisternal Injections

All injections were performed under anesthesia (isoflurane, 2.5-5% with oxygen by inhalation). Perineural administration of RTX to the infraorbital nerve was performed using a 25-gauge, 1.5 inch needle. The intersection of the zygomatic arch and the dorsal skull was located by palpation just medial to the eye on the top of the head. The needle was inserted at this point and carefully pressed along the bone of the skull until resistance was felt from the bone at the bottom of the arch, where the nerve passes through the infraorbital foramen. The fluid was injected and the needle was carefully removed.

Intracisternal injection of RTX or icilin was performed by dorsally flexing the rat's head at a 45 degree angle, inserting a needle near the base of the occipital bone, and stepping the needle

down until the atlanto-occipital membrane was breeched. A small amount of cerebrospinal fluid (CSF) was aspirated to confirm needle placement before drug administration.

Additional Behavioral Tests

Evaluation of wet dog shaking induced by icilin

Dose selection and timing of thermal testing was based on quantification of wet dog shakes (WDS). Sprague Dawley rats (n = 4-5 each dose, indicated in Table 3-1) were injected with icilin i.p. or i.c.m. and observed in holding cages for up to two hours following injection. WDS were counted in thirty minute intervals and the rate of WDS frequency was calculated for each rat by dividing the counts by the appropriate observation time, and averaged across rats. The following doses were used: 0.0025, 0.025, 0.25, and 2.5 mg of icilin (in DMSO). DMSO treated rats did not receive icilin. The other rats received icilin treatments, with at least 2 wash-out days between doses. Only the doses used for thermal preference testing are shown in Table 3-1.

Capsaicin eye-wipe test

Rats were taken into the observation room and allowed to acclimate for five minutes. Rats were removed from their cohort one at a time and placed in an observation cage, partially covered by a clear sheet of Plexiglas. Rats were gently restrained by the experimenter, 20µl of capsaicin solution (0.01%, dissolved in ethanol and diluted with PBS), was carefully delivered to one eye with a micropipetor, and the rat was immediately released. Wipes were counted by the experimenter; an "eye wipe" was considered either a pass of the forepaw caudo-rostrally across the treated eye, or scratching of the rostral corner of the eye by the hindpaw. Responses typically lasted from 30 seconds to one minute 30 seconds. The second eye was not treated until signs of discomfort in the first eye were gone. Specifically, after the rat no longer squinted or blinked the treated eye, and began engaging in exploratory investigation of the observation cage.

Statistical Analysis

Effects of menthol and vehicle on operant responses were evaluated using a one way ANOVA. The effect of icilin dose and delivery on the rate of wet dog shaking, as well as the effect of icilin dose on thermal preference, was determined with a Kruskall Wallace test and post-hoc Whitney Mann tests. The effects of capsaicin and RTX on operant response to cold stimuli were determined using unpaired t-tests. Thermal preference in either RTX or vehicle treated rats was determined using repeated measures ANOVA or paired t-test. Comparisons between RTX and vehicle treated rats for each stimulus in the preference task were made with unpaired t-tests. Significance was $p < 0.05$ for all analyses.

Results

Effect of the TRPM8 Agonist Menthol on Operant Responses to Cold

Male and female rats (n = 6 each sex) were tested at 24, 10, and -4°C fifteen minutes following injection of either menthol or vehicle. Each individual rat's outcome measures were compared to the baseline average for its sex to produce a percent increase or decrease from baseline for the menthol and vehicle treatment groups. There were no significant effects of sex, so the data from all rats was pooled. At 10°C, there was a significant increase in stimulus contacts ($F_{2,35} = 8.582$) and a significant decrease in success ratios ($F_{2,35} = 5.476$) for menthol treatment relative to vehicle (Figure 3-1). There was no significant change in licks for either treatment (data not shown). These changes are indicative of allodynia following menthol treatment. At 24 and -4°C there were no significant effects following menthol treatment (Figure 3-1). This data was previously reported in Molecular Pain (Rossi et al., 2006).

Effect of the TRPM8/TRPA1 Agonist Icilin on Wet Dog Shaking

Wet Dog Shaking increased in a dose dependent manner (Table 3-2). The bodily location and intensity of WDS differed depending on delivery method. When administered i.p., the

classic head-to-tail WDS was produced, sometimes so intense at the high dose (0.25mg) that the rat was briefly unbalanced. When administered i.c.m., the shakes tended to be more focused in the upper body, beginning in the head but usually ending in the upper abdominal region. There were dose- and administration-related differences in the rate of WDS across the two hour observation period (Table 3-2). The high dose (0.25mg) of icilin produced a regular rate of WDS that persisted throughout the two hour observation period. The rate was significantly higher for i.p. administration (two to three WDS/minute) than for i.c.m. (one to two WDS/minute), particularly in the middle hour of the observation period (Table 3-2). In contrast, the low dose icilin transiently elevated WDS in the first 30 minutes for i.c.m. administration (roughly one WDS every two minutes) and the second 30 minutes for i.p. administration (roughly one WDS every three minutes). In the remaining time, while WDS occurred with low dose icilin, they did not occur with significantly greater frequency than DMSO.

DMSO did not illicit regular WDS; in the entire two hour testing period rats exhibited an average of three WDS for both administration routes. Of the doses tested, 0.025mg was the highest dose that elevated WDS, but did not produce a persistent and regular rate of WDS. WDS remained elevated between 30 and 60 minutes for both high and low doses, so thermal preference testing was performed within this interval.

Effect of the Icilin on Thermal Preference

At baseline, rats strongly prefer 10°C over 48°C, licking more than 90% on that side and spending about 40% on the total test time on the 10°C thermode (Figure 3-2). Following i.c.m. administration of DMSO, although the baseline preference for 10°C was not altered there was a significant increase in the licking and time spent on the 48°C thermode. This indicates that DMSO itself may block some of the heat pain experience with 48°C stimulation, decreasing the rats' natural avoidance of this temperature. Following i.c.m. administration of a low dose of

icilin (0.025mg), the strong preference for 10°C exhibited at baseline and following DMSO administration was abolished. Roughly 65% of total licks occurred with 48°C stimulation, in contrast to about 5% at baseline or 20% with DMSO. Rats also spent about 20% of the total test time at 48°C. Intraperitoneal administration of DMSO and low dose icilin had no significant effects on thermal preference with facial stimulation (data not shown). Taken together, these data indicate that a low dose of icilin strongly decreased preference for 10°C in the face when administered i.c.m. Following high dose icilin (0.25mg), a preference for 10°C is maintained (about 60% of licks), but was not significantly different from DMSO or baseline. These data suggest that the two doses of icilin have different effects that may interact with the heat-blocking effect of DMSO.

Effect of the TRPV1 Agonist Capsaicin on Operant Responses to Cold

To examine the effect of TRPV1 activation on operant behaviors in the presence of cool or cold stimulation, a capsaicin cream (0.035%) was applied bilaterally to the face, left on for five minutes, and wiped away with water. Rats were tested immediately following removal of capsaicin at either 10 or -4°C (Figure 3-3). When tested with 10°C stimulation, licks where not significantly changed, while stimulus contacts were significantly decreased and success ratios were significantly increased with capsaicin than without. In contrast, when tested with -4°C stimulation, licks and success ratios were significantly decreased, while stimulus contacts were not significantly different. These data indicate that the moderate cold can alleviate the pain associated with capsaicin activation of TRPV1, while noxious cold enhances the discomfort of capsaicin.

Effect of TRPV1 Lesion with Resiniferatoxin on Operant Responses to Cold

It has been previously established that RTX eliminates sensitivity to noxious heat and can alleviate inflammatory and neuropathic pain. However, it had not been established what effect

the removal of TRPV1 could have on cold sensitivity. We evaluated the effect of peripheral or central application of RTX on operant responses to cold stimuli, thermal preference, and unlearned behaviors induced by menthol and icilin.

Peripheral versus Central RTX and Cold Sensitivity

Initial experiments with RTX targeted the peripheral end of the trigeminal nerve (infraorbital branch). This treatment method significantly reduces the capsaicin eye wipe response by a third and leads to a substantial loss of TRPV1 immunoreactive cells in the trigeminal ganglia as compared to vehicle treatment (Figure 3-4). There were also short term changes with respect to cold stimuli (2 and 10°C). One day following perineural RTX injection, stimulus contacts were dramatically reduced and success ratios were increased in the presence of 10°C stimulation relative to vehicle treated rats (Figure 3-5 B, C). However, by thirteen and fifteen days post-injection, responses to 10 and 2°C respectively returned to normal in the RTX-treated rats (Figure 3- 5). This could indicate that RTX treatment may lead to an immediate inflammatory response underlying increased cold seeking behavior (see responses to capsaicin with 10°C stimulation above) or that some functional recovery occurs following peripheral RTX. Either of these possibilities could involve the small percentage of TRPV1 positive neurons spared by peripheral injection. Therefore, we sought to use a method that would more thoroughly and permanently block TRPV1 function within the trigeminal system.

Effects of TRPV1 lesion by RTX on Cold Pain and Avoidance

The central application of RTX via i.c.m. injection targets the brainstem and cervical spinal cord. Following successful injection of RTX, capsaicin eye wipe response was entirely negative and inflammatory hyperalgesia is blocked (Wexel, 2008). Three female hairless Sprague Dawley rats treated centrally with RTX remained capsaicin eye wipe negative for up to a year, suggesting that no functional recovery of TRPV1 activity occurs. Unlike peripheral

application, TRPV1-positive cells remain intact in the ganglia, but TRPV1-positive fibers are absent in the spinal trigeminal nucleus (Wexel, 2008). Following injection, rats were allowed to recover for two weeks and tested in the single stimulus operant task with -4°C. While there were no significant effects on licks, RTX treatment significantly decreased stimulus contacts and increased success ratios, indicating insensitivity to cold pain (Figure 3-6).

A group of RTX- and vehicle-treated rats was also assessed for thermal preference (-4 or 48°C) post-injection. Recall from Chapter 2 that untreated rats prefer 48°C stimulation with this combination of stimuli. While RTX-treated rats are insensitive to both -4 and 48°C when these stimuli are presented individually, licking and testing duration indicated RTX-treated rats still prefer 48°C, although this preference does not reach statistical significance as it does for vehicle-treated rats (Figure 3-7 A, B). RTX-treated rats also had significantly fewer stimulus contacts and success ratios than vehicle-treated rats for the 48°C stimulus and for responses at both stimuli combined (Figure 3-7 C, D). This supports previous findings that RTX treatment renders animals insensitive to noxious heat. Thus, while cold sensitivity is highly impaired follow RTX treatment, some cold perception must still exist that contributes to a cold aversion. Recall from Chapter 2 that where both stimuli are non-painful, heat is preferred. Therefore, RTX lesion of TRPV1-expressing fibers likely renders the stimulus pair non-painful, but leaves the capacity for discrimination between warm and cold intact.

Effects of TRPV1 lesion by RTX on Thermogenic and Nocifensive Responses induced by TRPM8 Agonists

We have also assessed the effects of RTX treatment on innate behaviors induced or enhanced by agonists of TRPM8. We have observed that RTX-treated rats remain sensitive to icilin (0.25mg i.c.m.). Icilin is a TRPM8/TRPA1 agonist that induces wet dog shakes, which are thought to be the rodent analog of shivering and dependent on TRPM8 activity in the periphery

(Colburn et al., 2007; Dhaka et al., 2007). We have also observed that RTX application to the sciatic nerve can eliminate the ability of menthol to enhance nocifensive responses to acetone cooling of the hindpaw (general observations).

Taken together, these data provide behavioral evidence to support the idea that cold nociception represents a distinct subset of cells which express TRPV1. This nociceptive population is likely responsible for transmitting thermal pain regardless of modality (hot vs. cold), as well as agonist-related irritation, demonstrated by nocifensive behaviors. However, there are also cold receptive neurons not expressing TRPV1 that encode the cold quality of thermal stimulation. This population likely also plays an important role in the regulation of basal body temperature and thermogenesis, as well as affective judgments regarding thermal stimuli.

Discussion of TRP Channel Manipulation and Sensory Processing

Effects of Menthol on Cold Sensitivity

It was hypothesized that 10% menthol would have effects on all three cold stimuli because they are within the activation range for TRPM8, yet effects were only observed with 10°C stimulation. The lack of menthol induced allodynia with 24ºC stimulation likely has to do with sub-populations that may not be sufficiently activated by this level of stimulation. TRPM8 mRNA has been identified in both small and medium diameter rat DRG neurons *in vivo*, which are presumed to correspond to the cell bodies of C- and Aδ fibers, respectively (Kobayashi et al., 2005). This has also been demonstrated in mice expressing green fluorencence protein under the control of the TRPM8 promoter (Dhaka et al., 2008). In the periphery, 24ºC may not be of sufficient magnitude to activate TRPM8-containing C-fibers but could activate cold responsive Aδ fibers that block C-fiber activity and prevent the induction of menthol-induced allodynia (Liu et al., 1998). Within the spinal dorsal horn, cool-sensitive lamina I spinothalamic (STT) cells

have a specific sensitivity to temperatures between 34 and 15°C. The sensitivity of polymodal nociceptive HPC (for heat, pinch, cold) cells to noxious cold begins at about 24°C, and their response to cold accelerates at temperatures below 15°C. It has been suggested that an increase in HPC activity beyond that of cool-sensitive cells signals the sensation of burning pain (Craig, 2003). Even in the presence of menthol it seems 24°C does not provide input of sufficient magnitude to increase HPC STT cell activity beyond cool-sensitive STT cells in lamina I and thus could explain why menthol did not enhance sensitivity to 24ºC.

The lack of menthol-induced hyperalgesia with -4°C stimulation is likely due to the concentration used. Menthol is known to produce a burning sensation in humans at a concentration of 40% and also increases sensitivity to cold, presumably by its action on C-fibers (Wasner et al., 2004). It is possible that 10% menthol, while sufficient to induce allodynia at 10ºC, was not sufficiently concentrated to induce hyperalgesia at -4ºC. It is important to note that it also did not induce insensitivity to -4ºC, reducing the possibility that TRPM8-expressing cells were desensitized to further cold stimulation.

Effect of Icilin on WDS

We first characterized the WDS produced by different doses of icilin when administered either i.p. or i.c.m. A difference in temporal profile for i.p. versus i.c.m. administration was observed. Following i.c.m. administration of icilin, WDS elevation was evident within the first 30-minutes and was either maintained (high dose) or declined (low dose) over the following 90 minutes. Following i.p. administration, WDS elevation was evident in the first 30-minutes, but increased over the second 30-minute interval, rather than remaining steady or declining. This difference is likely related to the different local environment and distance of the icilin agonist. Intraperitoneal injection delivers the agonist acts to the cutaneous ends of primary afferent neurons, which are diffusely distributed in heterogeneous tissue. Once the agonist is in the

vicinity of a primary afferent, it must activate a sufficient number of channels to depolarize the cell. In contrast, i.c.m. injection delivers the icilin directly to the synapses between the primary afferent neurons and the second order neurons, increasing the chance that enough channels will interact with the agonist to alter the neuron's membrane potential.

Effect of Icilin on Thermal Preference

Based on the quantification of icilin-induced WDS, we chose two doses of icilin to manipulate thermal preference: a low dose that elevated WDS somewhat, but did not produce a persistent rate of shaking, and a high dose that produced a persistent rate of one to three WDS/minute. Thermal preference for 10°C was abolished when rats were administered low dose (0.025mg) icilin. This effect of low dose icilin is likely the consequence of increased sensitivity to cold due to icilin's action on TRPM8 channels and perhaps TRPA1 channels as well. Icilin potentiates the activation of TRPM8 by cold stimuli (Chuang et al., 2004) and also activates TRPA1, but with less potency (Doerner et al., 2007).. The capacity for TRPA1 to be directly activated by cold stimulation is still debated (Story et al., 2003; Jordt et al., 2004; Bautista et al., 2006; Kwan et al., 2006), although it clearly plays some role in nociceptive processing. There is evidence that TRPA1 may be involved in processing of mechanical stimuli (Kwan et al., 2006; Cahusac and Noyce, 2007; Kindt et al., 2007), and may therefore mediate pricking sensations induced by icilin.

Even if it is not directly activated by cold stimulation, TRPA1 may still be indirectly involved in cold nociception. It has recently been shown that calcium alone can activate the TRPA1 channel (Doerner et al., 2007), raising the possibility that this channel may act as a coincidence detector and modulator of nociception. There is evidence to suggest that there are other, unidentified cold receptors (Babes et al., 2002; Munns et al., 2007). If these are co-expressed with TRPA1, it might be possible that calcium influx via these receptors could further

activate TRPA1. Icilin could act on TRPA1 in these cells to increase their response in the presence of cold stimuli. Alternatively, because of its high degree of coexpression with TRPV1, activation of TRPA1 combined with activity in cold- responsive neurons could be responsible for the burning sensation sometimes reported by prolonged contact with cold stimuli. Additionally, TRPA1 has also been shown to facilitate excitatory synaptic transmission in the substantia gelatinosa (Kosugi et al., 2007). Thus, application of icilin could potentially enhance transmission of cold input from peripheral afferents by its actions on TRPA1 at the first synapse.

In contrast, the high dose of icilin did not significantly alter thermal preference relative to DMSO. This difference may be due to the escape option available to rats with the facial preference task, as well as potential interaction of DMSO with this dose of icilin. DMSO is commonly used as a solvent for application of agonists in electrophysiological and calcium imaging studies. It has also been used *in vivo* to sensitize A delta fibers (Wilson et al., 1999; Tzabazis et al., 2005) and has also been shown to block c fiber conduction (Evans et al., 1993). The mechanism for DMSO induced a delta sensitization has never been clarified, but the c fiber block may be a consequence of sensitized a delta fibers inducing long term depression in c fibers (Liu et al., 1998).

In this study, DMSO also increased licking and time spent with 48°C, but did not have a significant effect on 10°C repsonses, indicating a possible analgesic effect for heat pain. The inhibition of secondary pain conducted by c fibers may allow rats to recover from 48°C stimulation more readily. This would enable them to be somewhat more successful on the hot thermode. When DMSO is combined with a low dose of icilin that produces cold sensitivity, the combined effects of these compounds is complementary, and rats are even more successful at 48°C. Evidence suggests that TRPM8 is expressed in both A delta and c fibers, whereas TRPA1

is primarily expressed in c fibers. At low dose icilin, the activity of A delta fibers containing TRPM8 is likely enhanced by icilin's actions on TRPM8. When DMSO is combined with high dose of icilin, icilin's capacity to sensitize TRPA1-expressing c fibers may be blocked by DMSO. Conversely, any sensitizing effect that DMSO may have on TRPM8-expressing A delta fibers could potentially be inhibited by desensitization of TRPM8 that may occur in the presence of high dose icilin. Further experimentation is needed to resolve these issues.

A Role for TRPV1 in Cold Pain

The hyperalgesic responses to noxious cold induced by TRPV1 activity, and insensitivity to noxious cold following ablation of TRPV1 support the idea that TRPV1-expressing afferents can contribute to the perception of painful cold. The alleviation of capsaicin irritation by 10°C is supported by previous electrophysiological findings (Babes et al., 2002) and by menthol in oral irritation studies (Green and McAuliffe, 2000). The ability of RTX to eliminate menthol irritation further supports previous findings that hypothesized that menthol-induced irritation was dependent on capsaicin-sensitive nociceptors (Cliff and Green, 1996). There are two possible explanations for the effects of TRPV1 activation and lesion on cold mediated behaviors. The first possibility is that TRPV1 is expressed in cold nociceptors, activated by cooling below 10°C. The second is that TRPV1 expressing primary afferents provide convergent inputs with cold-responsive primary afferents onto the same second order neurons in the dorsal horn. These two possibilities are not mutually exclusive and likely both contribute to the effects of TRPV1 on cold perception.

With respect to the first explanation, TRPM8 and TRPA1 are co-expressed to some degree with TRPV1, as previously mentioned. Under normal conditions, the percentage of nociceptive cells expressing both TRPV1 and TRPM8 has been reported to be low (Xing et al., 2006; Dhaka et al., 2008), which has led some to suggestion that their contribution to normal cold pain may be

minimal (Dhaka et al. 2008). However, small population size may not be adequate reason to dismiss this population in normal cold nociception. TRPV1 knock-out mice were found to have intact withdrawal responses to painful heat within the activation range of TRPV1 (42-49°C) (Caterina et al., 2000). Later studies have attributed the maintenance of these responses to a small population of heat nociceptors that remain intact and provide input to lamina I (Eckert et al., 2006). In a similar manner, removal of the small TRPM8/TRPV1 co-expressing population may be sufficient to block cold nociception. Further behavioral testing of with TRPM8 and TRPA1 knockout mice, and treatment with RTX, could help determine if the TRPV1/TRPM8 plays a substantial role in normal cold pain perception.

Another candidate population of cold nociceptors is cells that express TRPV1 and TRPA1, which represents nearly 100% of the TRPA1 population *in vivo* (Kobayashi et al., 2005). The role of TRPA1 in cold nociception however has been highly debated. Early *in vitro* work demonstrated that TRPA1 expressed in Chinese hamster ovarian cells was responsive to cold <18°C, and that cells in mouse DRGs exhibited properties of TRPA1-expression (Story et al., 2003). Subsequently, this work has been both supported (Bandell et al., 2004; Macpherson et al., 2006; Sawada et al., 2007) and contested (Jordt et al., 2004; Nagata et al., 2005; Zurborg et al., 2007). Behavioral evaluation of TRPA1 knock-out mice developed in two different laboratories also yielded conflicting results. Kwan and colleagues demonstrated that female knockout mice exhibited a robust decrease in cold irritation (paw lifts with cold and duration of post-acetone responses), while males exhibited only slight, insignificant decrease in cold irritation (Kwan et al., 2006). In contrast, Bautista and colleagues did not reveal any effect of TRPA1 knock-out on cold irritation (acetone test), withdrawal thresholds, or shivering, although they did not make an effort to discriminate between sexes (Bautista et al., 2006), even within the

nociceptive range. However, even if TRPA1 is not directly activated by painful cold, it is known to be activated by intracellular calcium (Zurborg et al., 2007) and may serve as a coincidence detector for another unidentified cold receptor (Babes et al., 2006; Munns et al., 2007) that increases intracellular calcium.

The second explanation for the effects of TRPV1 on cold pain involves the convergent input of cold and hot nociceptors onto the same second order neuron in the dorsal horn of the spinal trigeminal nucleus. Cold-responsive units in the superficial trigeminal nucleus caudalis all responded to noxious heat and the majority of these also responded to menthol, with substantial overlap in menthol, cinnamaldehyde, and capsaicin responsivity (Zanotto et al., 2007), indicating convergent inputs of TRPM8, TRPA1, and TRPV1 expression afferents onto second order neurons. Furthermore, menthol and cinnamaldehyde are able to cross desensitize the responses of these units (Zanotto et al., 2008). These findings support the idea that cells expressing TRPM8 or TRPV1 only, and TRPV1/TRPA1 provide convergent input onto individual second order neurons within the trimeinal nucleus caudalis.

Conclusions

We demonstrate here that menthol and icilin can modulate cold-mediated behaviors, although these effects did not always follow expectations. Additionally, we demonstrated a role for TRPV1-expressing afferents in cold pain in a non-pathological state, although it is still unclear which molecular mediator(s) for cold sensitivity are coupled with TRPV1. Cold nociceptors are likely heterogeneous, which certain subpopulations and different proportions of activity encoding different aspects of cold sensation. Further manipulation of TRP channel activity in pathological states will allow us to further clarify the role of TRP channels in chronic pain.

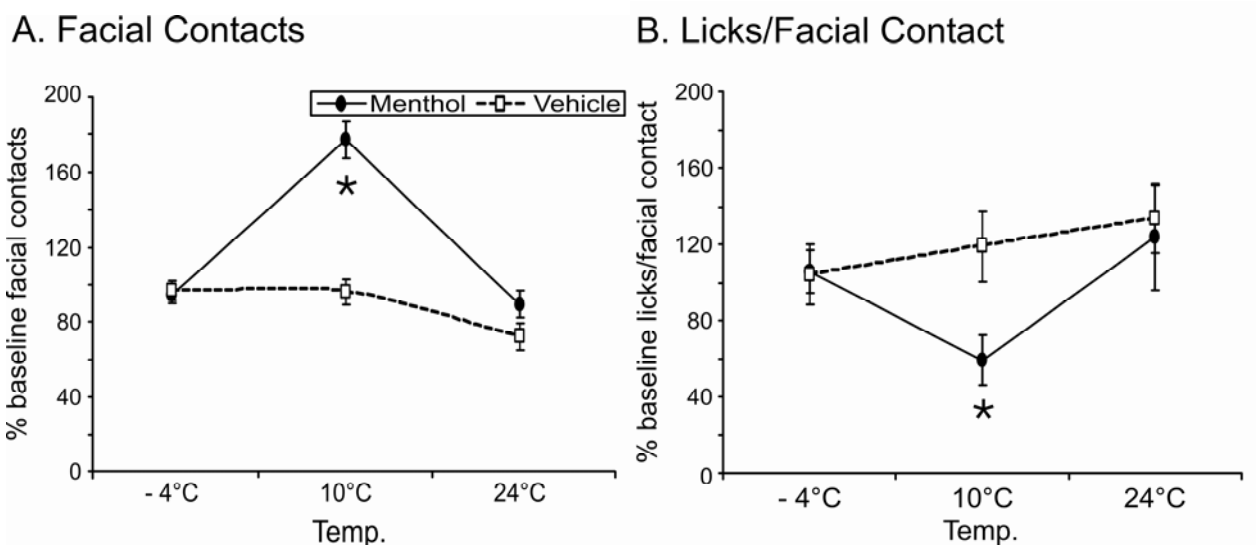


Figure 3-1 Effect of menthol (10%, s.c.) on operant responses to cold stimuli. A) Facial contacts are significantly increased by menthol treatment with 10°C stimulation, but not -4 or 24°C stimulation. B) Success ratios are significantly reduced by menthol treatment with 10°C stimulation, but not -4 or 24°C stimulation.* indicates $p < 0.05$ (one way ANOVA). Data are mean ± SEM.

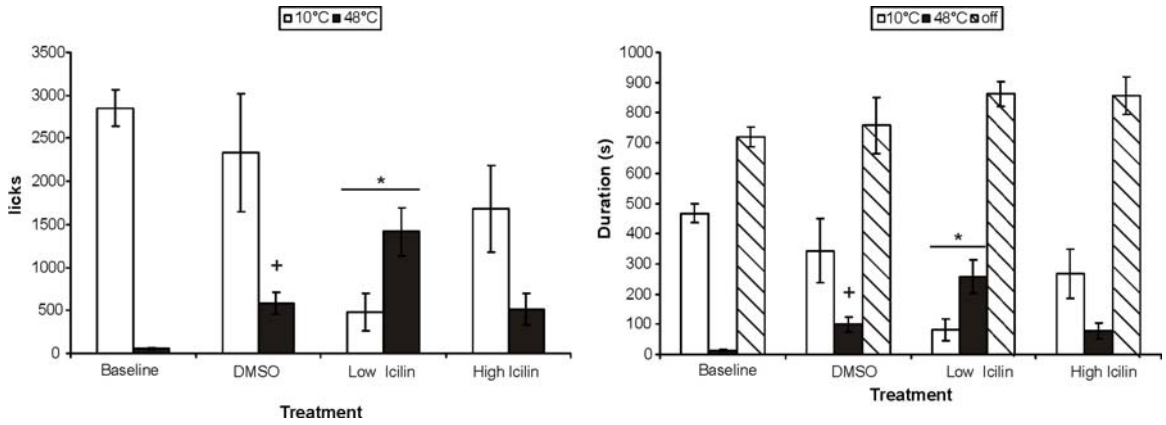


Figure 3-2. Effect of two doses of icilin and DMSO on thermal preference with facial stimulation at 10 and 48°C. The number of licks, A) and of the distribution of testing time, B) are shown for baseline (n = 12) and following DMSO (n = 6), 0.025mg (n = 8), or 0.25mg icilin (i.c.m., n = 6). Data are mean ± SEM. A) There was a significant effect of treatment on licking at 10°C (Kruskal- Wallis test, chi squared = 16.55, $p = 0.001$) and 48°C (chi squared = 20.93, $p < 0.001$). B) There was a significant effect of treatment on time at 10°C (chi squared =16.59, $p = 0.001$) and 48°C (chi squared =21.24, $p < 0.001$), but not on time off the thermodes (chi squared = 6.50, $p = 0.09$). Post-hoc comparisons were made using the Mann-Whitney U test. * indicates a significant effect of low dose icilin treatment as compared to all other treatments (baseline, DMSO, and high dose icilin). + indicates a significant difference between DMSO and baseline only. For all statistical tests $p < 0.05$.

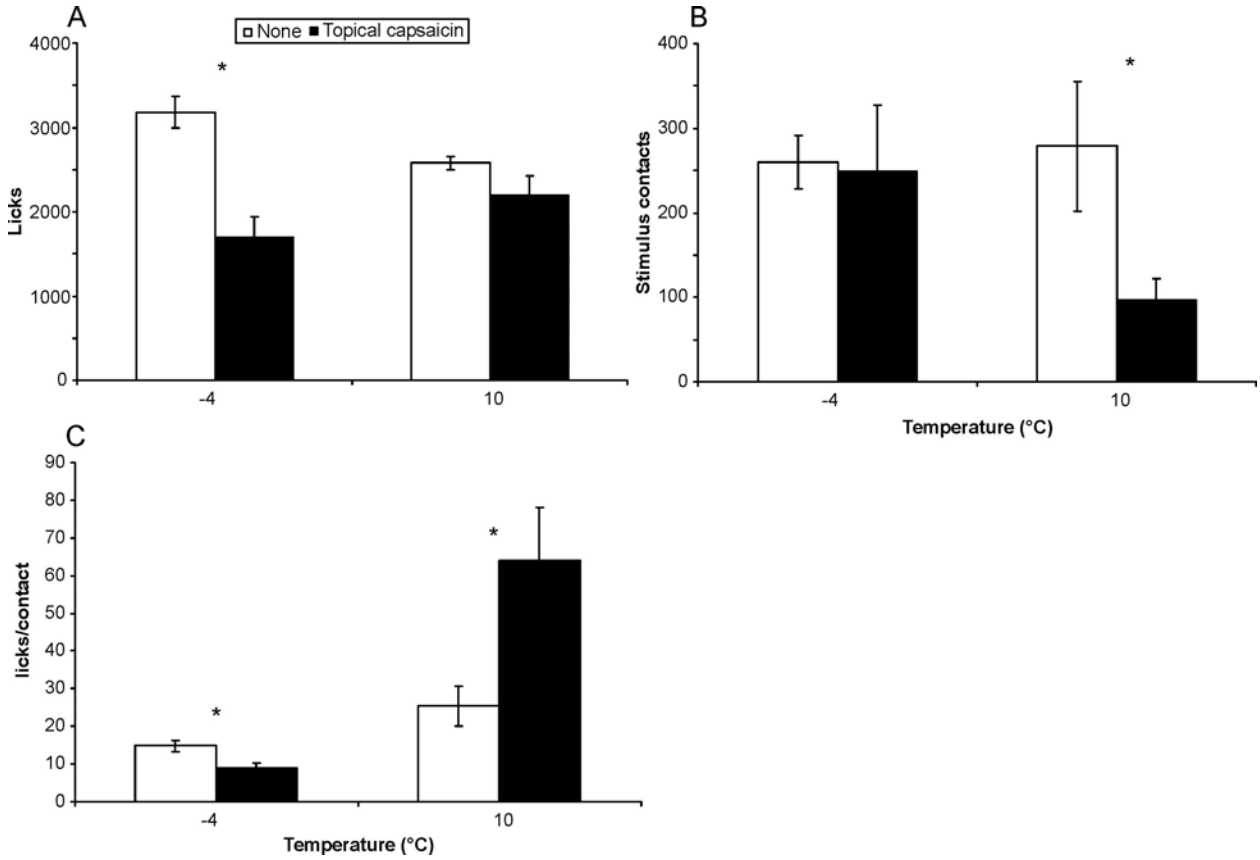


Figure 3-3 Effect of the TRPV1 agonist capsaicin on operant responses to moderate (10°C) and noxious cold (-4°C). A) Capsaicin (topical, 0.035-0.075%) significantly reduced licks with -4°C stimulation, but not 10°C. B) Capsaicin significantly reduced stimulus contacts with 10°C, but no -4°C stimulation. C) Capsaicin significantly reduced the success ratio (licks/contact) with -4°C stimulation and significantly increased it with 10°C stimulation. * indicates significant difference between capsaicin and no treatment, $p < 0.05$ (t-test). Data are mean ± SEM.

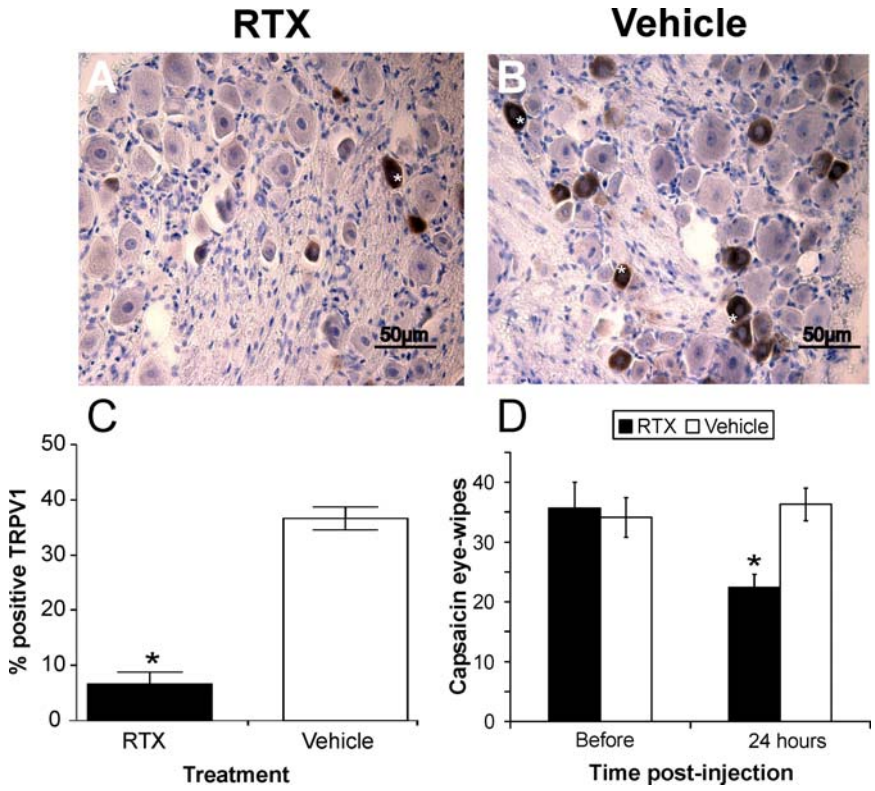


Figure 3- 4 Effect of peripheral resiniferatoxin (RTX) treatment on TRPV1 expression on in the trigeminal ganglia and TRPV1 function. A&B) Sections of trigeminal ganglia from an RTX (A) and vehicle (B) treated rats, labeled with a rabbit anti-TRPV1 antibody and counterstained with hematoxylin (200X). White astricies indicate TRPV1 positive cells (brown). C) Quantification of positive cells remaining in RTX versus vehicle treated ganglia. D) RTX significantly decreased wipes in response to intraocular capsaicin (0.1% in PBS), used to evaluate function of TRPV1. * indicates $p < 0.05$ (t-tests). Data are mean ± SEM. (Images courtesy of Alan Jenkins).

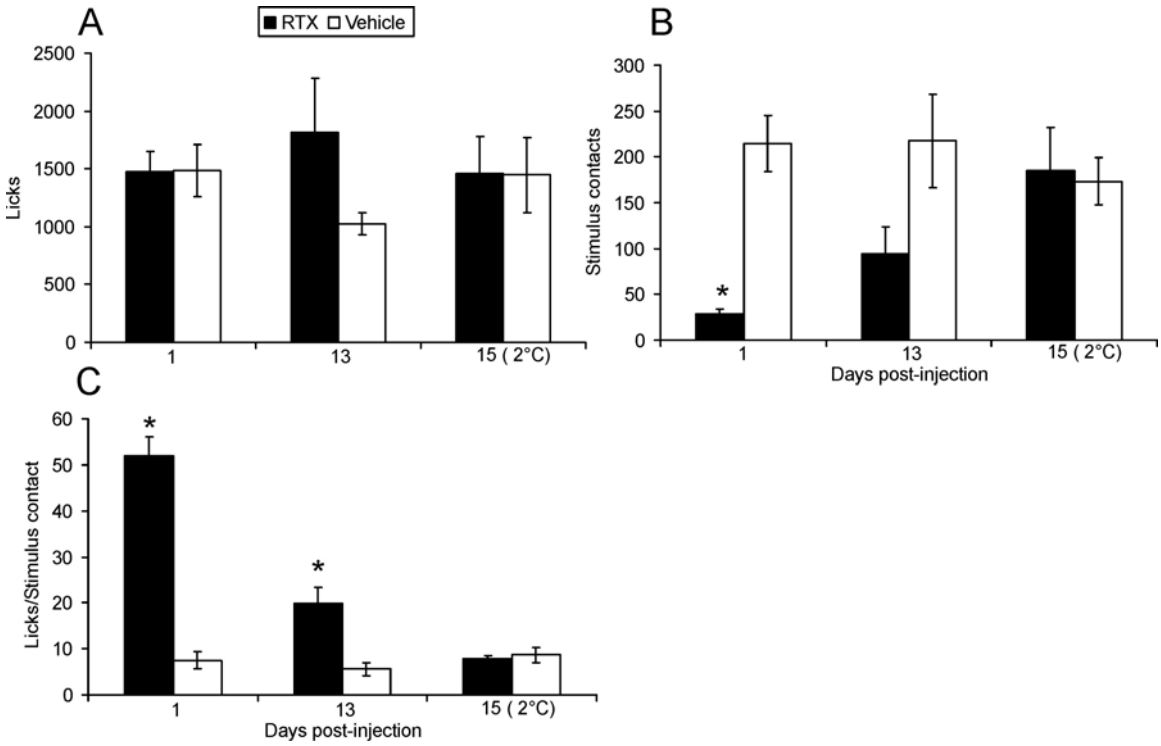


Figure 3-5 Effect of peripheral TRPV1 lesion by resiniferatoxin (RTX) on operant response to 10, 2°C stimulation. A) RTX had no effect on licks. B) RTX significantly reduced stimulus contacts with 10°C only one day post-injection. C) RTX significantly increased success ratios (licks/contact) up to thirteen days post-injection. * indicates significant difference between RTX (n = 3) and vehicle (n = 4), $p < 0.05$ (t-test). Data are mean ± SEM.

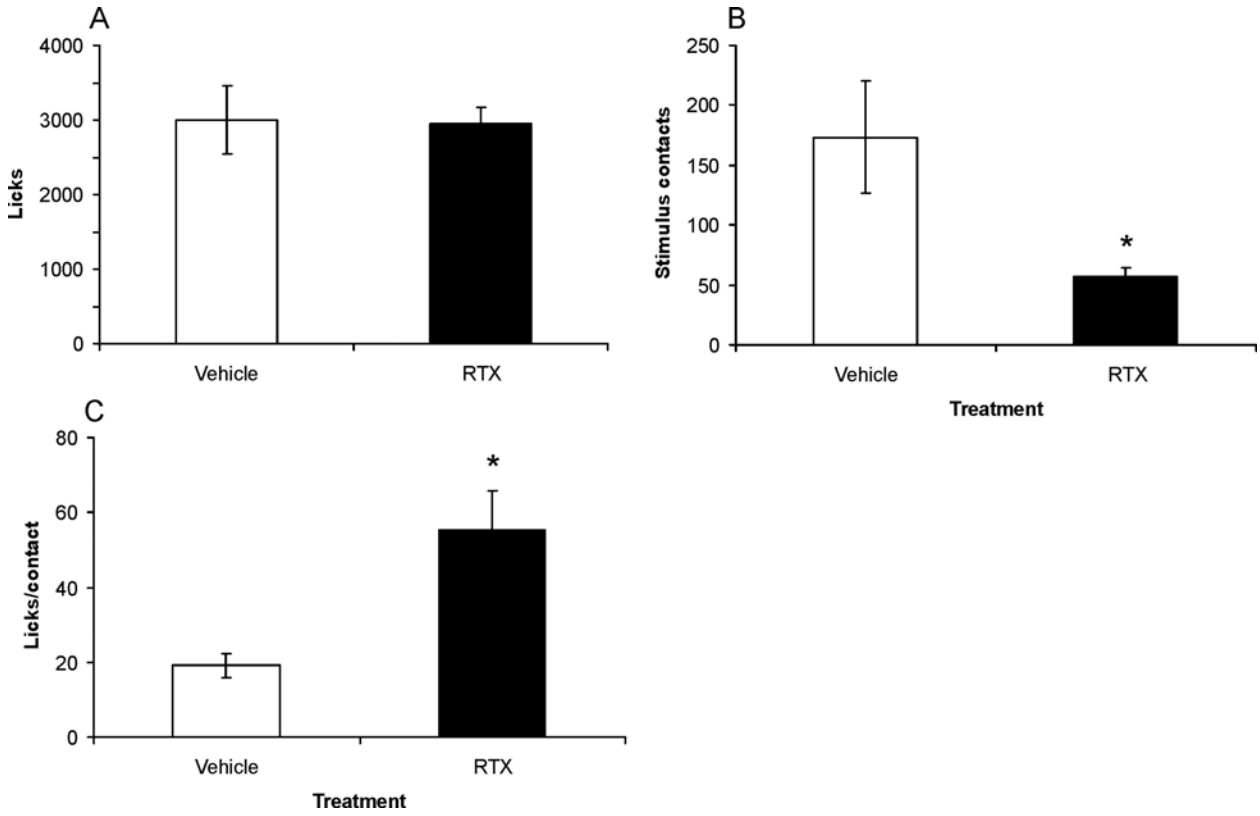


Figure 3-6 Effect of central TRPV1 lesion by resiniferatoxin (RTX) on operant response to -4°C stimulation. A) Licks were not significantly different between RTX- or vehicle-treated rats. B) RTX-treatment significantly reduced stimulus contacts. C) RTX-treatment significantly increased success ratios (licks/stimulus contact). * indicates significant difference, $p < 0.05$ (t-test). Data are mean ± SEM.

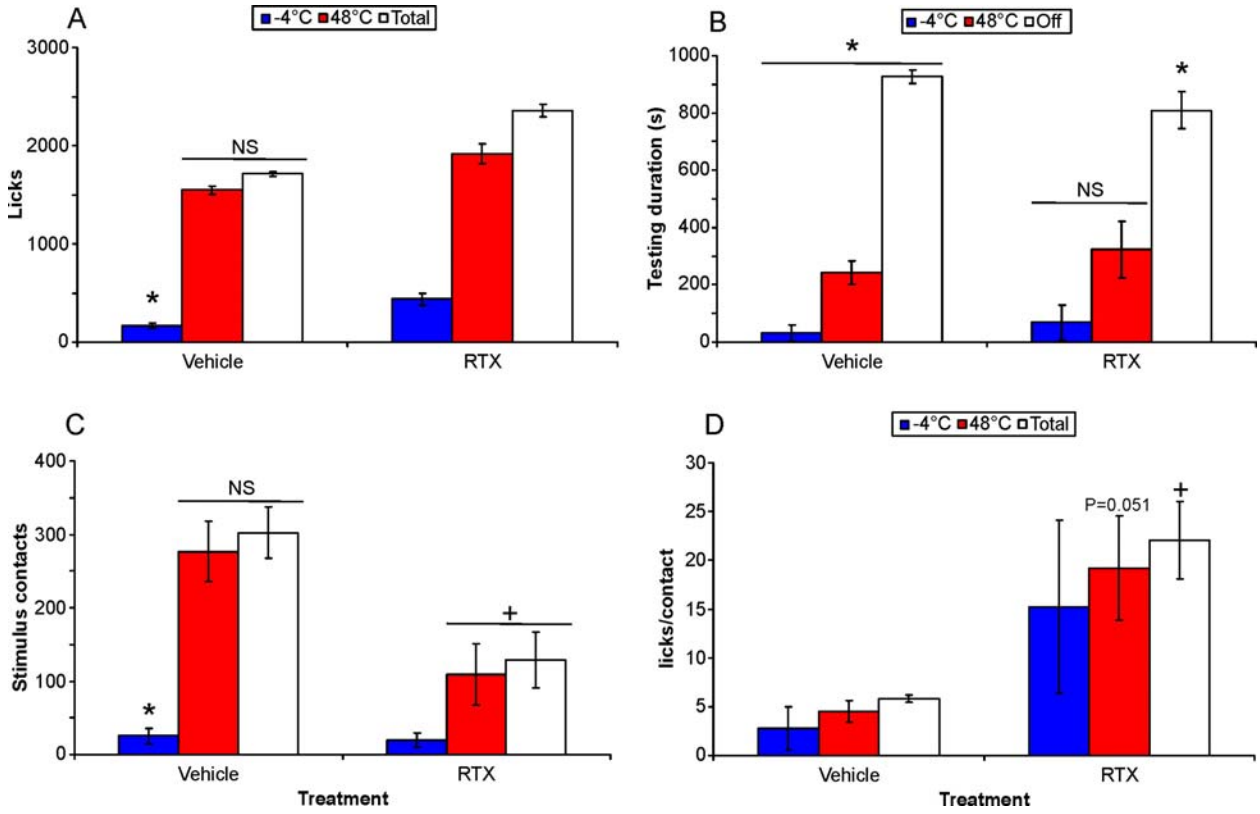


Figure 3-7 Effect of central TRPV1 lesion by resiniferatoxin (RTX) on thermal preference for -4 and 48°C stimulation. A) Licks occurred more with 48C stimulation for both treatment groups and total licks were slightly greater with RTX. B) Duration of stimulation also indicates 48C preference. C) Stimulus contacts were significantly reduced with RTX treatment relative to vehicle for 48C stimulation. D) Success ratios (licks/stimulus contact) for the combined stimuli were significantly greater with RTX-treatment than with vehicle. * indicates significant difference between stimuli or time distribution (paired t-test, repeated measures ANOVA for duration), + indicates significant difference between treatments (unpaired t-tests), $p < 0.05$. Data are mean ± SEM.

Table 3-2. Rate of Wet Dog Shaking (WDS per minute, mean ± SEM) calculated from observed counts over thirty-minute intervals following two doses of icilin or DMSO administered intraperitoneally (i.p.) or intracisternally (i.c.m.).

Route of Delivery	Dose	Rats (n)	Rate of WDS per Interval 0-30	30-60	60-90	90-120	Total Observation
i.p.	0.25 mg	4	3±0.6*	3.4±0.3*†	2.8±0.3*†	1.5±0.5*	2.7±0.2*†
	0.025 mg	4	0.1±0.05	0.3±0.04*	0.1±0.03	0	0.1±0.01*
	DMSO	5	0.08±0.02	0.01±0.01	0	0	0.02±0.01
i.c.m.	0.25 mg	4	2.2±0.3*	1.9±0.3*	1.2±0.3*	0.6±0.1*	1.5±0.2*
	0.025 mg	5	0.6±0.1*†	0.2±0.06	0.1±0.02	0.1±0.02	0.2±0.06*
	DMSO	4	0.03±0	0.03±0	0	0	0.04±0.004
	Total	26					
Chi squared values (Kruskal-Wallis test, df = 5, $p \leq 0.001$)			22.03	23.27	20.43	20.37	22.99

* Significant difference between icilin dose and DMSO vehicle value of same delivery type. † indicates significant difference between delivery type at the same dose. Significance is defined by $p<0.05$ (Mann-Whitney U test).

CHAPTER 4
EFFECTS OF NEUROPATHIC PAIN ON OPERANT RESPONSES TO THERMAL AND MECHANICAL STIMULATION

Neuropathic Pain

Despite the clinical prevalence of orofacial pain, basic research relating to trigeminal nociception is relatively limited as compared to other somatosensory systems in the body. The chronic constriction injury (CCI) is one model that has been used to mimic neuropathic pain states observed clinically, originally applied to the sciatic nerve (Bennett and Xie, 1988). CCI was first adapted for the trigeminal nerve by Vos and colleagues (Vos et al., 1994), and has been used by a handful of other groups to examine peripheral and behavioral changes associated with this injury (Imamura et al., 1997; Benoliel et al., 2001; Deseure and Adriaensen, 2004; Chichorro et al., 2006), as well as the effect of novel analgesics (Benoist et al., 1999; Chichorro et al., 2006; Lim et al., 2007; Ling et al., 2008). However, behavioral assessments in CCI-treated animals primarily rely on reflexive withdrawal and assessments of grooming behavior. Only three studies have used an operant assessment of pain following sciatic CCI (Vierck et al., 2005; Jabakhanji et al., 2006; Walczak and Beaulieu, 2006). In this chapter, the effect of bilateral CCI on operant behavioral measures is assessed.

Once we established the pattern of behaviors observed following CCI, we determined the effect of treatment with gabapentin and pregabalin on those behaviors. Gabapentin was initially used as an antiepileptic, then later found to be effective as an analgesic and was approved for the treatment of neuropathic pain (labeled Neurotin) (Wheeler, 2002). Despite the fact that it is an analog of the inhibitory molecule gamma-aminobutyric acid (GABA), it does not act on GABA receptors (Jensen et al., 2002). Pregabalin is a derivative of gabapentin, also approved for treating neuropathic pain (labeled Lyrica) (Selak, 2001). Both gabapentin and pregabalin bind and block the activity of alpha 2 delta subunit of voltage gated calcium channels at the first

synapse in the dorsal horn (Gee et al., 1996; Field et al., 2006). Following neuropathic injury, ectopic discharge of injured neurons contributes to the upregulation of the alpha 2 delta 1 subunit (Boroujerdi et al., 2008), which contributes to pain maintenance by increasing the excitability of neural activity within the dorsal horn (Li et al., 2006). In the current study, we demonstrate that both gabapentin and pregabalin improve success in the operant task with cold stimulation.

Methods

Induction of Neuropathic Pain, Monitoring Recovery, and Behavioral Testing

Rats were trained and tested with single thermal and mechanical stimuli as described in Chapter 2. Following training and baseline behavioral testing, rats received either a bilateral chronic constriction injury (CCI) or sham operation of the infraorbital portion of the maxillary trigeminal nerve. These surgeries were performed intraorally, as previously described (Imamura et al., 1997), with some modification. We chose this route rather than the more common external surgical site so that the incision site would not be within the stimulated portion of the skin. Briefly, rats were deeply anesthetized with a ketamine/xylazine cocktail (2:1, 8mg/kg ketamine, 4mg/kg xylazine, 1.2ml/kg cocktail). For local anesthesia, xylocaine (2%, 1:100,000 epinephrine) was applied to reduce operative sensation and for hemostasis. An incision was made in the buccal vestibule, beginning from the hard palate and extending approximately 1 cm rostrally, roughly parallel with the lip. The nerve was exposed from surrounding tissues by blunt dissection and gently elevated with a hooked instrument so that two ligatures (5-0 vicryl sutures) could be tied securely around the nerve. This procedure was the same for sham surgeries, except that sutures were passed twice under the nerve, but not tied. This process was repeated for the second side. All incisions were closed with 2-4 ligatures (5-0 vicryl suture).

Rats' weights, general behavior, facial swelling and scratching were monitored for one week after the surgeries to track the progress of recovery and note signs of excessive grooming

to the innervated area. A subjective swelling severity score (none, mild, moderate, severe; Table 4-2) was used to grade the amount of swelling in the orofacial region affected by surgical treatment. The location and extent of scratching within the innervated area was also noted. Behavioral testing began after the one week recovery period. Four sets of surgeries were conducted, for a total of 22 rats assigned to each treatment submitted for single stimulus testing. Following surgical treatment and recovery, rats were tested with one of the following weekly schedules: (1) three times with 10ºC and once with 37ºC; (2) once with rough mechanical, twice with 10ºC, once with 37ºC, and one with 48ºC; or (3) once each with rough mechanical, 10, 37, and 48ºC per week. One surgical group (n = 5 per treatment) was submitted for stimulus preference testing before and after surgical treatment. Fasting occurred three times per week on the nights prior to 10 and 48ºC stimulation, but not prior to 37 ºC, or mechanical simulation.

Evaluation of Innate and Aversive Behaviors during Operant Testing

We established a behavioral scoring system to assess additional innate and aversive behaviors occurring during the first five minutes of operant testing. Five innate behaviors (facial grooming with forepaws, facial grooming with hindpaws, forepaw shaking, head shaking, and wet dog shaking) and three aversive or aggressive behaviors (head tilting, wiping or pushing at the stimulus, and biting at the stimulus or sipper tube) were each given one point for a maximum possible combined score of 8 for the five minute period. We chose to assess the first five minutes of testing rather than the whole testing period because untreated rats attend to task completion immediately and typically do not engage in grooming or innate behaviors until they completed at least one bout of drinking. This and all behavioral testing described above was assessed by a treatment-blinded investigator.

Evaluating the Effect of Surgical Treatment, Novelty, and Pregabalin on Thermal Preference

One group of rats ("experienced") was trained and submitted for thermal preference testing both before and after surgical treatment (see above), rendering them experienced with this task before and after pain induction. Another group of rats ("inexperienced") was not exposed to the thermal preference task until after surgical treatment and four weeks of single stimulus testing, primarily with 10 and 37°C stimulation. By this time, CCI-treated rats in this group no longer exhibited allodynia, nor did they suffer from heat hyperalgesia. At five weeks post-surgery, this group was first exposed to the neutral (37/37°C) place preference task for two sessions, then evaluated for thermal preference with 10 and 48°C stimulation twice with two days separating each exposure (hot and cold sides were switched). Preferences with each surgical group were consistent and therefore the two days were pooled. At six weeks post-surgery, they were evaluated for thermal preference again following treatment with pregabalin (45 minutes prior, 10mg/kg i.p.). Both the experienced and inexperienced groups consisted of CCI-, sham-, and un-treated rats.

Histology

Tissue preparation

Rats were euthanized by isoflurane asphyxiation and decapitation at several post-operative time points (2, 5, 6, 7, and 12 weeks). The infraorbital trigeminal nerves and trigeminal ganglia were dissected free and post-fixed in 10% neutral buffered formalin for 48 hours before being transferred to 70% ethanol. Tissues were embedded in paraffin blocks and 10μm sections were cut with a microtome and mounted on slides. At least two non-adjacent sections, separated by 50μm, were taken from each pair of nerves and ganglia. Tissues were de-parafinized and rehydrated in a graded series of alcohol washes into PBS. Nerves were stained

with Gill's hematoxylin and eosin Y (Fischer, Kalamazoo, MI) to visualize tissue for evaluation of inflammation as described below. Immunohistochemistry was performed on sections of trigeminal ganglia sections to visualization TRPV1 and TRPM8 proteins and quantify immunoreactivity.

Immunohistochemistry

Target retrieval was used for immunostaining of TRPV1 only; it interferes with TRPM8 immunostaining. Sections were incubated in 100ml target retrieval solution (Dako Cytomation North America, Carpinteria, CA) for 15min in boiling water, then incubated in an oven overnight at 60°C overnight. On the second day, sections were cooled and washed with 0.1% Tween20 in PBS. They were then blocked with 10% normal goat serum (NGS) for 30 minutes at room temperature, and incubated with rabbit anti-VR1 antibody (1: 500, Affinity Bioreagents; Golden, CO) in 5% NGS in 0.1% Tween20 in PBS (the antibody diluent) overnight at 4°C. This process was the same for immunostaining of TRPM8 with the following changes: target retrieval was not performed and the chicken anti-TRPM8 antibody (1:500, Aves, Tigard, OR) was incubated for two days at 4°C.

After incubation in the primary antibody, sections were washed and incubated with the appropriate secondary antibody in the antibody diluent at 1:1,000 (for TRPV1; goat anti-rabbit IgG AlexaFluor 488 and for TRPM8; goat anti-chicken IgY Alexa Fluor; Invitrogen). Slides were then washed in PBS and coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burliugame, CA). Immunostained sections of trigeminal ganglia were viewed with a Micro-Leica DMLBZ microscope at 200x magnification, and images were captured using the Q Imaging Micropublisher 5.0 camera and Q Capture Pro software. The area of the trigeminal ganglion innervated by the infraorbital nerve was surveyed and cell counts were made using three representative images from each ganglion from each individual (n = 6 per rat). While no

difference was observed in the percentage of TRPM8 positive cells, a difference in the intensity of TRPM8 staining between treatment groups was noted qualitatively (contrast conditions were the same for all groups). Thus additional images (n = 6 per rat) consisting of TRPM8 positive cells were taken and the intensity of staining was analyzed using ImageJ 1.4g (National Institutes of Health).

Assessment of nerve inflammation and injury

Assessment of nerve inflammation and injury was made by a blinded investigator. Two longitudinal, non-adjacent sections of the left and right nerves from each individual were assessed in the approximate surgical area. A scoring system was used to assess inflammation and injury to the nerve (Figure 4-1), and the presence of suture and foreign body reaction (giant cells) was noted. Each of the four sections per rat received an inflammation score and an injury score. These were then added to produce a composite score for each rat (maximum of 24), used for statistical analysis. If suture and foreign body reaction was noted in any of the four sections, then the rat was considered to have suture.

Statistical Analysis

For analysis of operant data following surgical treatment, outliers were first removed from all data sets using a box plot analysis (SPSS, v.16), on an outcome by outcome basis. Outliers are defined as any value beyond one and a half times the interquartile range. General linear models were used to assess the effects of surgical treatment, time and their interaction on operant outcome measures and behavior scores for all stimuli tested, the effects and interaction of drug and surgical treatment on operant responses to 10°C, as well as scores assessing inflammation, percentage of TRPV1 expression, and TRPM8 intensities. One way- Analysis of Variance (ANOVA) was used to assess the effect of time within each surgical group, followed by post-hoc Tukey's test. Unpaired t-tests were used to make comparisons between behavioral

outcome measures of CCI- and sham-treated rats at each post-operative time point. One way ANOVA was also used to determine significant effects of experience and pregabalin treatment on thermal preference following surgical treatment. Statistical significant was set at $p<0.05$ for all analyses.

Results

General Observation of Immediate Post-Surgical Recovery and Behavior

All rats' weights were recorded before surgery and monitored daily for one week after surgery to track recovery. CCI-treated rats lost an average of 3% of their body weight at 24hrs post-op, but recovered to their preoperative weights or greater by the seventh post-operative day. Most sham-treated rats lost less than 3% or maintained their pre-operative weight at 24hrs post-surgery. Those shams that did lose more than 3% of their pre-operative weight gained it back by the fourth post-operative day. Although CCI-treated rats had a trend for lower body weights than their sham-treated counterparts, no significant differences were observed between the two groups.

All operated rats exhibited moderate to severe swelling in the surgical region for the first two post-operative days, which was more severe and longer lasting in CCI-treated rats than in their sham-treated counterparts. It took six days for swelling to disappear in half of the CCI-operated rats (Table 4-1). In contrast, it took only three days for swelling to disappear in half of Sham-operated rats (Table 4-1). Swelling was completely gone in all CCI-operated rats by seven to nine post-operative days and in all sham-operated rats by six post-operative days.

All surgically treated rats were observed engaging in isolated facial grooming on recovering from anesthesia. In particular, some CCI-treated rats would swat at the rostral portion of their faces with their forepaws, in a motion reminiscent of normal face washing, but without contact to the face and often interspersed with forepaw shakes.

Some CCI-treated rats were also observed jerking or jumping as if in response to little or no apparent facial contact with their environment. These behaviors might be indicative of spontaneous pain, but jerking and jumping were not observed consistently after the first two postoperative days. Scratching was noted in the surgical area of 15 CCI-operated rats, and in 9 sham-operated rats (n=19/treatment). This self-injurious behavior was more persistent in the CCI-operated rats. However, these injuries were superficial and healed by the end of the one week recovery period.

Effect of Surgical Treatment on Operant Responses to Cold, Neutral, and Hot Facial Stimulation

Operant responses to 10, 37, and 48°C stimulation were assessed pre and post-operatively and the effects of treatment, time, and their interaction were evaluated using a general linear models (Figure 4-2 inset table). For both 10 and 37°C stimulation, general linear models revealed only a significant effect of time on licks, and within group ANOVAs indicated that these effects were only present in the sham-treated rats, not their CCI-treated counterparts. The increases within the sham group at two, three (10°C), and four weeks (37°C) post-surgery are likely the consequence of normal appetitive increase that occurs with time (Figure 4-2A, diamonds and squares). For 48°C stimulation, general linear models revealed a significant effect of treatment and time, and a significant interaction between the two on licks (Fig. 2 inset table). However, no within group differences from baseline were revealed, nor were any between group differences at any of the given post-operative time points (Figure 4-2A, circles).

For 10 and 37°C stimulation, general linear models reveal significant effects of treatment and time on stimulus contacts, as well as significant interactions between the two (Figure 4-2, inset Table). Within group differences revealed that the contacts of CCI-treated rats were significantly greater than baseline from one to three weeks post-surgery, with between group

differences also apparent in this time frame (and at four weeks for 37°C stimulation). There were no within group differences for sham-treated animals (Figure 4-2B, diamonds and squares). In contrast, for 48°C stimulation a general linear model revealed only a significant effect of time on stimulus contacts, but was not supported by within group analysis (Figure 4-2, inset Table). The only significant difference in stimulus contacts with 48°C stimulation was between the treatment groups at four week post-surgery (Figure 4-2B, circles). Taken together, these finding indicate that following CCI-treatment, rats contact the 10 or 37°C stimulus more frequently in order to obtain the milk reward. This effect is not observed in sham-treated rats, nor is it the case for 48°C stimulation.

For 10°C stimulation, a general linear model revealed a significant effect of treatment and time, as well as a significant interaction on success ratios (Figure 4-2, inset Table). CCI-treated rats had lower success ratios at one and two weeks post surgery, and between group differences were apparent at all post-operative time points (Figure 4-2C, diamonds). For 37°C stimulation, a general linear model revealed a significant effect of treatment on success ratios, no significant effect of time, but a significant interaction between the two (Figure 4-2, inset table). CCI-treated rats had lower success ratios for all post-operative time points, with between group differences apparent as well (Figure 4-2C, squares). For 48°C stimulation, a general linear model revealed the same pattern of significance as with 37C stimulation. However, there were no difference from baseline within either treatment group and only between group differences observed at two and three weeks post-surgery (Figure 4-2C, circles). Taken together, these findings indicate that CCI-treated rats are less successful at obtaining the milk reward in the presence of 10 and 37°C stimulation, but task completion with 48°C stimulation is not rendered much more difficult with injury than without.

In summary, for 10 and 37°C stimulation, the lack of an injury effect on licks coupled with a post-injury increase in stimulus contacts (i.e. reward attempts) indicates that the CCI-treated rats must try harder post-operatively, to obtain the same amount of reward they could pre-operatively. As a result, their post operative success ratios are reduced. These effects are specific to CCI treatment and are not observed with sham injury. In contrast, for 48C stimulation, changes in licks, stimulus contacts and success ratios were not observed following injury. Therefore, CCI treatment was able to induce allodynia to cold and neutral stimulation, with maximal cold allodynia observed two weeks post-injury and lasting allodynia to neutral stimulation following injury. It was not able to induce heat hyperalgesia.

Effects of Surgical Treatment on Operant Response to a Rough Stimulus

The findings with respect to neutral stimulation could be the consequence of mechanical, rather than thermal allodynia. Therefore, surgically treated rats were also tested pre- and post-operatively with a rough stimulus to assess mechanical sensitivity. No preoperative differences in operant responses were observed between future sham- or CCI-treated rats A general linear model revealed significant effects for treatment, time, and significant interaction between the two (Figure 4-3, inset Table). There were no significant effects or interaction for licks, while there were significant effects and interaction for bouts and licks per bout (Figure 4-3, inset Table). Within-subjects analysis revealed no significant effect of time on any outcome measure in the sham-treated rats. In contrast, CCI-treated rats exhibited significantly elevated bouts at one week post-surgery as compared to baseline, and significantly reduced licks per bout at one week post-surgery as compared to all other time-points (Figure 4-3 B,C). Between-group differences were also apparent for these time points. Taken together, these findings indicate that neuropathic injury produced a transient allodynia to the brush of a rough stimulus.

Effect of Surgical Treatment on Innate and Aversive Behaviors in the Presence of Thermal and Mechanical Stimulation

In addition to the operant outcome measures, general behavior in the testing box was observed for each session. Early in post-operative testing, it be came apparent that many of the CCI-operated rats were exhibiting behaviors likely indicative of stimulus-specific pain. In particular, many CCI-operated rats adopted an upward head-tilt, not observed in their sham-operated counterparts during this time period (Figure 4-4, Objects 4-1 and 4-2). Rats exhibiting this strategy would initially place their faces with the dermatome innervated by ION against the thermode as they had been trained, but as contact with the 10°C thermode continued, the rat would tilt his head upward, shifting contact to more caudle portion of the face not innervated by the constricted portion of the nerve. Early in testing some individuals exhibiting this head tilt would jerk back on first contact. This head tilt was also accompanied by paw wiping, pushing, or in some cases biting at the thermode.

A behavioral scoring system was used to assess the occurrence of innate and aversive behaviors occurring in the presence of the thermal and mechanical stimuli during the first five minutes of operant testing. In order to establish a normal pattern of scores for the stimuli tested, five naïve rats were scored along with surgically treated rats. There were no significant effects of time on the innate, aversive, or combined scores of the naïve rats, thus the data were pooled for each stimulus for comparison across stimuli (Table 4-2). All scores were significantly greater in naïve rats with 10°C stimulation than with 37 or 48°C. For the innate component this is likely due to the greater frequency of head and wet-dog shaking that occurs with 10°C stimulation (Table 4-2). Head tilting did not occur in naïve rats, but thermode wiping was observed, particularly with 10°C and rough stimulation (Table 4-2). Biting did not occur with thermal stimulation, but was observed with the rough stimulus. Effort was made to distinguish

between exploratory nibbling and aversive biting of the velcro, but false identification of a bite as aversive may still account for these responses in naïve rats.

We also assessed changes in the behavior scores following surgical treatment. General linear models revealed a significant effect of treatment on the innate score with 10 and 48°C stimulation, but not 37°C or rough mechanical stimulation (Figure 4-5 inset table). Post-hoc analysis revealed that CCI-treated rats exhibited greater scores than their sham-treated counterparts at one and three weeks post-surgery with 10°C stimulation, and at one and two weeks with 48°C stimulation (Figure 4-5 A). General linear models also indicated there were no significant effects of time with any of the stimuli, although within-subjects analysis revealed that CCI-treated animals also had significantly greater innate scores with 10°C stimulation at one and three weeks relative to pre-surgical scores (Figure 4-5 A). There were no significant within-subjects effects for sham-treated rats with any stimulus. There was also a significant interaction between treatment and time with 10°C stimulation only. Taken together, these findings indicate that CCI-treatment can intermittently increase the occurrence of innate behaviors with cold stimulation, but does not substantially alter innate responses with 37, 48°C, or rough mechanical stimulation.

For the aversive component of the behavioral score, general linear models revealed a significant effect of both treatment and time, as well as a significant interaction for all stimuli (Figure 4-5 inset table). Post-hoc analysis revealed that CCI-treated rats had significantly greater aversive scores than their sham-treated counterparts for all post-operative time points and all thermal stimuli, and from one to three weeks for rough stimulation (Figure 4-5 B). Within-subjects analysis revealed that aversive scores were significantly greater in CCI-treated rats post-operatively and not changed in sham-treated animals. Taken together, these findings indicate

that aversive behaviors are increased following injury for all of the stimuli tested. Closer examination of the aversive behaviors indicates that this increase in score is due primarily to the induction of head tilting behavior following surgical treatment and to a lesser extent an increased frequency in thermode wiping, regardless of stimuli (Table 4-3). Head tilting occurs more frequently and persistently in CCI-treated rats than their sham counterparts. Thermode wiping occurs in both groups post-operatively, although this behavior is generally more frequent in the CCI-treated animals. Biting never occurred with 37°C stimulation and rarely with 10 or 48°C stimulation.

In conclusion, nerve injury was able to produce a persistent occurrence of aversive behaviors with all stimuli tested, and only transiently effected innate behavior exhibited with cold stimulation. These findings are in agreement with the decreased success observed in the operant task with 10 and 37°C stimulation. However, the robust induction of aversive behaviors with 48°C stimulation would appear to contrast with the lack of effect on success in the operant task. This may suggest that the changes within peripheral and subcortical structures following injury do not lead to a substantial change in operant response to a stimulus that is already noxious.

Effect of Surgical Treatment on Nerve Inflammation and Injury

Nerves from CCI- and sham-treated rats were assessed for degree of inflammation and nerve injury. The knots of the vicryl ligatures were visibly present only around nerves taken two weeks post-surgery (Figure 4-6 A); at all later time points suture material was identified microscopically (Figure 4-6 B). Of the two sham-treated rats with suture present (Figure 4-6 inset table), one scored unusually high and appeared as an outlier when the scores were submitted to box plot analysis. A re-examination of this individual's behavior revealed a consistent occurrence of head tilting and reduction in success ratios with all stimuli post-

operatively. This individual was therefore excluded from the sham treatment group for subsequent analysis. For nerves taken at 2-7 weeks post-surgery, a general linear model was used to assess effects of and interaction between treatment and time on the composite score. There was a significant effect of treatment ($F_1 = 15.217$, $p = 0.001$), but no significant effect of time ($F_3 = 0.272$, $p = 0.845$) and no significant interaction ($F_3 = 0.404$, $p = 0.752$). Thus, all nerves collected at 2-7 weeks have been pooled to illustrate the difference between each group (Figure 4-6 inset table). Injury, inflammation, and composite scores were all significantly greater in constriction injured nerves taken within 2-7 weeks post-surgery as compared to time-matched sham treated nerves or nerves taken at 12 weeks post-surgery of either surgical treatment (Figure 4-6 inset table). As a negative control, nerves from two naive individuals were included and consistently assigned scores of zero for all assessments and declared "healthy" by the blinded investigator.

Effect of Surgical Treatment on TRPV1 and TRPM8 expression at Two Weeks Post-Treatment

Trigeminal ganglia from CCI- and sham-treated rats were taken at two and seven weeks post-surgery and sections were either stained with antibodies against TRPV1 or TRPM8. Assessment of immunoreactivity was restricted to the area innervated by the infraorbital (treated) region of the trigeminal nerve. An increased percentage of TRPV1 positive cells were noted in ganglia taken at two weeks post-CCI, as compared to all other treatment groups, even ganglia taken seven weeks post-CCI (Figure 4-7). While no changes in the percentage of TRPM8 positive cells was noted, the intensity of TRPM8 staining was greatest in ganglia taken two weeks post-CCI compared to all other treatment conditions (Figure 4-8). Ganglia from CCI-treated rats also exhibited more intense TRPM8 staining than time-matched sham tissue at seven weeks post-surgery. Taken together, these findings indicate that TRPV1 expression is detectable

in more cells following CCI, but this expression wanes between two and seven weeks post-surgery. TRPM8 expression may also be up-regulated in cells that normally express the channelor the protein may be more concentrated on the plasma membrane following CCI. Peak intensity of TRPM8 staining also occurs at two weeks post surgery, and while it wanes by seven weeks, is still significantly greater than sham at this time point.

Effect of Pregabalin and Gabapentin Treatment on Operant Responses to Cold Facial Stimulation in Surgically-Treated and Untreated Rats

A subset of rats was treated with pregabalin (10 mg/kg), gabapentin (30mg/kg), or vehicle (water) and tested with 10ºC stimulation at two week post-surgery, when cold allodynia is maximal. To control for drug effects in the absence of surgical treatment, naïve rats were also included for each drug treatment. A general linear model revealed significant effects of surgical treatment on stimulus contacts and success ratios, but not licks, as was reported above. There was also a significant effect of drug treatment on all three out come measures and a significant interaction between surgical and drug treatment for stimulus contacts and success ratios (Figure 4-9, inset table).

There was a significant effect of gabapentin on licks in surgically treated and naïve rats relative to no drug treatment, and a significant effect of pregabalin on sham-treated and naïve rats relative to no drug treatment (Figure 4-9 A). Within group effects of drug treatment on stimulus contacts were near the significance cut off for CCI- and sham-treated rats ($p = 0.05$ and 0.047 respectively), and were not significant for naïve rats (Figure 4-9 B). Between-group effects on stimulus contacts were not observed for any drug treatment. Within group effects revealed a significant effect of gabapentin on success ratios for both surgically treated and naïve rats, and a significant effect of pregabalin on success ratios for surgically treated rats only. There were no significant between group effects on stimulus contacts (Figure 4-9 C).

Taken together, these data suggest that both gabapentin and pregabalin can enhance successful task completion in surgically treated rats, and gabapentin can also enhance task completion in naïve rats. The elevation in licks with drug treatment suggests that some of these effects may be appetitive in nature, particularly with respect to the sham-treated and naïve rats. However the trend for decreased stimulus contacts in CCI-treated rats with drug treatment suggests that the significant increase in success ratios observed in this group is the result of drug-related analgesia. The significant pregabalin effect observed in sham-treated rats may also indicate that although cold allodynia is not present in this group, the less-severe injury is sufficient to cause changes that render them sensitive to the effects of the drug. In conclusion, we demonstrate that gabapentin and pregabalin are able to ameliorate the cold allodynia experienced by CCI-treated rats and that effects in sham-treated and naïve rats indicate affective or appetitive effects of these drugs on operant behaviors with a 10ºC stimulus.

Effect of Surgical Treatment on Mechanical Preference

When the effect of time on mechanical preference was evaluated for each surgical group, no significant effect of time was observed for licks or testing duration for either stimulus. Thus, all post-operative time points were pooled to compare the effects of treatment on licks and duration for each starting stimulus condition. As with naïve rats in chapter 2, preference was influenced by the starting stimulus in surgically treated rats. For both CCI and sham-treated rats, soft stimulation was greatly preferred when this was the starting stimulus (Table 4-4). However, when rough stimulation occurred first, soft preference occurred more consistently among CCI-treated rats post-operatively, than among sham-treated rats (Table 4-4, parenthetical values). Of the three groups, CCI-treated rats exhibited a soft stimulus preference the most frequently (Table 4-4).

When rough stimulation occurs first, CCI-treated rats exhibited a pronounced preference for soft stimulation not observed in sham or naïve rats. Interestingly, CCI-treated rats also had significantly greater total licks and significantly lower unstimulated time relative to sham-treated and naïve rats (Figure 4-10 A,B). When soft stimulation occurs first, all three surgical groups exhibit a strong preference for the soft stimulus. In this case, CCI-treated rats also exhibit significantly greater total licks than naïve rats (Figure 4-10 C). They also spent significantly more time on the soft stimulus and significantly less time unstimulated as compared to naïve rats (Figure 4-10 D). These findings indicate that CCI-treated rats may have a more pronounced aversion to the rough stimulus post-operatively than do sham or naïve rats. The difference in total licks and unstimulated time likely indicates that the increased drive in CCI rats to avoid rough stimulation leads to increased exploration and more reward intake than the other two groups not strongly motivated away from the rough stimulus. It is also possible that the chronic pain state experience by CCI-treated rats may enhance sensitivity to reward.

Effect of Surgical treatment on Thermal Preference and the Influence of Task Novelty and Drug Treatment

To evaluate the effect of surgical treatment on thermal preference (10 and 48°C) when cold allodynia is maximal (two weeks post-surgery), one group of rats was evaluated both pre- and post-operatively, along with naïve controls. It was expected that the pronounced cold allodynia and lack of heat hyperalgesia exhibited by CCI-treated rats in the single stimulus task might mean that CCI-treated rats would avoid 10°C and preference to 48°C at two weeks post-surgery. However, this was not the case in this experienced group of rats. CCI-treated rats still exhibited a preference for 10°C stimulation, also demonstrated by sham-treated and naïve rats (Figure 4-11). Also contrary to expectation was the decrease in unstimulated time exhibited by CCI-treated rats as compared to both other treatment groups. The pattern of stimulus contacts

and success ratios, as well as the presence of aversive behaviors indicate that CCI-treated rats were suffering from pain (Figure 4-12). The lack of a substantial influence of CCI-treatment on preference suggests either that 10°C in a neuropathic state is still more desirable than the noxious 48°C stimulus, or that experienced CCI-treated rats are able to modulate their behavior to get the most reward with a tolerable degree of pain.

A second, inexperienced group underwent novel thermal preference testing at five weeks post-surgery, when pain was no longer evident in the CCI-treated rats. In contrast to the experienced CCI-treated rats, inexperienced CCI-treated rats did not exhibit a thermal preference, while their sham-treated counterparts exhibited a slight preference and naïve rats exhibited no difference in preference from their experienced counterparts (Figure 4-11). This suggests that although CCI-treated rats may no longer exhibit signs of pain in the single operant task, preference can be influenced by previous pain. When inexperienced rats were treated with pregabalin, cold preference was enhanced in both CCI-treated and naïve rats, but not affected in their sham-treated counterparts (Figure 4-11). This would suggest that pregabalin may be able to modulate affective pain processing in CCI-treated and naïve rats with respect to cold stimulation. The lack of effect in sham-treated rats could be due to a subtle deficit in sensory discrimination or alteration in affect towards cold and heat that may accompany surgical treatment.

Discussion of Neuropathic Pain and Operant Behavior

We show here that rats receiving CCI of the trigeminal nerves develop pronounced sensitivity to 10°C stimulation that is maximal at two weeks post-surgery. They also exhibit a robust, long term allodynia to 37°C stimulation, which was somewhat unexpected and did not follow the pattern of behavior exhibited toward the rough mechanical stimulus. This sensitivity is indicated by both decreased success with the operant task and persistent occurrence of aversive behaviors in the presence of these stimuli. In contrast, CCI-treatment did not hinder successful

task completion in the operant task with 48°C stimulation, even though aversive behaviors persisted. Sham-treated rats did not demonstrate lasting changes in operant performance and aversive behaviors were observed transiently in some individuals. We also show here that treatment with pregabalin and gabapentin can alleviate cold allodynia in neuropathic animals, and also produce effects in sham-treated and naïve rats suggestive of affect-modulation. While the poor success in the single stimulus task was transient for rough stimulation, CCI-treated rats still exhibited a pronounced preference for soft stimulation not shown in sham or naïve rats. Finally, experience with the thermal preference task led to a nearly normal pattern of preference in CCI-treated rats, despite signs that they were experiencing allodynia. In contrast, inexperienced CCI-treated rats who had previously been in pain exhibited a lack of thermal preference, despite the fact that their sham and naïve counterparts did exhibit a preference. Pregabalin was able to "reset" normal preference in CCI-treated rats.

Effects of CCI on Behavioral Responses to 10, 37°C and Mechanical Stimulation

According to the current body of literature, CCI to either the trigeminal or the sciatic nerve is typified by allodynia and/or hyperalgesia to all stimulus modalities, as established by measuring reflexive withdrawal or assessment of grooming behavior evoked by stimulation applied by the experimentor. For cold stimulation, this could involved a cooling compound such as acetone (Chichorro et al., 2006; Walczak and Beaulieu, 2006; Lim et al., 2007), or a cold floor (Bennett and Xie, 1988; Allchorne et al., 2005). Cold allodynia has also been demonstrated following both unilateral and bilateral CCI using operant methods based on time spent escaping cold stimulation (Vierck et al., 2005; Jabakhanji et al., 2006; Walczak and Beaulieu, 2006). Particularly, the findings of this study are in agreement with that of Vierck and collegues, who reported both post-operative increases in lick-guard responses and escape duration with cold stimulation following a bilateral sciatic CCI.

However, our findings with respect to 37°C stimulation were somewhat surprising. Neuropathic pain studies typically focus on cold allodyna, heat hyperalgesia, and mechanical allodynia (assessed typically with Von Frey filaments); most do not even test responses to thermal stimuli at body temperature, or don't report it if they do. At least one other group reported reduced latency to withdraw from 38°C stimulus in the streptozotocin model of diabetic neuropathy (Beyreuther et al., 2006) and in a model of chemotherapeutic pain (Beyreuther et al., 2007). The fact that these animals and our injured animals experience alterations in mechanical sensitivity may still indicate that this effect is primarily a consequence of mechanical allodynia. Dynamic mechanical allodynia is a common feature of neuropathic pain (Bowsher, 2005; Lang et al., 2006; Samuelsson et al., 2007) and also a consequence of animal injury models (Field et al., 1999), usually assessed by brushing a soft bristled brush or cotton swab across the affected skin. The lack of a prolonged decrease in success with rough stimulation suggests that the profound sensitivity to 37°C is not simply mechanical allodynia, but thermal allodynia.

Additionally, mechanical sensitivity does not explain the different behavior profiles exhibited with 10 or 48°C stimulation. It has been noted in normal human subjects that dynamic mechanical stimulation provided by a thermal probe can reduce pain sensations elicited by moderate cooling (termed innocuous cold nociception or ICN) and by menthol during ICN (Green and Pope, 2003; Green and Schoen, 2005; 2007), but it remains to be seen if such a phenomenon occurs in individuals suffering from neuropathic pain. The capacity for a delta or c fiber activity to mutually suppress evoked cortical potentials to the other fiber type (Tran et al., 2008) may contribute to this phenomenon. If this phenomenon of ICN is intact or only transiently disrupted following neuropathic injury, it could contribute to an apparent recovery of normal cold responses over time.

Another explanation for the lasting allodynia apparent with 37°C is that CCI-treated rats are hyper-reactive to the operant testing chamber because of repeated testing and first post-operative exposure to exquisitely painful stimuli. However, if that were the case we would likely expect that this exaggerated response would be apparent with the same time course for other stimuli and it is not. Also, CCI-treated rats continue to make attempts to obtain reward, even without fasting (as is the case with 37°C and rough stimulus testing), so we may rule out a decrease in the hedonic value of sweetened condensed milk in the presence of pain.

Effects of CCI on Hot-Mediated Behaviors

Withdrawal from radiant heat is typically enhanced following unilateral sciatic CCI (Bennett and Xie, 1988), which has also been demonstrated following unilateral CCI of the trigeminal nerve (Imamura et al., 1997). In contrast, in a study comparing unlearned behaviors and operant behaviors in response to heat, bilateral CCI of the sciatic nerves did not produce increased lick-guard to or escape from nociceptive heat (up to 47°C) (Vierck et al., 2005). The operant responses to 48°C reported are in agreement with Vierck and collegues; there was no significant effect of bilateral CCI-treatment on success ratios post-operatively (Vierck et al., 2005). In contrast to Vierck and collegues, we did observe a persistent post-operative elevation of unlearned aversive behaviors in the CCI-treated rats and a transient elevation in these behaviors within sham-treated rats post-operatively (Vierck et al., 2005).

It is possible that the aversive behaviors reflect an exquisite sensitivity to the smooth metal thermode, rather than a response to the heat of the stimulus. However, enhancement of withdrawal to radiant heat, which lacks a mechanical component, has been observed following trigeminal CCI (Imamura et al., 1997; Liang et al., 2007). Both the aversive behaviors exhibited here and reflexive withdrawal directly measure segmental processing of painful stimulation, whereas the operant assessment reflects a direct measure of cortical integration and assessment

of signals from the periphery. Thus the differences observed between unlearned and operant behaviors here may indicate that there is an enhancement of heat nociception at the level of the brainstem, but this enhancement does not result in cortical changes necessary to impair successful performance of the operant task with respect to painful heat.

The different effect of neuropathic injury on cold versus heat related operant behaviors may be related to differential gating of these stimuli by the thalamus. Lesioning various thalamic nuclei can induce transient analgesia with differing efficacies to different stimulus modalities in a neuropathic state (Saadé et al., 2006) and patients with specific thalamic lesions have been shown to exhibit impairments in cold sensitivity, but normal heat perception (Kim et al., 2007). The lack of effect on heat-mediated operant behaviors could also be related to the convergent processing of the affective, cognitive, and intensity aspects of painful stimulation. For both cold and heat testing the rats are fasted to motivate performance. In the case of heat stimulation the neuropathic injury does not appear to conflict sufficiently with the drive to satisfy hunger, while cold stimulation does. Perhaps if the rats were not fasted prior to heat testing, we might observe a robust difference between sham-treated and CCI-treated rats in the operant task. We have also shown that noxious heat is preferred over noxious cold in intact rats (Rossi et al., 2006).

Effects of CCI on Cold-mediated Behaviors and TRPM8 Expression

The peak in cold sensitivity at two weeks post surgery exhibited by CCI-treated rats is accompanied by an increased intensity, although not increased cell counts of TRPM8 immunoreactivity in the trigeminal ganglia. TRPM8 has a primary role in the perception of both innocuous and painful cold stimuli (McKemy et al., 2002; Peier et al., 2002), however, results are mixed with respect to the role of this channel in the development of cold sensitivity in a neuropathic state. Some have shown that TRPM8 mRNA expression is not changed at one or two weeks (Obata et al., 2005; Katsura et al., 2006) following spinal nerve ligation in rats, nor is

protein expression at one week (Katsura et al., 2006). In contrast, others have shown that TRPM8 mRNA (Frederick et al., 2007) and protein expression are increased in DRGs and in the dorsal horn of the spinal cord ipsilateral to sciatic CCI at two weeks post-injury (Proudfoot et al., 2006).

The increased intensity of TRPM8 immunoreactivity we observed following CCI could be reflective of increased TRPM8 expression observed by others (Proudfoot et al., 2006; Frederick et al., 2007). However, the lack of change in total number of TRPM8 positive cells would seem in contrast with the findings of Proudfoot and colleagues (Proudfoot et al., 2006). This discrepancy may be due to differences in antibody sensitivity and/or inclusion criteria for positive TRPM8 immunoreactivity. They report that TRPM8 immunoreactivity occurred primarily in c fibers (indicated by small, peripherin positive cells) of naïve DRGs (Proudfoot et al., 2006). In contrast we observe that TRPM8 is expressed in wide range of cell sizes in naïve TG, likely indicating a mix of positive c and a delta fibers. It has also been noted that there is a greater percentage of TG cells expressing TRPM8 mRNA than in the DRG (Kobayashi et al 2005), which could also underlie slight differences in our findings. An alternate explaination for the increased intensity of staining is that TRPM8 channels already present on cells become clustered together on the surface of the cell following injury, rather than being diffusely distributed within the plasma membrane.

Cold Sensitivity and TRP Expression

In addition to increased aversive behaviors to nociceptive heat, we observe an increase in TRPV1 immunoreactivity in CCI-treated rats at two weeks post-surgery. Most reports indicate an increased expression of either mRNA, protein, or both in DRG, TG, or spinal cord dorsal horn ipsilateral to the model injury (Ma et al., 2005; Wilson-Gerwing et al., 2005; Biggs et al., 2007). In particular a recent study indicated that TRPV1 is upregulated in uninjured neurons within the

ipsilateral TG following mental nerve transection, appearing more frequently in medium sized cells than in the absence of injury (Kim et al., 2008). Another study, however, did not observe any change in mRNA expression following unilateral CCI using a novel multiplex ribonuclease protection assay to assess expression of multiple TRP channels in a single sample (Frederick et al., 2007).

TRPV1 has been long implicated in the development of both enhanced heat nociception and mechanical allodynia following neuropathic injury. Several studies have shown alleviation of neuropathic pain symptoms following treatment with TRPV1 antagonists like BCTC (Pomonis et al., 2003) and capsazepine (Walker et al., 2003). However, such compounds are now known to also act on TRPM8 and may act on other TRP channels. More specific silencing of TRPV1 with antisense RNA has been shown to alleviate neuropathic pain, including cold pain (Christoph et al., 2006; Christoph et al., 2007). Another study also implicated TRPV1 in the development of cold allodynia following sciatic CCI; these authors observed an increase in neurons responding to both capsaicin and menthol (i.e. containing both TRPV1 and TRPM8) in ipsilateral DRGs (Xing et al., 2006; Xing et al., 2007). They also observed that these cells exhibited enhanced responses to menthol and cold stimulation, relative to what is observed in naïve DRG cells (Xing et al., 2007). Unfortunately, due to limitations in our staining procedures, we are unable to accurately assess co-labeling of TRPM8 and TRPV1 directly, but the appearance of TRPV1 immunoreactivity in larger cells that often exhibit TRPM8 reactivity may indicate an increase in co-labeling following CCI. The increased expression of TRPV1 and enhanced intensity of TRPM8 immunoreactivity correspond to the maximal enhancement of cold sensitivity at two week post-surgery, suggesting that both channels may play a role in peripheral sensitization to cold stimuli.

Effect of Drug Treatment on Cold Allodynia

We also demonstrate that pregabalin and gabapentin have a significant effect on operant behaviors (including success) not only for CCI-treated rats, but in sham-treated and naïve rats as well. In the CCI-treated rats, stimulus contacts following gabapentin and pregabalin were reduced by about half and a quarter respectively relative to both baseline and vehicle, although these do not reach statistical significance due to the greater variability in drug treated animals. In contrast, stimulus contacts were not changed in naïve rats. This suggests that these compounds, which are currently FDA approved for the treatment of neuropathic pain, do provide pain relief to injured animals. This also supports previous findings regarding the anti-allodynic potential of pregabalin (Ling et al., 2008; Tanimoto-Mori et al., 2008) and gabapentin (Ling et al., 2007) with respect to cold stimulation, evaluated using standard reflex testing in models of neuropathic pain and using a cold preference task (Walczak and Beaulieu, 2006).

There were significant effects of licks for both drug treatments in surgically treated and naïve animals, which suggests either that these drugs may have appetitive effects not previously noted or this may be a consequence of affect modulation. In studies that more closely resemble our paradigm, a lever press is paired with food reward and additional foot shock punishment in some trials, pregabalin (10 – 30mg/kg) significantly increase lever press during painful punishment (Field et al., 2001; Evenden et al., 2006). Both pregabalin and gabapentin (100mk/kg) treatment also decreased escape behavior in a fear conditioning assay (Nicolas et al., 2007). A recent animal model of post-traumatic stress disorder showed short term anxiolytic effects of pregabalin, in higher doses than presented here (100 & 300mg/kg) (Zohar et al., 2008). Gabapentin reduced both anxiety-like behaviors and increased mechanical withdrawal thresholds in rats with sciatic CCI, although unlike the current study, they did not observe anxiety-related effects in sham-treated animals (Roeska et al., 2008). This difference could be related to

differences in severity of the two sham procedures or differences in the stressfulness of the open arm test versus our facial stimulation task. In dental patients, pregabalin (600 mg oral) has also been shown to reduce anxiety within a 3-4 hour period following administration (Nutt et al., 2008). Thus our findings support animal models reporting anti-allodynic effects, as well as anxiolytic effects in both rodents and humans. The influence of pregabalin on affective pain judgment is also supported by our finding that it can re-establish normal preference in CCI-treated rats with minimal experience in a thermal preference task.

Modulation of Preference After Injury and Affective Aspects of Pain

Despite the fact that apparent sensitivity to a single rough stimulus is transient (although impairments in success could be underestimated), preference for the soft stimulus was apparent in CCI-treated rats when starting on the rough stimulus, suggesting that the rough stimulus was avoided. This was not exhibited in naïve or sham treated rats tested concurrently. It should also be noted that aversive behaviors were exhibited in these CCI treated rats and were observed with both stimuli. This indicates that neuropathic injury can cause changes not only in absolute sensitivity to stimuli, but can influence decision making processes related to stimuli.

With respect to preference for 10°C versus 48°C, we found that previous experience may have led CCI-treated rats maintain preference for 10°C, despite the fact that their increased contacts, decreased success, and presence of aversive behaviors indicates that they clearly experience pain with this stimulus at two weeks post-surgery. As observed in the single stimulus condition, there was no reduction in success for the 48°C stimulus, but as before, aversive behaviors were noted.

In contrast, inexperienced CCI-treated rats, who had previously experienced pain with 10°C, did not exhibit a preference, despite the fact that their sham and naïve counterparts were able to do so within the same limited number of trials. These rats were repeatedly exposed to

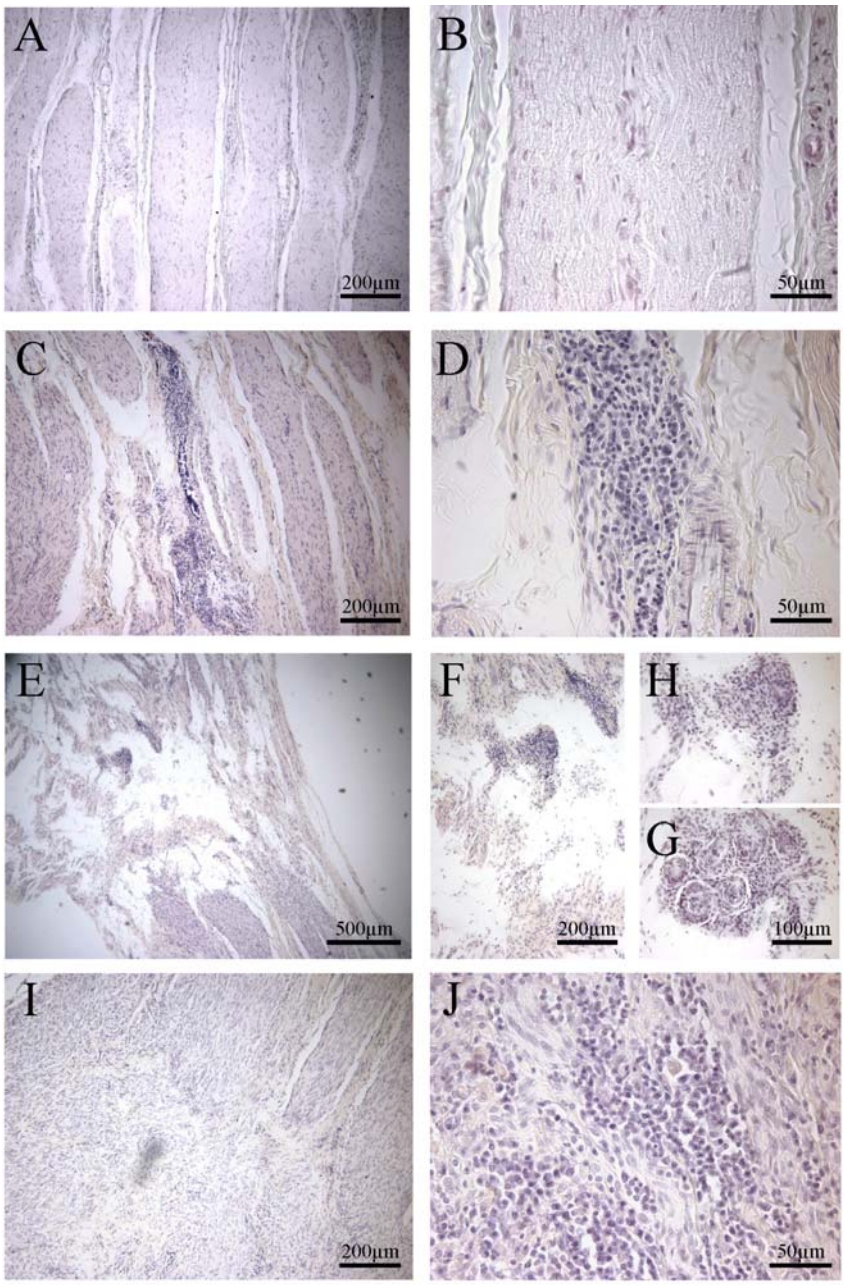
10°C stimulation for the four weeks prior to thermal preference testing, and the CCI-treated rats in this group exhibited deficits in operant success and aversive behaviors indicative of pronounced pain as reported above. By four weeks their responses were normal and aversive behaviors were no longer consistently observed. This should be confirmed by testing preference inexperienced animals two weeks post-surgery when pain is maximal.

As discussed in chapter 2, inflammatory pain models can be used to condition aversion to a compartment (Vaccarino et al., 1992; Colpaert et al., 2006; van der Kam et al., 2008). These findings may indicate that previous experience of cold allodynia following neuropathic injury could condition aversion to 10°C in our preference assay. Conditioned changes in pain sensitivity have also been demonstrated in humans using intrinsic reinforcement of pain by coupling ratings with changes in stimulus intensity (Hölzl et al., 2005; Becker et al., 2008). Additionally, the role of memory in the maintenance of chronic pain has been recently raised by a report of two individuals that experienced a cessation of chronic pain accompanying amnesia (Choi et al., 2007). While further follow up is needed to determine the preference and behavior of inexperienced rats tested at 2 weeks post-surgery, the findings reported here suggest that the stimulus novelty and previous experience of neuropathic pain may be used to study the relationship between memory and pain in experimental animals.

Conclusion

We show here that operant responses to cold stimulation are in agreement with previous studies using reflex and unlearned behavioral assessment of neuropathic allodynia, while operant responses to heat are not significantly altered by neuropathic injury. We also show for the first time that both operant and unlearned behaviors to neutral stimulation are altered following neuropathic injury in a manner suggestive of lasting allodynia. Cold allodynia was reversible with pregabalin and gabapentin. Mechanical preference and thermal preference changes were

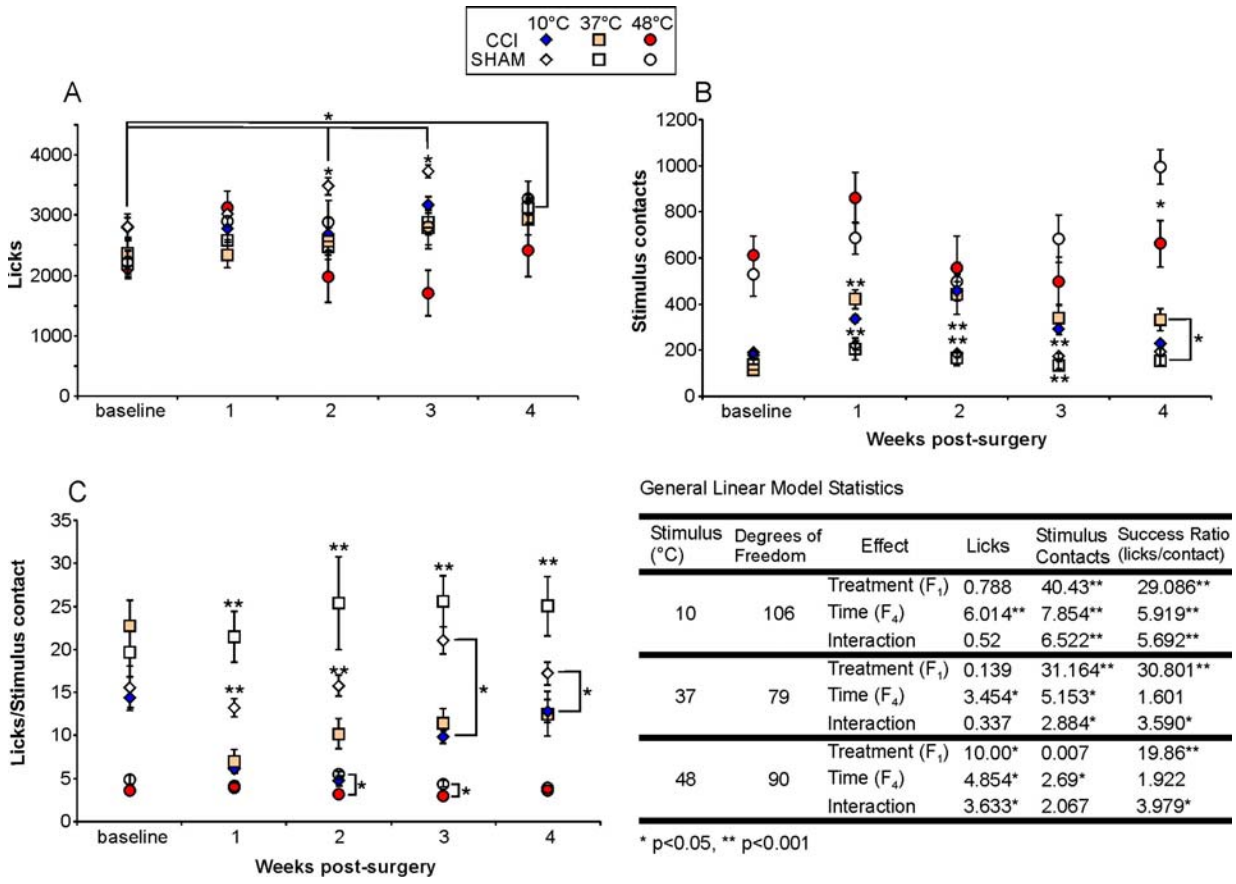
also noted, reflecting the influence of experience on operant behaviors in a neuropathic state. These behavioral changes are also accompanied by changes in both TRPV1 and TRPM8 expression at two weeks post-surgery, which may underlie cold allodynia observed at this time point. These findings indicate that operant assessment of facial pain can provide insights not indicated by reflex based methods, potentially indicative of changes in cortical processing of pain. This may lead to a better understanding of the relationship between peripheral sensitization and central processing that lead to enhanced, and sometime intractable, chronic pain.



	Injury Score		Inflammation Score
0	None, A	0	None, B
1	Scattered inflammation, C	1	Scattered inflammatory cells, D
2	Inflammation around nerves, but not dense, E	2	Moderate infiltrate of cells in discrete foci, F-H
3	More that two foci of dense inflammation surrounding nerves, I	3	Wide-spread dense infiltrate of cells, J

Figure 4-1. Explanation of injury and inflammation scores used to assess the health of surgically treated infraorbital nerves. A-B are images of an untreated nerve, with scores of 0 for

both injury and inflammation. C-J are examples of varying degrees of injury and inflammation apparent in chronic constriction injured nerves, as indicated in the table below. A, C, E, I (left panels) are lower magnification to illustrate nerve injury, as indicated by the infiltration of chronic inflammatory cells, lymphocytes. B,D, F-G, J (right panels) are higher magnification to show the degree of inflammation. . G depicts giant cells/foreign body reaction and inflammatory cells. Injury and inflammation scores for the four sections viewed were added together, with a maximum possible composite score of 24/animal. All nerves were stained with hematoxylin and eosin.

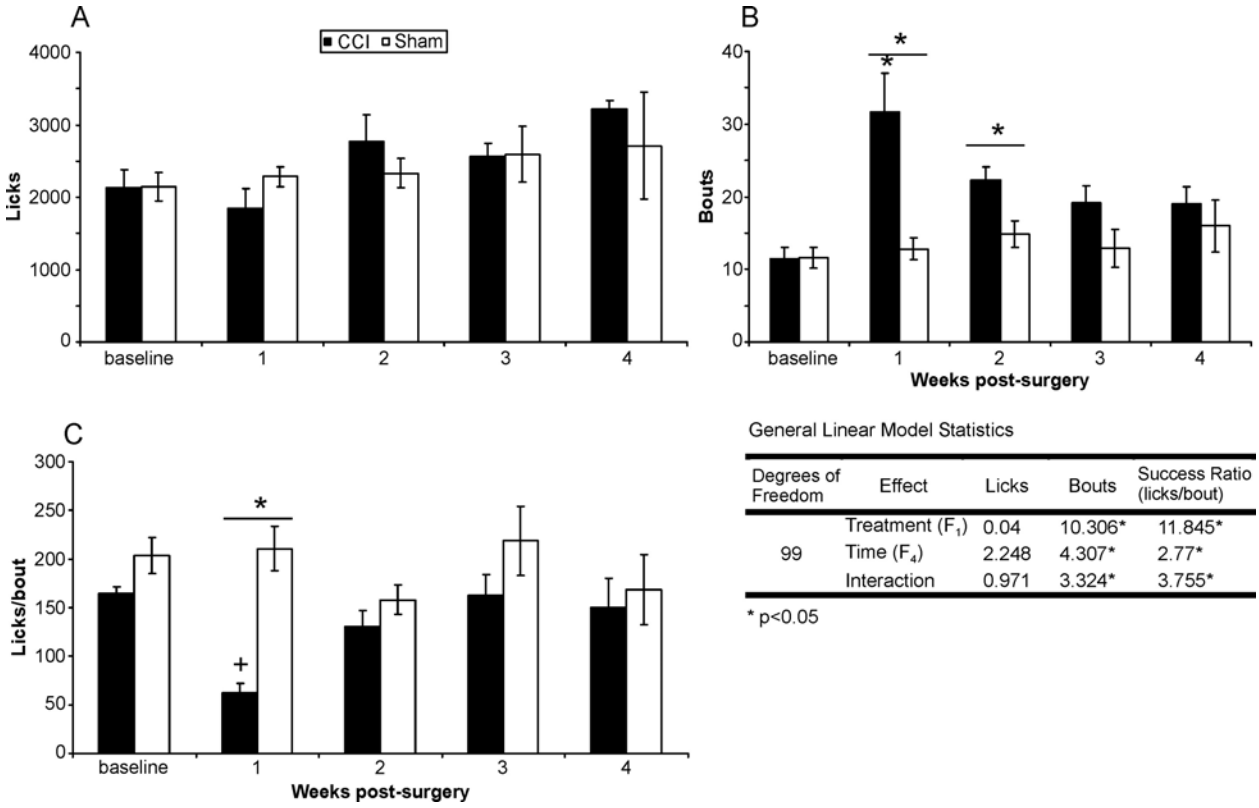


General Linear Model Statistics

Stimulus (°C)	Degrees of Freedom	Effect	Licks	Stimulus Contacts	Success Ratio (licks/contact)
10	106	Treatment (F_1)	0.788	40.43**	29.086**
		Time (F_4)	6.014**	7.854**	5.919**
		Interaction	0.52	6.522**	5.692**
37	79	Treatment (F_1)	0.139	31.164**	30.801**
		Time (F_4)	3.454*	5.153*	1.601
		Interaction	0.337	2.884*	3.590*
48	90	Treatment (F_1)	10.00*	0.007	19.86**
		Time (F_4)	4.854*	2.69*	1.922
		Interaction	3.633*	2.067	3.979*

* p<0.05, ** p<0.001

Figure 4-2. Effect of surgical treatment on operant responses with 10, 37, and 48°C stimulation**.** Diamonds = 10°C, squares = 37°C, circles = 48°C. A) Licks were not significantly affected by CCI treatment (filled symbols) for any stimuli. Sham-treated rats (open symbols) exhibited time related increases in licks for 10 and 37°C stimulation, as indicated by lines. Between group differences occurred with 10°C. B) Stimulus contacts were significantly affected by CCI-treatment with 10 and 37°C, but not 48°C stimulation. The only effect observed for sham-treated rats was a significant increase at four weeks with 48°C stimulation. Between group differences occurred for the majority of post operative time with 10 and 37°C stimulation, and only at four weeks with 48°C stimulation. C) Success ratios (licks/contact) were significantly affected by CCI treatment with 10 and 37°C, not 48°C stimulation. There was no effect of sham treatment. Between group differences occurred for all post-operative time points with both 10 and 37°C stimulation and at two and three weeks with 48°C. * indicates a significant between group difference for the time point and stimulus indicated (unpaired t-test). ** indicates both a significant between group difference at the indicated time point, and a within group difference for the CCI-treated rats relative to baseline (ANOVA). Significance is $p < 0.05$. All data is mean ± SEM. Table indicates statistical outcome of general linear models evaluating time and treatment effects and interactions for the three stimuli shown.



General Linear Model Statistics

Degrees of Freedom	Effect	Licks	Bouts	Success Ratio (licks/bout)
99	Treatment (F_1)	0.04	10.306*	11.845*
	Time (F_4)	2.248	4.307*	2.77*
	Interaction	0.971	3.324*	3.755*

* $p<0.05$

Figure 4-3. Effect of surgical treatment on operant responses with rough mechanical stimulation. A) Licks were not significantly effect by treatment or time. B) Bouts were significantly increased by CCI-treatment at one week post-surgery, and significant between group effects were observed at one and two weeks. C) Success ratios, approximated by licks/bout, were significantly reduced by CCI-treatment at one week post-surgery. Inset table shows the statistical results of a general linear model evaluating the effects and interaction of treatment and time. * indicates significant between group different or significant difference from baseline. + indicates significant difference from all other time points. Significance is $p < 0.05$ and all data are mean ± SEM.

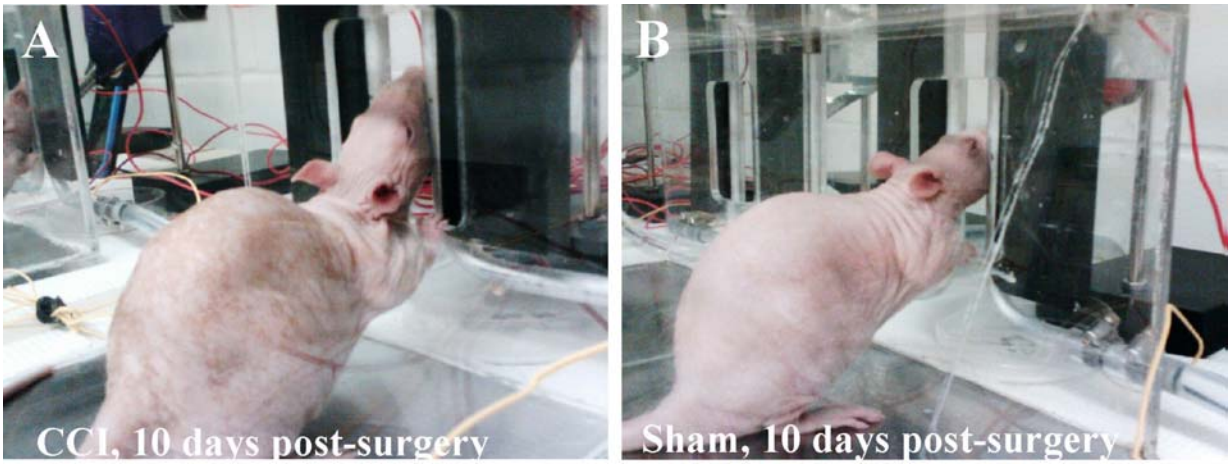
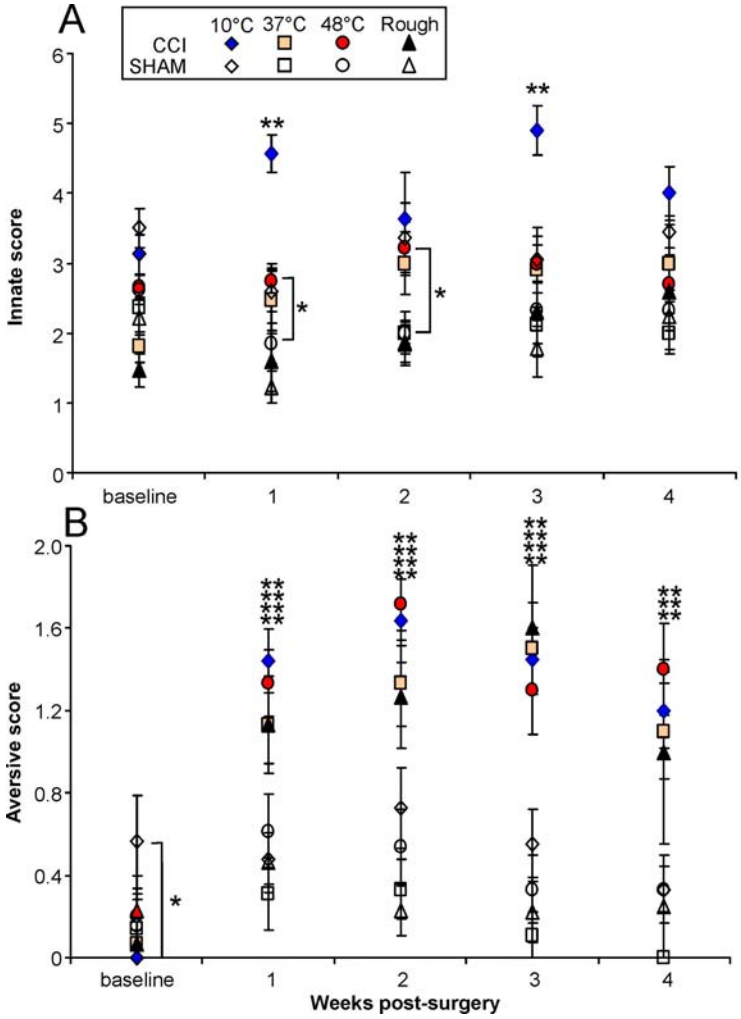


Figure 4-4. CCI-treated rats exhibit aversive behaviors towards the stimulus not observed in Sham-treated rats. (A) An example of a CCI-treated tilting his heat upward, shifting contact away from the mystacial pad affected by the constriction injury. (B) This sham-treated rat, which underwent surgery on the same day as the rat on the left and was also tested within the same session. Naïve animals have the same posture as the sham-treated animals (*not shown*).

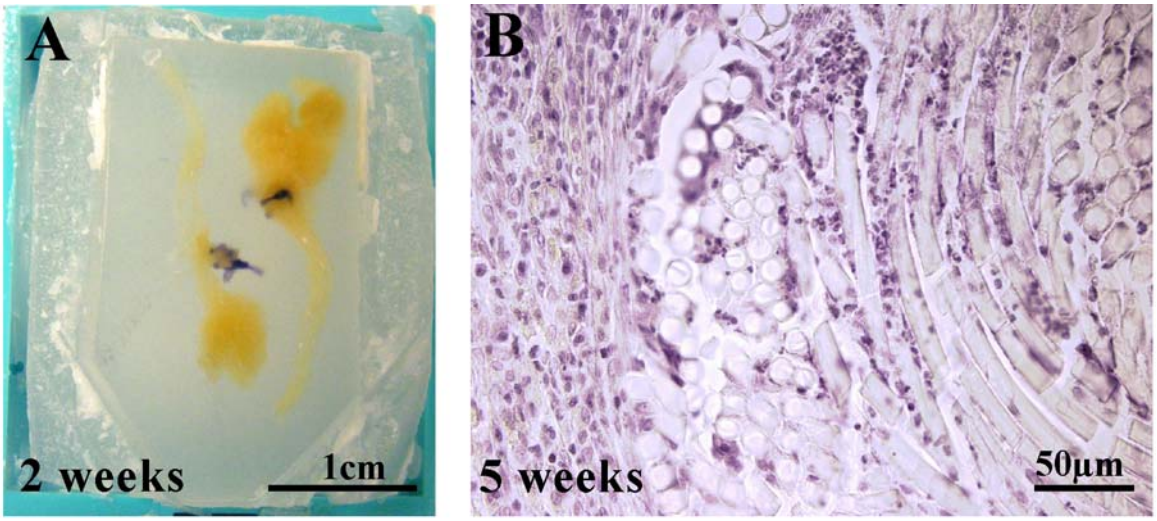


General Linear Model Statistics

Stimulus (°C)	Degrees of Freedom	Effect	Innate	Aversive
10	181	Treatment (F_1)	14.035**	33.907**
		Time (F_4)	0.993	7.791**
		Interaction	4.449*	7.712**
37	120	Treatment (F_1)	3.451	62.409**
		Time (F_4)	0.706	8.076*
		Interaction	1.227	4.242*
48	120	Treatment (F_1)	12.943*	55.08**
		Time (F_4)	0.674	10.913**
		Interaction	1.481	4.021*
Rough	99	Treatment (F_1)	0.146	27.283**
		Time (F_4)	1.409	5.041*
		Interaction	1.053	4.510*

* p<0.05, ** p<0.001

Figure 4-5. Effect of surgical treatment on innate and aversive behavior scores with thermal and mechanical stimuluation. Table indicates general linear model statistics for each stimulus. A) Innate behaviors were only transiently elevated following CCI-treatment with cold stimulation. B) In contrast, aversive behaviors were persistently elevated for all thermal stimuli following CCI-treatment and from one to three weeks for mechanical stimulation. ** indicates both between and within group differences from baseline (one way ANOVA with post-hoc Tukey's test). * indicates a between group difference only (unpaired t-test). $P < 0.05$ and all data are mean ± SEM.



Weeks post-op.	Treatment	n	Suture	Scores Injury	Inflammation	Composite
2-7	CCI*	19	15	1.0 ± 0.1	2.0 ± 0.1	14.7 ± 1.5
	Sham	11	2	0.2 ± 0.1	0.6 ± 0.1	2.9 ± 0.9
12	CCI	8	2	0.5 ± 0.1	1.0 ± 0.2	5.9 ± 1.7
	Sham	8	0	0.3 ± 0.1	0.6 ± 0.2	3.4 ± 1.9

Figure 4-6 Presence of suture and quantification of inflammation in surgically treated infraorbital trigeminal nerves. A) Example of grossly visible suture (violet fibers) around nerve removed two weeks post-surgery. B) Example of microscopically visible suture (striate whirls of transparent material with infiltrating lymphocytes) within nerve removed at five weeks post surgery (hematoxylin and eosin). Table indicates number of animals with suture present in the nerves and inflammation, injury, and composite scores (mean ± SEM). *indicates $p < 0.05$ for CCI-treated rats at 2-7 weeks post-surgery relative to all other groups for all scores.

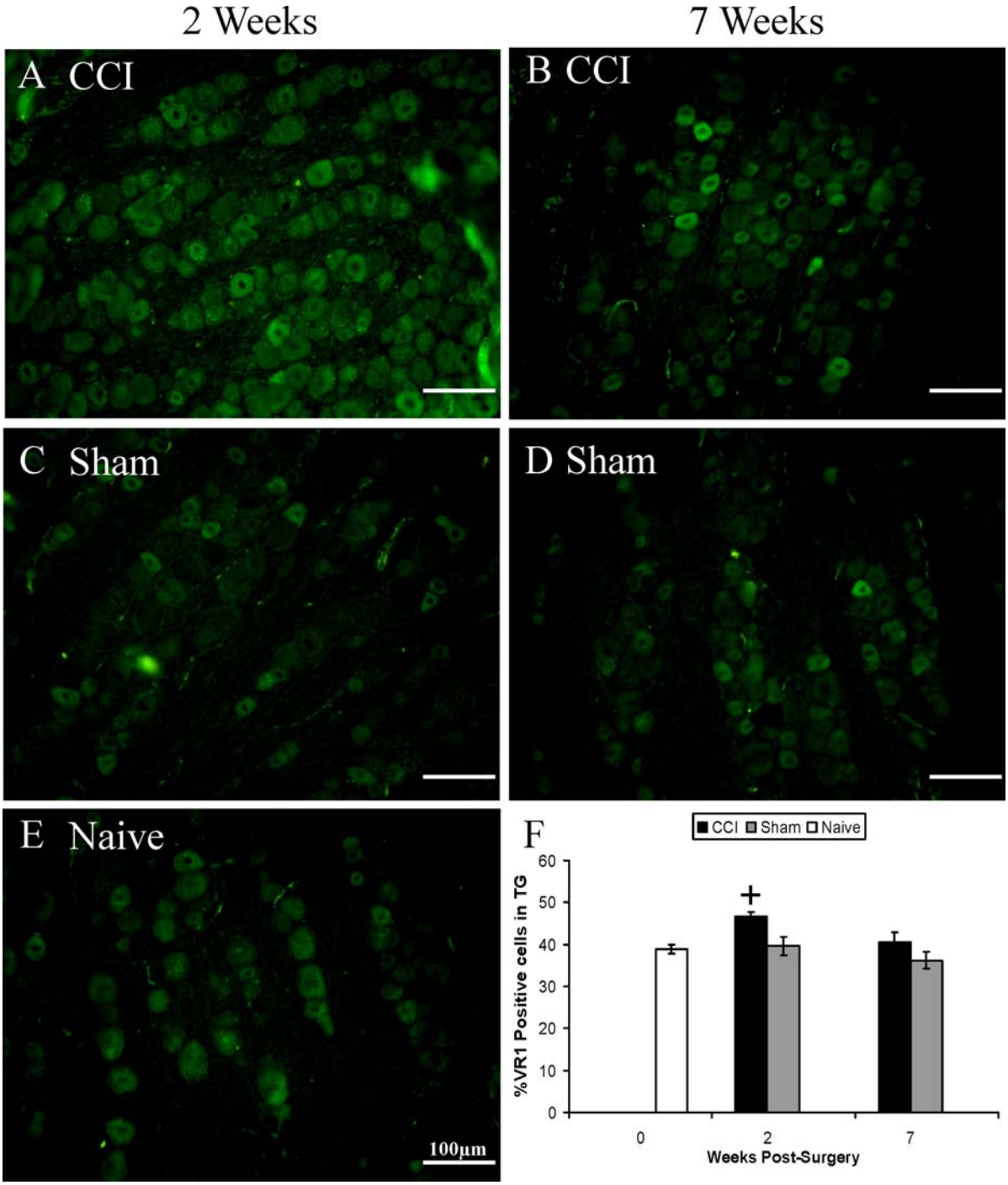


Figure 4-7. TRPV1 immunoreactivity in trigeminal ganglia from naïve and surgically treated rats. A-E are exemplars of TRPV1 immunoreactivity in different treatment groups, as indicated, at two (left) or seven (right) weeks post-surgery. F is graph quantifying the number of TRPV1 positive cells in the trigeminal ganglia for the three treatment groups. + indicates significant difference from all other groups, where $p < 0.05$. Data are mean ± SEM.

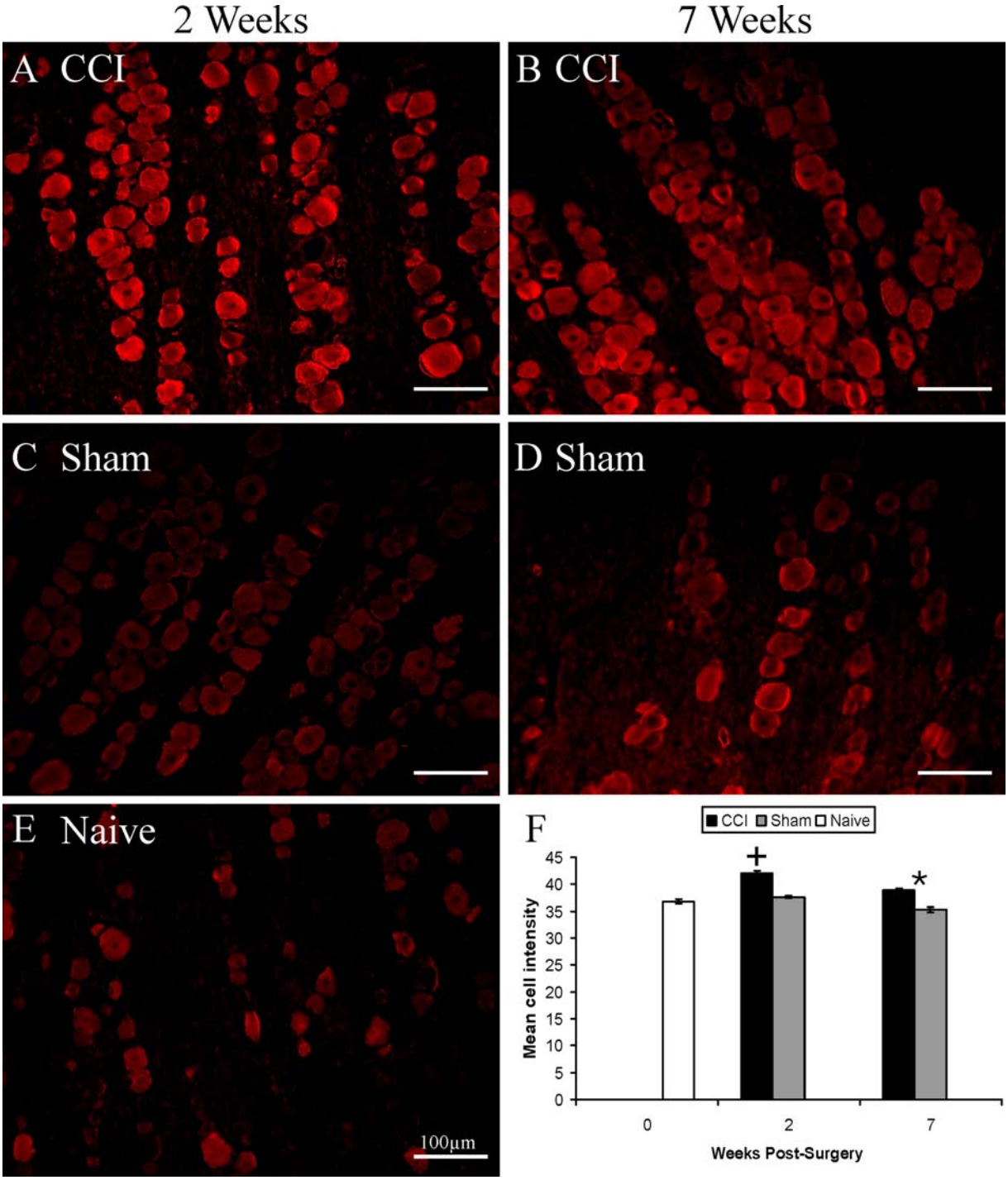
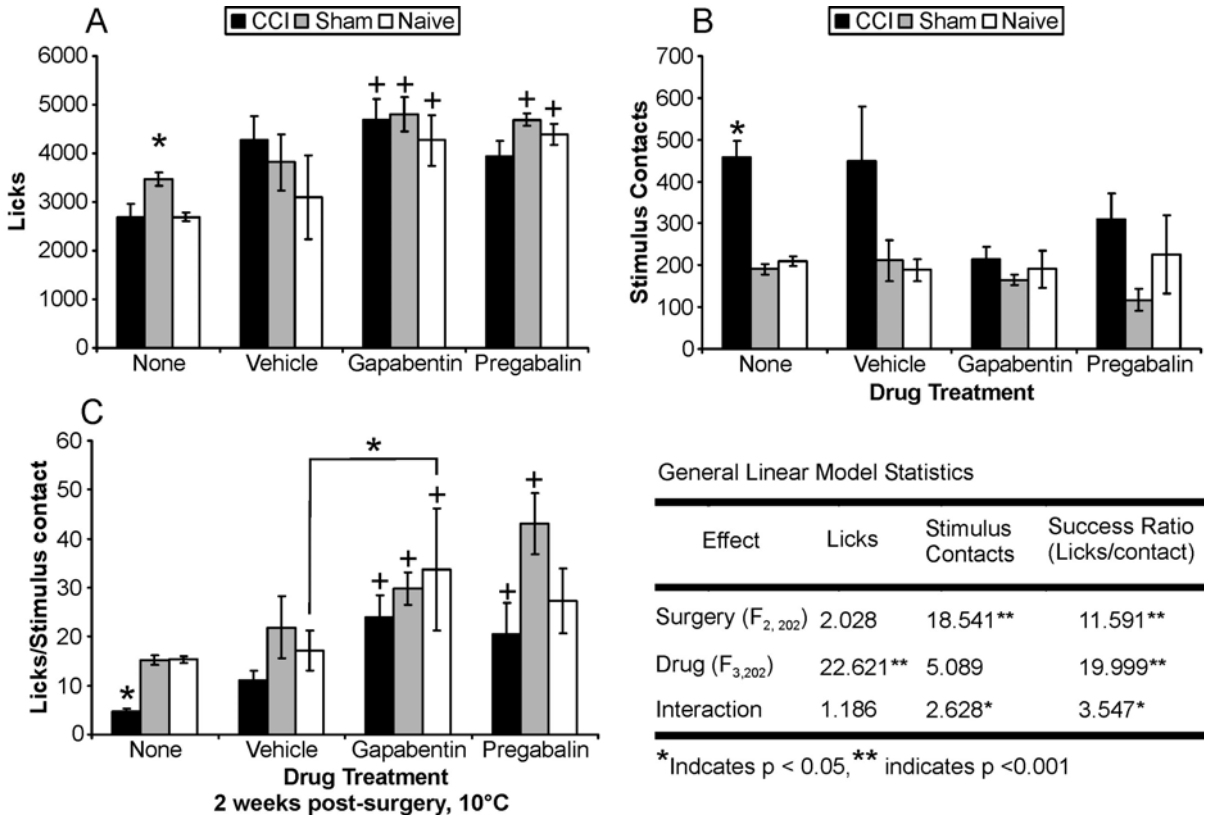


Figure 4-8. TRPM8 immunoreactivity in trigeminal ganglia from naïve and surgically treated rats. A-E are exemplars of TRPM8 immunoreactivity in different treatment groups, as indicated, at two (left) or seven (right) weeks post-surgery. F is graph quantifying the intensity of TRPM8 staining for the three treatment groups. + indicates significant difference from all other groups, * indicates significant difference between the two surgical treatments at seven weeks post-surgery, where $p < 0.05$. Data are mean ± SEM.



General Linear Model Statistics

Effect	Licks	Stimulus Contacts	Success Ratio (Licks/contact)
Surgery ($F_{2, 202}$)	2.028	18.541**	11.591**
Drug ($F_{3,202}$)	22.621**	5.089	19.999**
Interaction	1.186	2.628*	3.547*

*Indcates $p < 0.05$, ** indicates $p < 0.001$

Figure 4-9. Effect of drug treatment on operant behavior in surgically treated and naïve rats with 10ºC stimulation at two weeks post-surgery. Inset table shows statistics for general linear model evaluating the effect of surgical treatment, the effect of drug treatment, and their interaction. DF = degrees of freedom. A) Licks were significantly increased in all three surgical treatment groups with gabapentin (30mg/kg, i.p.), and in sham-treated and naïve rats with pregabalin treatment (10mg/kg, i.p.). There was no effect of vehicle (water, i.p.) on licks in any group. B) The effect of drugs on stimulus contacts did not reach significance for any group. C) Success ratios (licks/stimulus contact) were significantly increased in all three surgical groups with gabapentin, and in surgically-treated rats with pregabalin. * indicates significant between group effects, + indicates as significant within-group increase relative to no drug treatment (determined by one-way ANOVA). Significance is $p < 0.05$ and all data are mean ± SEM.

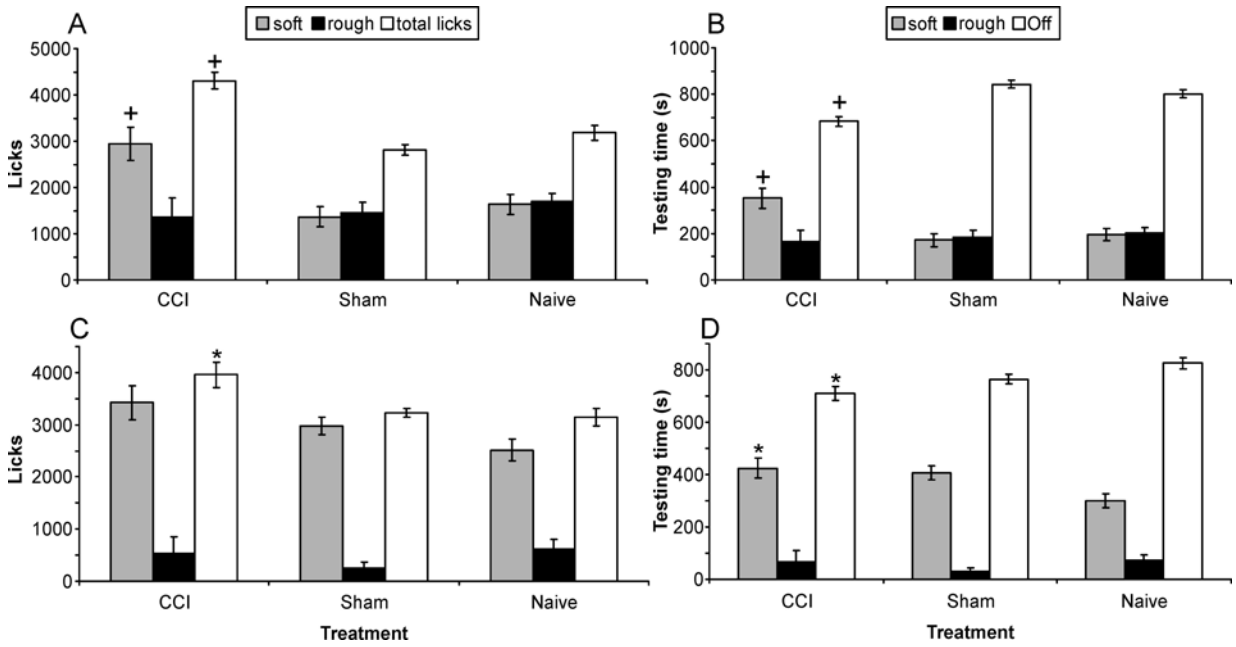


Figure 4-10. Effect of surgical treatment and starting stimulus on mechanical preference, total licks, and time spent unstimulated. When rough is the starting stimulus (A & B), CCI-treated rats lick (A) and spend significantly more time (B) on the soft stimulus than either sham or naïve rats. CCI-treated rats also have significantly greater total licks and lower unstimulated time than the other groups. When soft is the starting stimulus (C & D), CCI-treated rats have more total licks than naïve rats (C) and spend more time on the soft stimulus and less time unstimulated than the naïve rats (D). + indicates significant difference compared to all other treatments and * indicates significant difference from naïve rats only (as determined by one way ANOVA and Tukey's test post-hoc). $P<0.05$. All data are mean ± SEM.

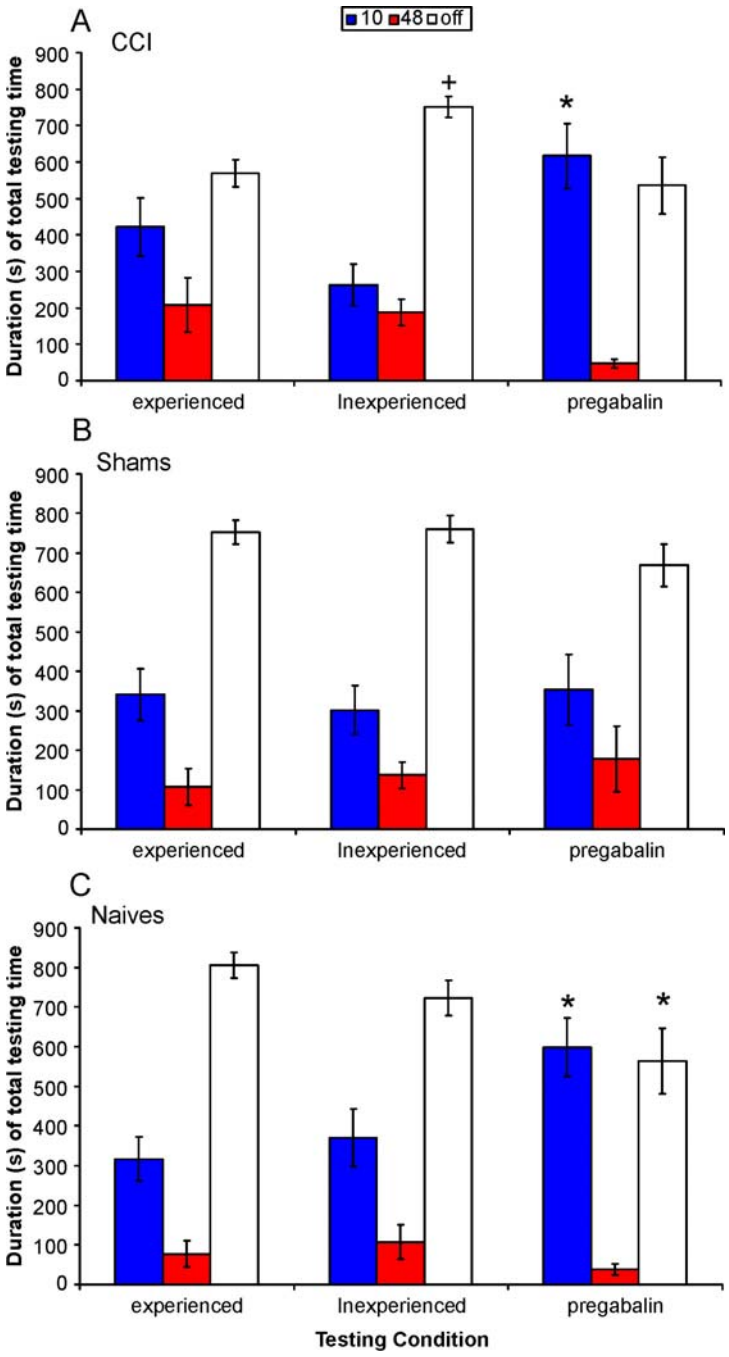


Figure 4-11. Effect of surgical treatment and experience on thermal preference and unstimulated time. A) CCI –treated rats that are experience, but in pain still exhibit a cold preference, while inexperience rats have no preference, which is restored to normal by pregabalin treatment. B) Sham-treated rats exhibit similar preferences despite different levels of experience or drug treatment. C) Both experience and inexperience naïve rats exhibit a pronounced cold preference, which is enhanced by pregabalin treatment. + indicates significant difference compared to all other treatments and * indicates significant difference from the lowest value (as determined by one way ANOVA and Tukey's test post-hoc). $P<0.05$. All data are mean ± SEM.

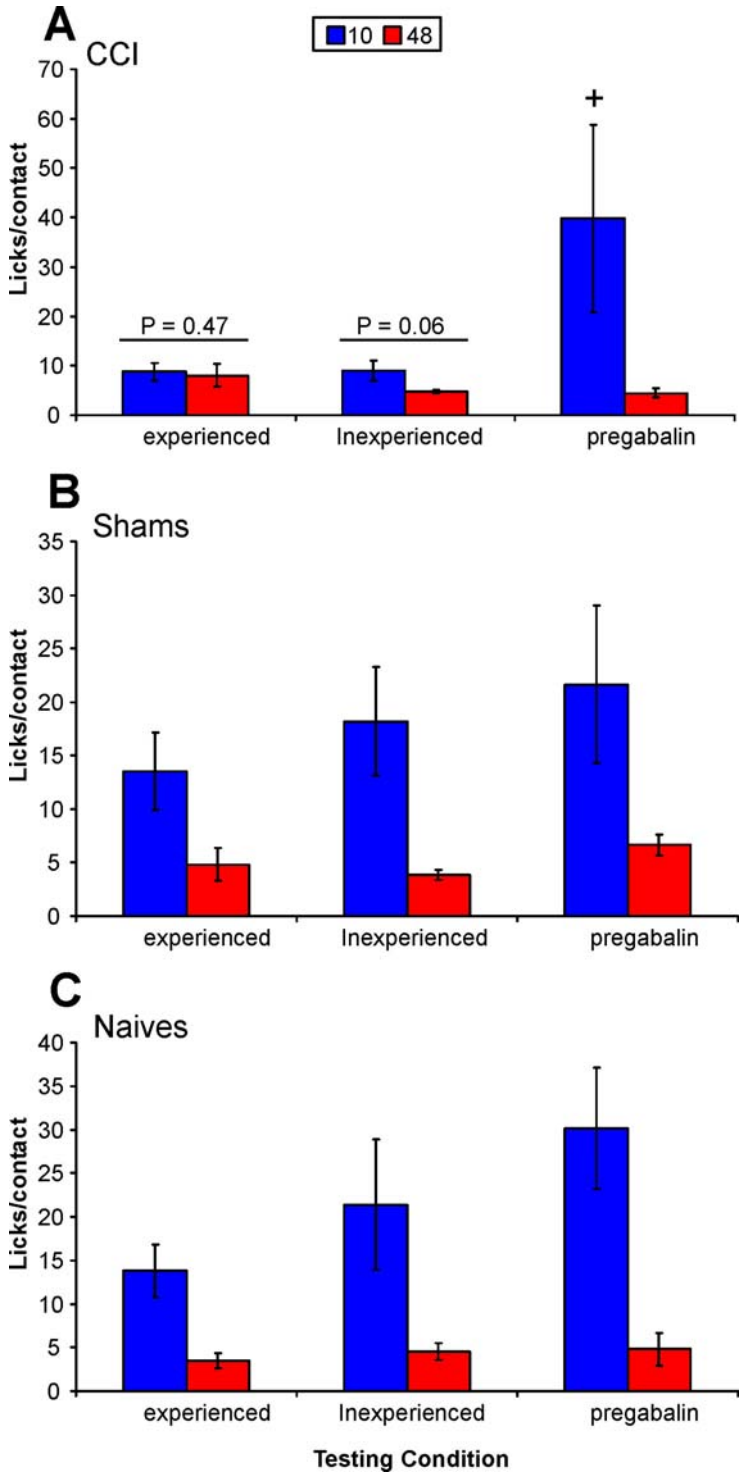


Figure 4-12. Effect of surgical treatment and experience on success for each stimulus in the thermal preference taks. A) Experience and inexperience CCI –treated rats exhibit reduced success ratios on the cold stimulus as compared to all Sham-treated (B) and naïve (C) rats. This is ameliorated by pregabalin treatment. + indicates $p<0.05$ significant difference compared to all other treatments (one way ANOVA and Tukey's test post-hoc). All data are mean ± SEM.

Table 4-1. Seven day post-operative recovery progress for surgical groups as indicated by number of rats with swelling in the surgical area ranging from severe to none.

	Swelling severity	1 day	2 days	3 days	4 days	5 days	6 days	7 days
CCI	severe	9	3	4	2	0	0	0
N = 19	moderate	9	12	9	8	7	2	0
	mild	1	4	5	7	6	6	6
	none	0	0	1	2	6	11	13
SHAM	severe	8	0	0	0	0	0	0
N = 19	moderate	8	10	2	2	0	0	0
	mild	3	6	7	6	4	0	0
	none	0	3	10	11	15	19	19

Table 4-2. Percentage of occurrence of behaviors and behavioral scores (mean ± SEM) with 10°C, 37°C, 48°C, and rough stimulation for naïve rats (n = 5, across multiple sessions).

Stimuli	FGF	FGH	FS	HS	WDS	HT	Wipe	Bite	Total n
10°C	97	91	34	66	46	0	26	0	35
37°C	92	80	32	32	8	0	0	0	25
48°C	92	100	28	32	12	0	4	0	25
Rough	90	35	35	25	10	0	20	5	20

	Innate score	Aversive score	Combined score
10°C	3.3 ± 0.1*	0.3 ± 0.1*	3.6 ± 0.1*
37°C	2.4 ± 0.2	0	2.4 ± 0.2
48°C	2.6 ± 0.1	0	2.7 ± 0.1
Rough	2.0±0.3	0.3±0.1	2.2±0.3

* $p<0.05$. FGF = facial grooming with forepaws, FGH = facial grooming with hindpaws, FS = forepaw shaking, HS = head shaking, WDS = wet dog shaking, HT=head tilting, wipe = wiping at the stimulus, bite = biting the stimulus

Table 4-3. Percentage of CCI- and Sham-treated rats exhibiting head tilting (HT) or thermode wiping (wipe) with thermal and mechanical stimulation, or biting with mechanical stimulation pre- and post-operatively.

Stimulus Temp. (°C)/Mechanical	Surgical Treatment	Aversive Behavior	Weeks Post-Surgery				
			Baseline	1	2	3	4
10°C	CCI	HT	0	77	100	75	55
37°C	CCI	HT	0	67	80	90	80
48°C	CCI	HT	0	87	80	70	60
rough	CCI	HT	7	80	73	90	60
10°C	Sham	HT	0	19	9	5	5
37°C	sham	HT	0	3	3	0	0
48°C	sham	HT	0	23	8	33	0
rough	Sham	HT	0	0	0	0	0
10°C	CCI	wipe	0	58	55	65	65
37°C	CCI	wipe	7	47	53	60	30
48°C	CCI	wipe	20	47	80	60	70
rough	CCI	wipe	0	38	58	56	0
10°C	Sham	wipe	31	31	64	40	25
37°C	sham	wipe	0	8	8	11	0
48°C	sham	wipe	8	38	50	0	22
rough	Sham	wipe	8	15	17	22	0
rough	CCI	bite	0	0	7	20	40
rough	Sham	bite	15	31	8	0	25
10°C		n	16	26	11	20	20
(both 37&48°C)		n	13-15	13-15	12-15	9-10	9-10
rough		n	13-15	13-15	12-15	9-10	4-5

For samples sizes, lower number indicates number of rats in sham group and higher in the CCI group.

Table 4-4. Percentage of surgically treated rats exhibiting soft, rough, or no stimulus preference post-operatively (n = 30 per treatment).

Treatment group	Stimulus preference		
	Soft	Rough	None
CCI	73 (13/20)	17	10
Sham	53 (8/22)	40	6

Numbers in parentheses indicate the fraction of surgically-treated rats with a soft preference when starting with rough stimulation.

Object 4-1. Video clip of aversive head tilting behavior exhibited by a CCI-treated rat at post-operative day 10, with 10°C stimulation.

Object 4-2. Video clip of normal operant behavior exhibited by a sham-treated rat at post-operative day 10, with 10°C stimulation.

CHAPTER 5
FUTURE DIRECTION

In the current work, an operant method for evaluating facial sensitivity to thermal and mechanical stimuli in rodents has been described. This method was used to evaluate the effect of TRP channel agonists on thermal perception and finding support a role for a TRPV1-expressing population in cold pain, as well as menthol irritation, but not in the maintenance of a putative thermogenic behavior, wet dog shaking. The loss of TRPV1-expressing population also does not affect discrimination of cold from warmth. This work also characterized both unlearned and operant behaviors towards mechanical and a range of thermal stimuli following neuropathic injury. Alleviation of cold allodynia by pregabalin and gabapentin was demonstrated and the influences of affect, experience, and memory on neuropathic pain behaviors were discussed. While this work contributes to and supports the current knowledge regarding the role of TRP channels in thermal processing, normal, and pathological pain, it does not represent an end point. Scientific inquiry is like a small child; the answer to a question often leads to another series of questions. In this final chapter, future directions of this work are discussed.

Adaptation of the Assay to Evaluate Pain in Mice

The importance of incorporating behavioral evaluations of pain that require a decision making process has been emphasized in this work. Operant and thermal preference assessments, with the exception of TRPM8 (Dhaka et al., 2007) and TRPV4 (Lee et al., 2005) knock-out mice, are lacking with respect to the behavioral characterization of TRP channel knock-out mice. To address this gap in the literature, the assays described in this work have been scaled for use in mice, making all of the assessments in the current study possible for various strains of wild type and genetically modified mice. The lab is currently in the process of characterizing the full range of thermally-mediated operant behaviors in TRPM8 and TRPV1 knock-out mice, and may

do so with TRPA1 knock-out mice in the future. Thermal preference in various strains of knock-out mice will also be assessed, particularly with respect to the discrimination between hot and cold stimuli. There is potential for co-expression among TRP channel populations, as previously mentioned, and since these channels can be modulated by local voltage changes, as well as intracellular signaling cascades, interactions among channels, as well as their intracellular milieu, under different stimulating conditions must be considered (Belmonte and Viana, 2008). The next step in TRP channel research should be aimed at evaluating the additive effects of these channels in encoding normal and pathological thermal perception.

Thermal preference assessments may be one way to accomplish this. We showed in the current work that although pharmacological "knock-out" of TRPV1 eliminates cold pain, it does not affect cold avoidance. In TRPM8 knock-out mice, with profound impairment in cold sensitivity, but no heat related impairments, what profile of behavior would we observe if cold is paired with moderate heat or with noxious heat? Colburn and colleagues demonstrated that these mice prefer 5 over 45°C and do not distinguish between 15 and 25°C in contrast to wild type mice (Colburn et al., 2007), suggesting that the aversive quality of very cold pain is impaired in the absence of TRPM8. We would like to determine if this is true for facial stimulation as well. Likely it is, but the contribution of TRPM8 to cold perception and pain in the face and head may be slightly different in the trigeminal nervous system than in the sciatic, as differences have been noted in the expression of TRPM8 in the TG versus DRGs (Kobayashi et al., 2005). An additional method to evaluate the additive effects of multiple channels is to selectively remove them via genetic knock-out and pharmacological means or RNA silencing methods. For example, treatment of TRPV1 knock-out mice with a TRPM8 antagonist, such as capsazepine

(also an antagonist for TRPV1, but not an issue in this case), could also address the contribution of TRPM8 versus other cold-activated channels to cold perception and pain.

We can also use knock-out mice to assess the contribution of different TRP channels to the development and modality specificity of neuropathic pain. TRPV1 knock-out mice exhibit differences from wild type in various models of chronic pain (Bölcskei et al., 2005), but CCI was not assessed, nor was cold sensitivity. If the abnormal expression of TRPV1 with TRPM8 or other cold sensing channels underlies cold allodynia following neuropathic injury, then cold allodynia should not be observed in TRPV1 knock-out mice with a neuropathic injury. CCI to the sciatic nerve in TRPM8 knock-out mice failed to produce increased responsiveness to the acetone spray test, which could not be explained by a lack of mechanical hypersensitivity (Colburn et al., 2007). However, responses to direct cold stimulation were not assessed. There is evidence for other cold activated receptors (Babes et al., 2006; Munns et al., 2007), but do any of these contribute to the cold allodynia that characterizes neuropathic pain?

Thermal Preference, Conditioned Aversion, and Drug Treatment

In Chapter 2, we presented findings to indicate that the thermal preference task could be used to condition an aversion or preference for one thermode, suggesting that this assay could be used to evaluate the effect of analgesic and anxiolytic compounds on pain-related decision making. To address this issue, I propose a series of experiments (Table 5-1). We would need to establish how many times and with what inter-testing period conditioned aversion/preference can be reliably reproduced in the same group of animals. If multiple testing in a single group of animals is possible, then the drug treatments proposed in Table 5-1 could use a within-individual design, as long as sufficient recovery period was allowed between treatments. If multiple testing in a single group is not possible, then the experiments in Table 5-1 would need to be replicated for each drug treatment applied to unique groups of animals. If this work is to be conducted with

mice, the work of chapter 2 would need to be repeated in mice to establish similarities or differences that may exist between mice and rats. The addition of video tracking will also allow us to measure movement and general activity in the preference box, which can be used to assess sedation or loss of motor control or balance that might accompany drug treatment.

Stimulus Novelty and Neuropathic Injury

Chapter 4 presented data suggesting that experience and novelty can effect how CCI-treated rats perform on the thermal preference task. We have also generally observed that CCI-treated rats also are less successful than their sham or naïve counterparts when exposed to a novel stimulus that is not part of their normal routine. This could reflect injury-related alterations in cortical regions associated with affective procession of painful stimuli. A controlled study is needed to confirm that CCI-treated rats exhibit reduced performance in the operant task with respect to novel stimuli. This could be done by training rats without collecting baseline data, surgically treating as subset of rats with either CCI or sham, then testing surgically treated and untreated rats at two weeks post surgery with any stimulus other than the training stimulus. This process could be repeated in subsequent groups for different stimuli, or at a later time post surgery. The latter condition could help address the possibility that behavioral manifestations of neuropathic pain subside as a consequence of habituation to the pain evoked by testing conditions. If CCI-treated rats consistently respond poorly to novelty, this may allow for studies evaluating the effect of electrolytic or chemical lesion of cortical regions, such as the anterior cinglulate cortex, that could be involved in affective judgments about painful stimuli.

General Conclusion

This work characterizes an operant method for evaluating facial pain in experimental animals that is relevant to the human condition. In combination with histological, functional imaging, and other molecular techniques, this behavioral evaluation method can provide insights

regarding both peripheral and central pain processing. These insights will lead to more effective pain treatment, or perhaps prevention, that could profoundly enhance the quality of life for future generations.

Table 5-1. Experimental schedule for evaluating the effect of different classes of drugs on conditioned aversion in the thermal preference task.

	Left Thermode	Right Thermode
Day 1: conditioning aversion	52°C *	18°C
Day 2: measuring aversion (OR)	18°C	18°C
Day 1: conditioning aversion	45°C	-4°C*
Day 2: measuring aversion	45°C	45°C
Where * indicates the experimenter controlled starting side, the avoided side.		
On "Day 2" drug treatment would be applied prior to testing.		
Treatment Classification	Potential Drug(s)	Expected Outcome
Untreated	N/A	Aversion to * side
Vehicle (injection control)	PBS	No change in aversion
Anxiogenic	Yohimbe	Enhanced aversion
Anxiolytic/non-analgesic	Valium, Diazepam	Aversion abolished, success no different
Anxiolytic/analgesic	Morphine, pregabalin, Cymbalta	Aversion abolished, success increased
Analgesic only	Lidocaine, NSAID, RTX	Aversion intact, success increased

At end, confirm 50/50 preference for Day 2 state in the absence of conditioning or treatment. NSAID = non-steroidal anti-inflammatory drug

APPENDIX
DOSE DETERMINATION OF PREGABALIN USED FOR TREATMENT OF CHRONIC CONSTRICTION INJURY

The literature typically reports an effective analgesic dose for pregabalin between 30-100 mg/kg in animals (Field et al., 1999; Hong-Ju et al., 2004; Beyreuther et al., 2006; Ling et al., 2008). However, these assessments are made using reflex based assays. Pregabalin also has the potential for anxiolytic properties that could contribute to analgesia (Field et al., 2001; Nicolas et al., 2007; Nutt et al., 2008; Zohar et al., 2008). We sought to determine if using an operant method of pain evaluation revealed a similar or different effective analgesic dose, which we used in later studies (see Chapter 4) to treat animals following neuropathic injury.

Evaluation of General Activity by Rearing

In order to evaluate changes in general activity induced by pregabalin, rearing (vertical locomotion) data was collected as previously described (Neubert et al., 2007; Rossi and Neubert, 2008). Briefly, rats were placed in a cylindrical chamber and the number and duration of rears were recorded for a 15-minute period. This information was used to calculate the duration per rearing event reported here. Rearing data was recorded for four consecutive days to allow rats to become accustomed to the chamber prior to pregabalin administration.

Administration and Dose Determination of Pregabalin

In order to determine which dose to use following neuropathic injury, rats (n = 10, not part of the surgically treated animals described in Chapter 4) were injected intraperitoneally (i.p.) with different doses of pregabalin (PG) or PBS vehicle and tested in the rearing chamber 30 minutes later. After rearing assessment, capsaicin cream (0.035%, Capzasin P; Chattem, INC; Chattanooga, TN) was applied to the faces of gently restrained rats, left on for 5 minutes, and removed with water. Immediately after capsaicin removal, rats were placed in the operant thermal chambers and their responses to a 45°C stimulus were recorded. The doses of pregabalin

used were: 1, 5, 10, and 100 mg/kg (n = 5 rats per dose). Because these experiments were performed on multiple groups of rats on different days, data was converted to a percentage of the rats' baseline values and subsequent statistical analysis was performed on the percentage values.

Pregabalin Dose Determination Based on Rearing and Alleviation of Capsaicin-Induced Heat Hyperalgesia

We also wished to examine how neuropathic behavior revealed by the operant task could be altered by commonly used analgesic treatments, such as pregabalin and gabapentin. Before administering pregabalin in neuropathic animals, we assed the effects of several doses of pregabalin on general activity and heat hyperalgesia in naïve rats to determine the lowest effective dose. The rearing assay was used to assess the effects of pregabalin on general activity. The highest dose examined, 100 mg/kg (i.p.), had significant effects on rearing behavior (Fig. 3A). At this dose, rats were hyper active in the rearing chamber, with ataxia and difficulty balancing on their hindpaws. This resulted in many short rearing events as compared to baseline. None of the other doses tested had significant effects on rearing behavior.

Following assessment of general behavior, we evaluated the ability of these doses to alleviate capsaicin-induced heat hyperalgesia. We found that the two highest doses tested, 10 and 100 mg/kg, significantly increased licks and the success ratio as compared with PBS and lower doses of pregabalin, but had no significant effect on stimulus contacts (Fig. 3 B-D). The number of licks and the success ratio produced in the presence of 10 and 100mg/kg with capsaicin was not significantly different from those outcomes produced by 45°C alone. In other words, 10 and 100mg/kg were able to strongly alleviate capsaicin-mediated hyperalgesia. However, rats that received 100mg/kg pregabalin were initially ataxic, then became increasingly lethargic as testing progressed. In contrast, rats that received 10 mg/kg did not show signs of

sedation. Based on these findings, we chose 10mg/kg of pregabalin for subsequent treatment of CCI- and sham-operated rats.

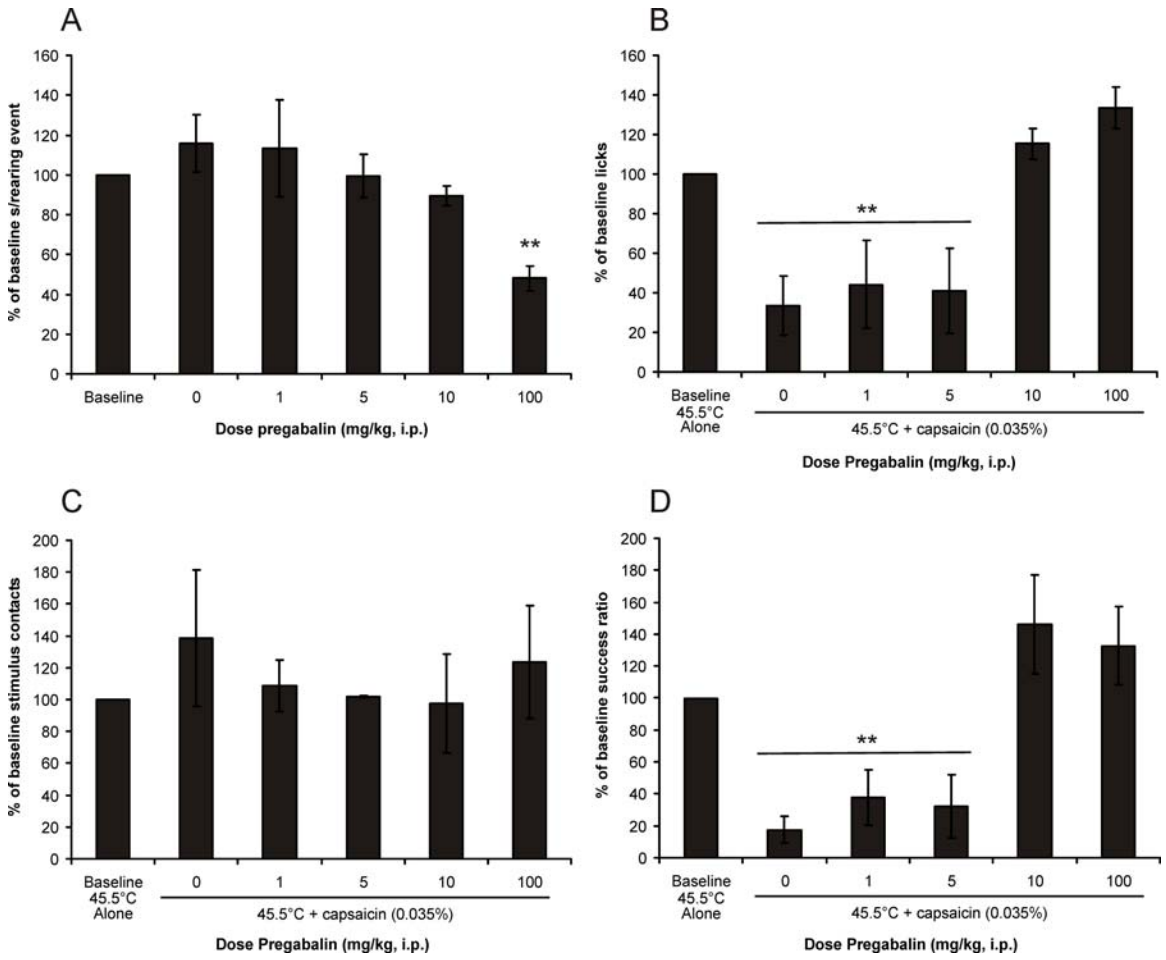


Figure A-1. Determination of lowest analgesic dose of pregabalin without significant effects on general activity, measured by rearing behavior. Rats (n = 5 each dose) were injected with pregabalin or PBS i.p. and their rearing behavior was recorded 30 minutes later. A. The duration per rearing event (s/rear) was calculated for each dose and is expressed as a percentage of the baseline value to normalize across multiple testing sessions. B-D. Immediately following rearing, rats were tested at 45.5ºC with topical capsaicin (0.035%). The percentage change from baseline licks (B), stimulus contacts (C), and the success ratio (licks/contact, D) are shown for each dose. Baseline for stimulus testing refers to behavior recorded in the presence of the 45.5ºC stimulus without capsaicin treatment. Data are mean ± SEM. Main effect of pregabalin treatment for each outcome, as determined by ANOVA: (A) $F_{5,34} = 6.755$, (B) $F_{5,43} = 14.283$, (C) $F_{5,43} = 0.665$, (D) $F_{5,43} = 15.215$. ** indicates $p<0.05$, as determined by post-hoc Tukey's test.

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BIOGRAPHICAL SKETCH

Heather Rossi graduated *cum laude* from the University of North Carolina at Asheville in May 2003, with a bachelor's degree in biology. Later that fall, she enrolled in the Interdisciplinary Program in the Biomedical Sciences at the University of Florida. She joined the laboratory of Dr. John Neubert in January 2005, and successfully advanced to doctoral candidacy that October. She has been a member of the Society for Neuroscience since 2003 and has presented a poster at the last three annual meetings. She also has two first-authored and four co-authored publications from her time in Dr. Neubert's laboratory and plans to submit several manuscripts before she graduates in December 2008. She will be the first member of her family to receive a doctoral degree.