CHARACTERIZATION OF ITCH-RELATED BEHAVIOR AND PHYSIOLOGY OF TRIGEMINOTHALAMIC TRACT NEURONS IN RATS, INCLUDING THE ROLE OF OPIOIDS

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Dedication

This work is dedicated to the best person I know, Leif Moser…
To his loyalty as my husband, his unfailingly gentle nature, his unending forgiveness, his commitment to love his neighbor as himself, his whole-hearted enthusiasm in the pursuit of happiness, and his ability to laugh at the absurdity of it all. Not a day goes by that I am not thankful he is my partner.

Abstract

The sensations of itch and pain are processed by the same neural systems, yet produce distinct perceptual experiences and behaviors. Therefore, it is important to study itch in animal models which exhibit differential behaviors to itchy versus painful stimuli. In rodents, application of pruritogens to the face produces scratching with the hindlimb while application of algogens produces wiping with the forelimb. Here, we use the face model in rats to characterize behaviors produced by the peripheral pruritogen serotonin, the central pruritogen morphine, and combined delivery of these two drugs. Intradermal injection of serotonin or intracisternal injection of morphine elicits dose-dependent scratching. Serotonin-induced scratching is attenuated by naloxone while serotonin-induced wiping is attenuated by morphine. Combined delivery of serotonin and morphine results in a superadditive increase in scratching. In humans, itch and pain sensations require spinal projection neurons which terminate in somatosensory thalamic nuclei. Here, we characterize responses to pruritogens in trigeminothalamic tract (VTT) neurons in rats which may be involved in carrying information about facially-applied pruritogens to the forebrain for producing the itch sensation and related behaviors. VTT neurons respond to a variety of pruritogens; all pruriceptive VTT neurons also respond to noxious mechanical and/or thermal input. We show that intrathecal application of morphine excites pruriceptive VTT neurons and inhibits nociceptive-only VTT neurons. Responses to serotonin are increased in the presence of morphine, providing a possible explanation for the superadditive increase in facial scratching seen upon combined delivery of these two drugs. Our results provide evidence against a labeled line for itch information within the central nervous system and suggest that the brain may decode itch signaling via spike patterns or a population code. These experiments lay the groundwork for use of the face model and the VTT system in rats for the future study of salient questions which remain in the field of itch.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction

Acute itch which can be easily relieved by scratching is a commonly experienced sensation for most humans. However, chronic pruritus (itch) due to skin disorders, neuropathy, or systemic disease can be severely debilitating. It is estimated that 20-27 percent of people suffer from chronic pruritus worldwide (Dalgard et al. 2007; Matterne et al. 2009; Weisshaar and Dalgard 2009), and for most of these people, itch is not treatable with histamine receptor antagonists, the classic treatment prescribed for acute itch (Kjellberg and Tramer 2001; Szarvas et al. 2003). The severity of itch experienced by chronic pruritus sufferers can lead to self-inflicted skin damage due to scratching and in the worst cases, depression or even suicide (Dalgard et al. 2007; Weisshar et al. 2008; Tessari et al. 2009). While some types of itch are due to underlying conditions which can be treated directly, thus alleviating the itch, other types of itch are primary symptoms of dermatologic or neurologic pathologies. For the latter, a better understanding of the neural mechanisms underlying itch is necessary for the development of more effective treatments.

In addition to itch caused by chronic pathological conditions, itch can be experienced as a side effect of drug therapy. Opioid-induced pruritus is a well-documented adverse side effect of opioid treatments for pain and is especially frequent upon intrathecal or epidural administrations, with reported incidences of 20-100 percent (Ballantyne et al. 1988; Ganesh and Maxwell 2007). The itch can be so debilitating that patients may choose to receive less pain relief, rather than endure the severe itch. Furthermore, endogenous opioid systems have been

implicated in producing chronic pruritic conditions including atopic dermatitis, psoriasis vulgaris, chronic urticaria, and cholestatic and uremic pruritus (Phan et al. 2010). For example, patients with hepatic cholestasis have significantly increased levels of endogenous opioids and often experience severe itch, which is partially relieved by treatment with μ-opioid receptor antagonists or κ-opioid receptor agonists (Bergasa et al. 1992, 1999; Dawn and Yosipovitch 2006). These clinical studies strongly suggest a role for opioid receptors in opioid-induced pruritus as well as chronic pruritus and emphasize the need for a better understanding of the relationship between itch and pain.

Rodent models of human itch

Historically, a lack of fully characterized animal models has posed a challenge for performing basic research on the underlying neural mechanisms of itch. A valid animal model of human itch or pain should exhibit responses to itchy stimuli (pruritogens) or painful stimuli (algogens) which are analogous to human responses (LaMotte et al. 2011). In humans, itch and pain are experienced as distinct sensations. Therefore, a valid animal model should exhibit distinct behavioral responses to pruritogens versus algogens. Itch is defined as an unpleasant sensation which produces the desire to scratch, thus scratching is the behavioral endpoint for animal studies of itch. In studies of pain, behavioral endpoints most often involve protective behaviors such as withdrawal from or removal of a painful stimulus.

In rodent experiments, the nape of the neck has traditionally been the most common site for application of pruritogens to the skin. This part of the body can only be accessed by the hindlimb (e.g. for scratching) and therefore does not allow study of other potentially relevant behaviors, such as those that may be associated with pain. For example, both histamine, a predominantly itchy stimulus in humans, and capsaicin, a distinctly painful stimulus in humans, each elicit scratching when these chemicals are applied to the nape of the neck in mice (Shimada and LaMotte 2008). Furthermore, several studies have shown that rats do not reliably scratch in response to histamine when it is applied to the nape of the neck (Thomsen et al. 2001; Jinks and Carstens 2002). In addition to not correlating well with human psychophysics, an important aspect lacking from these previous behavioral studies is the demonstration of a differential response to itch-evoking versus pain-evoking stimuli. Therefore, application of pruritogens to the nape of the neck is not an ideal method for the study of itch in rodents.

More recently it has been reported that rodents show distinct behavioral responses to pruritogens versus algogens when these stimuli are applied to the face. This “face model” of itch has been used in mice to show that histamine produces robust scratching with the hindlimb directed to the site of injection while capsaicin produces a site-directed wiping response with the forelimb (Shimada and LaMotte 2008). This finding has been replicated and expanded to include a variety of pruritogens (e.g. serotonin, BAM8-22, chloroquine, cowhage) and algogens (e.g. mustard oil) in mice (Akiyama et al. 2010). Likewise in rats, facial

application of the pruritogens serotonin or chloroquine elicits primarily hindlimb scratching while the algogen mustard oil elicits forelimb wiping without scratching. Application of histamine or capsaicin to the rat face causes a mixture of wiping and scratching, depending on the concentration (Klein et al. 2011). Thus, application of pruritogens to the face in rodents appears to be a valid model for the study of human itch.

Pruritogens for experimental study

The face model of itch in rodents can be used to study stimuli which produce scratching via actions in the peripheral nervous system when delivered to the skin as well as those which cause scratching via actions in the central nervous system when delivered to the spinal cord or brainstem. Peripheral pruritogens used in the experiments discussed here include serotonin, chloroquine, BAM8-22, histamine, capsaicin, and cowhage (Table 1). The peripheral receptors, human psychophysics, and behaviors characterized using the rodent face model corresponding to each of these chemicals are listed in the table and will be discussed in more detail at appropriate sections throughout subsequent chapters. Importantly, note that for each chemical which causes itch in humans, itch can be accompanied by pain. Likewise, for each chemical which causes scratching in rodents, scratching can be accompanied by wiping. The only exception is chloroquine which causes scratching without wiping in rats; the corresponding human psychophysics cannot be directly compared since there

		Response to pruritogen		
Pruritogen	Receptor	Human	Mouse	Rat
Serotonin	5-$HT_{1D,2}$[1,2]	Itch>Pain[3-6]	Scratch>Wipe[7]	Scratch>Wipe[8,9]
BAM8-22	MrgpRC11[10]	Itch>Pain[11]	Scratch[12]	N.R.
Chloroquine	MrgpRA3[10]	N.R.	Scratch>Wipe[7,12]	Scratch[8]
Histamine	H1,4[13,14]	Itch>Pain[15-17]	Scratch>Wipe[7,18]	Wipe>Scratch[8]
Capsaicin	TRPV1[19]	Pain>Itch[16,17,20-22]	Wipe>Scratch[7,18]	Wipe=Scratch[8]
Cowhage (mucunain)	PAR-2,4[23]	Itch>Pain[17,23,24]	Scratch=Wipe[7]	None[8]

Table 1. *Pruritogens used in human and rodent studies.* Neural receptors implicated in the production of itch by each pruritogen are noted in column 2. Columns 3-5 indicate the sensations elicited in human psychophysics studies and behaviors elicited in animal experiments using the rodent face model. **1**: Berendsen and Broekkamp 1991; **2**: Yamaguchi et al. 1999; **3**: Weisshaar et al. 1997; **4**: Thomsen et al. 2002; **5**: Hosogi et al. 2006; **6**: Rasul et al. 2012; **7**: Akiyama et al. 2010; **8**: Klein et al. 2011; **9**: Spradley et al. 2012; **10**: Liu et al. 2009; **11**: Sikand et al. 2011; **12**: Wilson et al. 2011; **13**: Bell et al. 2004; **14**: Dunford et al. 2007; **15**: Simone et al. 1987; **16**: Schmelz et al. 2003; **17**: Sikand et al. 2009; **18**: Shimada and LaMotte 2008; **19**: Caterina et al. 1997; **20**: Simone et al. 1989; **21**: LaMotte et al. 1991; **22**: LaMotte 1992; **23**: Reddy et al. 2008; **24**: LaMotte et al. 2009.

exist no reports of chloroquine causing itch in humans when it is delivered to the skin. Rather, chloroquine is known to cause itch when administered orally as a treatment for malaria (Mnyika and Kihamia 1991; Sowunmi et al. 2000).

Several chemical stimuli induce scratching when administered to the central nervous system in rodents. These include bombesin (Lee et al. 2003; Su and Ko 2011), gastrin-releasing peptide (GRP) (Sun and Chen 2007; Su and Ko 2011; Mishra and Hoon 2013), natriuretic polypeptide B (Nppb) (Mishra and Hoon 2013), neuromedin B (Su and Ko 2011), and opioids such as morphine (Thomas and Hammond 1995; Lee et al. 2003). In rats, intracisternal injection of morphine causes small but significant dose-dependent increases in body scratching (Lee et al. 2003), and injection of morphine into the spinal trigeminal nucleus causes increased facial scratching (Thomas and Hammond 1995). These behavioral studies suggest that the rat facial somatosensory system may serve as a good model for providing insights into the mechanisms underlying opioid-induced pruritus.

Anatomical substrates for itch

In the peripheral nervous system, itch is mediated via unmyelinated or lightly myelinated nociceptive primary afferent fibers. In humans, itch can be evoked by activation of individual C fibers (Ochoa and Torebjörk 1989). Histamine activates a subset of mechanically insensitive, slowly conducting C fibers in humans with similar timing and intensity to the sensation of itch

produced by histamine (Schmelz et al. 1997, 2003; Namer et al. 2008). A subset of these histamine-responsive C fibers is also activated by serotonin (Schmelz et al. 2003). Nociceptors which respond to histamine and/or serotonin also respond to capsaicin (Schmelz et al. 2003; Namer et al. 2008). In rats, serotonin activates a subset of small diameter C fibers in dorsal root ganglia and trigeminal ganglia with a time-course similar to that of serotonin-induced scratching (Hachisuka et al. 2010; Klein et al. 2011). The majority of serotonin-responsive C fibers is activated by pinch; none is mechanically insensitive (Hachisuka et al. 2010). The algogens mustard oil and capsaicin also produce a response in the majority of C fibers activated by serotonin in rats (Klein et al. 2011).

Pruriceptive primary afferent fibers synapse onto neurons in the dorsal horn of the spinal cord. Subsets of neurons in superficial and deep layers of the dorsal horn in rats respond with an increase in discharge rate to chemical stimuli including histamine, serotonin, and capsaicin (Carstens 1997; Jinks and Carstens 2000, 2002). Responses to histamine are reduced by scratching as well as noxious heat or cold (Carstens 1997; Jinks and Carstens 1998a,b), stimuli which also reduce the sensation of itch in humans (Ward et al. 1996; Yosipovitch et al. 2007; Kosteletzky et al. 2009). Klein et al. (2011) describe neurons in the spinal trigeminal subnucleus caudalis which are responsive to the pruritogen serotonin applied to the rat cheek. The axonal projections of the neurons in these previous studies were not determined, making it difficult to interpret whether the studied cells play a role in producing the itch sensation.

Through the use of genetic and pharmacologic manipulations, significant advances have been made in elucidating the cellular and molecular mechanisms underlying itch circuitry within the dorsal horn. Scratching due to a variety of pruritogens is reduced in mice lacking the gastrin-releasing peptide receptor (GRPR) (Sun and Chen 2007). Scratching is also reduced by ablation of GRPR-containing neurons in the spinal cord (Sun et al. 2009). Pain behaviors were not affected in these studies, leading to speculation that GRPR-containing neurons constitute an itch-specific neural pathway. However, there is no direct evidence that GRP, the agonist for GRPR, is released from primary afferent terminals in the presence of pruritic stimuli. Koga et al. (2011) showed that activation of GRP-responsive dorsal horn neurons by somatosensory input is blocked by glutamate receptor antagonists. More recently, Nppb has been identified as a neurotransmitter expressed by primary afferent pruriceptors (Mishra and Hoon 2013). Dorsal horn neurons containing the Nppb receptor Npra are required for scratching induced by intradermal injection of histamine or intrathecal application of Nppb; scratching induced by GRP and pain-related behaviors remain intact. These data suggest that Nppb is released by primary afferent pruriceptors to activate Npra-containing interneurons in the dorsal horn, which in turn release GRP to activate GRPR-containing neurons to produce the sensation of itch. In addition to GRPR-containing neurons, cells containing receptors for other agonists such as neuromedin B or bombesin have been implicated in producing itch (Su and Ko 2011). The peptide substance P may also be important for itch

sensation, as ablation of neurons which contain the substance P neurokinin-1 (NK-1) receptor in the caudal medulla and rostral cervical spinal cord resulted in reduced scratching evoked by intradermal injection of serotonin in rats (Carstens et al. 2010). NK-1 receptors are located primarily in superficial layers of the dorsal horn, including within the spinal trigeminal subnucleus caudalis. Approximately one-fourth of rat spinal trigeminal subnucleus caudalis neurons which project to the thalamus contain NK-1 (Li et al. 1996). GRPR-containing neurons are also found primarily in superficial layers of the dorsal horn (Sun and Chen 2007; Liu et al. 2011b; Fleming et al. 2012), though it has not been demonstrated whether these neurons are projection neurons or local circuit neurons.

Spinothalamic tract (STT) neurons in the dorsal horn of the spinal cord carry information about noxious stimuli to the thalamus via the ventral lateral funiculus. Ventral lateral cordotomies block both itch and pain sensations in humans (White and Sweet 1969), suggesting an important role of the STT in carrying information used to produce the sensation of itch. A subset of nociceptive STT neurons in the monkey which responds to noxious mechanical and/or chemical stimuli, also responds to pruritogens applied to cutaneous receptive fields (Simone et al. 2004; Davidson et al. 2007, 2009, 2012). These pruriceptive STT neurons are inhibited by noxious stimulation of the receptive field during an ongoing response to a pruritogen (Davidson et al. 2009), and therefore likely contribute to producing the itch sensation for receptive fields on

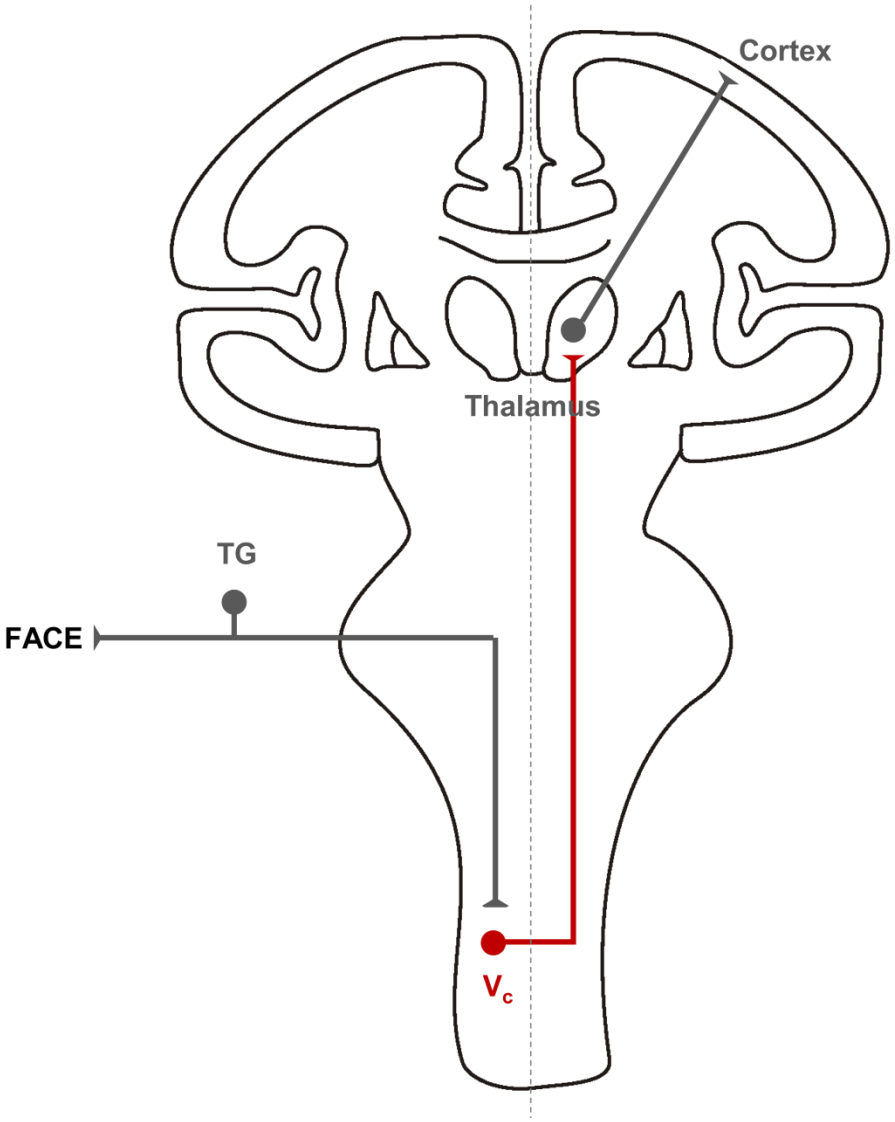

Figure 1. Simplified diagram of the neural pathway for information regarding facial application of noxious stimuli to reach the forebrain, including trigeminothalamic tract neurons (red). Additional processing by local interneurons in the medullary dorsal horn and thalamus likely occurs. TG: trigeminal ganglion; V_c: spinal trigeminal subnucleus caudalis.

the lower body. In the face, information about noxious stimuli is carried by the trigeminal nerve (cranial nerve V) to several targets in the brainstem, including the spinal trigeminal subnucleus caudalis. A subset of neurons in the spinal trigeminal nucleus carries information about noxious stimuli to the thalamus via the trigeminothalamic tract (VTT) (Willis et al. 1995) (Fig. 1). In awake monkeys, responses of VTT neurons in spinal trigeminal subnucleus caudalis to noxious stimuli correlate well with relevant behaviors elicited by these stimuli (Bushnell et al. 1984), indicating a role for these neurons in producing the sensation of pain and related behaviors. VTT neurons have not been tested for their responsiveness to pruritogens.

STT and VTT neurons terminate within the contralateral thalamus. Histamine and cowhage-responsive pruriceptive STT neurons in monkeys terminated within the posterior and ventrobasal thalamus, including ventroposterior lateral (VPL), posterior, supragenicular, and medial geniculate nuclei (Davidson et al. 2012). Nociceptive VTT neurons send projections to these same areas, though facial somatosensory input terminates in the ventroposterior medial (VPM) rather than the VPL nucleus. STT terminations within VPL are somatotopically organized and there exist neurons within VPL and VPM nuclei with relatively small receptive fields, suggesting that these areas may play a role in discriminating the location of noxious stimuli (Price and Dubner 1977). The ventroposterior nuclei contain neurons which can be antidromically activated by cortical stimulation (Kenshalo et al. 1980), indicating that STT and VTT input can

reach the cerebral cortex via neurons in the VPL or VPM nuclei, respectively. Nuclei from the posterior thalamus, including suprageniculate and medial geniculate nuclei, also send direct projections to the cortex (Kurokawa et al. 1990; Linke 1999; Linke and Schwegler 2000; Gauriau and Bernard 2004). Neurons in the posterior thalamus have large, often bilateral receptive fields with no somatotopic organization (Price and Dubner 1977). Spinal projection neurons which end in posterior thalamus may be involved in producing affective responses to noxious stimuli, as posterior thalamic nuclei project to subcortical limbic structures such as the amygdala (LeDoux et al. 1985; Kurokawa et al. 1990; Linke 1999; Linke and Schwegler 2000; Gauriau and Bernard 2004), a brain area implicated in fear conditioning in rats (LeDoux et al. 1986a,b; Shi and Davis 1999). The locations of axon termination for pruriceptive spinal projection neurons within the thalamus have not been identified in rodents.

Theories for decoding itch from pain

There are several theories which have been used to form hypotheses regarding the ability of the nervous system to decode itch from pain: labeled line theory, intensity theory, spike pattern theory, and population code theory. Labeled line hypotheses are based on the premise that there exists a complete neural circuit for contributing specifically to production of the itch sensation, and not contributing to production of the pain sensation. Important early discoveries to support the labeled line theory include primary afferent fibers in humans which

respond to histamine but not noxious mechanical input (Schmelz et al. 1997) and mechanically insensitive STT neurons in cats which responded to histamine but not mustard oil (Andrew and Craig 2001). The latter was based on a very small number of histamine-responsive STT neurons (n=2) that were not also responsive to mustard oil, and the distinct and powerful algogen capsaicin was not tested in the mechanically insensitive neurons in either study. A later study demonstrated that mechanically insensitive histamine-responsive C fibers were also activated by capsaicin (Schmelz et al. 2003), indicating that these putative itch-specific neurons may also contribute to pain. More recently, labeled line hypotheses have gained traction from evidence that GRP/GRPR may be involved in itch-specific signaling in the spinal cord (Sun and Chen 2007; Sun et al. 2009).

In contrast to the labeled line concept, hypotheses based on the remaining three theories (intensity, spike pattern, and population code) include neurons which process information about both itch and pain. According to intensity theory, low-frequency discharges signal itch while higher frequency discharges signal pain; this theory is not supported by findings that electrical stimulation of specific C fibers elicits itch which increases in intensity with increased stimulation, but does not change to pain (Tuckett 1982; Ikoma et al. 2005). In contrast to intensity theory, spike pattern theory does not take into account the intensity of stimulation or subsequent neural discharge, but rather posits that itch or pain is elicited by distinct patterns of action potentials within the same neuron. There is currently no

evidence to indicate that spike pattern theory can explain the coding of itch versus pain, though few studies have attempted to test relevant hypotheses (Tuckett 1982; Davidson et al. 2012). A final theory of itch signaling is based on population coding whereby the brain interprets incoming signals as either itch or pain signals based on the population(s) of neurons activated by a stimulus. A very important study by Han et al. (2013) showed that specific activation of itch-selective polymodal nociceptors, even by the normally distinctly painful stimulus capsaicin, results in itch-related behaviors in mice. These results are in accordance with a population code theory in which activation of the entire population of nociceptors results in pain, while activation of the pruriceptive subpopulation of nociceptors is interpreted by the brain as signaling itch, not pain.

Introduction to current work

A more complete characterization of pruriceptive versus nociceptive spinal neuron responses to the various neurotransmitters and neuromodulators involved in itch and pain processing would contribute toward resolving the discrepancies between the various models proposed for itch sensation. The rodent face model of itch is an ideal system for use in such experiments, as it allows the differentiation between itch versus pain-related behaviors. The following chapters include experiments designed to test hypotheses regarding possible mechanisms underlying itch versus pain-related behaviors in rats.

Chapter 2 describes data from experiments in which the face model of itch is used to characterize itch-related behaviors elicited by the peripheral pruritogen serotonin, the central pruritogen morphine, and combined delivery of these two stimuli. Chapter 3 examines pruriceptive and nociceptive responses in VTT neurons which may be involved in producing the behaviors examined in Chapter 2, and characterizes the recording locations, axon projection locations, and receptive fields of these neurons. Finally, Chapter 4 utilizes the VTT system to test hypotheses regarding the ability of morphine to induce itch while reducing pain, including its interactions with serotonin. The relationship between pain and itch pathways in the central nervous system has been a source of controversy. Results from our studies contribute to a better understanding of both the differences and similarities between these two sensory systems.

CHAPTER 2

Characterization of behaviors elicited by intradermal injection of serotonin, intracisternal injection of morphine, and combined delivery of each using the rat face model of itch and pain

Introduction

The unpleasant sensations of itch and pain are each mediated by nociceptive neurons which respond to a variety of noxious stimuli, yet the sensations are distinctly perceived. Itch causes the desire to scratch and pain causes discomfort which results in withdrawal from or removal of a noxious stimulus. Therefore, when studying itch it is important to employ an animal model that allows the differentiation of itch- versus pain-related behaviors. Until recently, an important aspect lacking from behavioral studies of itch in rodents has been the demonstration of a differential response to pruritogens versus algogens. For example, in mice the pruritogen histamine injected into the nape of the neck produces scratching, but scratching is also elicited by capsaicin (Shimada and LaMotte 2008), a stimulus which in humans more commonly causes a distinct pain sensation (Simone et al. 1989; LaMotte et al. 1991; LaMotte 1992). To further complicate studies of itch in rodents, rats do not scratch in response to histamine when it is injected intradermally into the nape of the neck (Kuraishi et al. 1995; Thomsen et al. 2001; Jinks and Carstens 2002). Nevertheless, the nape of the neck has been the typical site for application of pruritogens in rodent studies. Given the discrepancies between human psychophysics and rodent behaviors described above, it has been speculated that the nonhuman primate is the ideal animal model for the study of human itch. However, it has more recently been shown that pruritogens and algogens elicit distinct behaviors when applied to the rodent cheek. In mice, when chemicals are applied to the skin on the face,

histamine elicits scratching with the hindlimb while capsaicin elicits wiping with the forelimb directed to the site of chemical application (Shimada and LaMotte 2008). This distinction between itch-evoked scratching and pain-evoked wiping has been replicated with an array of other pruritogens in mice (Akiyama et al. 2010; Wilson et al. 2011) and rats (Klein et al. 2011; Spradley et al. 2012).

One of the most effective pruritogens found using the rat face model is serotonin (Klein et al. 2011). In humans, the itch sensation can be produced by application of serotonin to the skin (Fjellner and Hägermark 1979; Weisshaar et al. 1997; Thomsen et al. 2002; Hosogi et al. 2006; Rasul et al. 2012). In several conditions involving itch, including allergic contact dermatitis and atopic dermatitis the skin of patients exhibits increased levels of serotonin (Lundeberg et al. 1999; Soga et al. 2007). Serotonin can also elicit pain in humans (Schmelz et al. 2003). Accordingly, when applied to the rat face, serotonin elicits scratching with the hindlimb as well as wiping with the forelimb (Klein et al. 2011). It has been suggested that serotonin may cause itch in humans via serotonin-induced release of histamine from mast cells (Weisshaar et al. 1997), though administration of antihistamines failed to result in a significant reduction of serotonin-induced itch compared to placebo treatment (Hosogi et al. 2006). In rats, mast cells contain very little histamine but instead degranulate to release a large amount of serotonin into the skin (Wallengren 1993). There is evidence that serotonin-induced scratching in rodents maybe involve both 5-HT_{1D} and 5-HT_2 receptors in the skin (Berendsen and Broekkamp 1991; Yamaguchi et al. 1999).

Together, these data support the use of serotonin as a peripheral pruritogen in humans and rodents.

In addition to pruritogens which cause itch via activation of peripheral nociceptors, there are a number of agents which cause itch-related behaviors when administered to the central nervous system in animal models (Koenigstein 1948; Thomas and Hammond 1995; Lee et al. 2003; Sun and Chen 2007; Su and Ko 2011; Mishra and Hoon 2013). One of the most frequently studied central pruritogens is morphine. Morphine is commonly prescribed for relief from chronic pain, but side-effects, including severe itch, can limit the maximum tolerable dose and thus the effectiveness of morphine for producing analgesia. Opioid-induced pruritus is often localized to facial regions of patients (Scott et al. 1980; Baraka et al. 1981; Collier 1981; Bromage et al. 1982), suggesting the value of using the rodent face model of itch to study this phenomenon. The highest incidence of opioid-induced pruritus in human patients (20-100%) occurs following intrathecal administration (Baraka et al. 1982; Bromage et al. 1982; Ballantyne et al. 1988; Szarvas et al. 2003; Ganesh and Maxwell 2007). Intracisternal injection of morphine in rats causes robust body and facial scratching (Lee et al. 2003) as does injection of morphine within the spinal trigeminal nucleus (Thomas and Hammond 1995). Thus, the rat trigeminal system appears to be valuable for studies of the mechanisms underlying opioid-induced pruritus.

Morphine and other pharmacological agents which act at μ-opioid receptors appear to modulate itch caused by other stimuli. Spradley et al. (2012)

showed that the μ-opioid receptor antagonist naltrexone reduced scratching caused by facial application of pruritogens while morphine reduced wiping caused by algogens in rats. This finding suggests that μ-opioid receptor activation has opposite effects on processing of itchy versus painful stimuli applied to the face. Opioids such as morphine likely also play a role in producing other itch-related sensory phenomena such as hyperknesis (increased itch caused by pruritogens) (Fjellner and Hägermark 1982; Onigbogi et al. 2000) and alloknesis (itch caused by innocuous mechanical stimuli that normally do not cause itch) (Koenigstein 1948; Heyer et al. 2002).

Here, we utilize the face model of itch to establish dose-response curves for the induction of scratching by intradermal injection of serotonin and intracisternal injection of morphine in rats. We hypothesize that scratching induced by serotonin involves activation of opioid receptors. We predict that activation of opioid receptors by intracisternal injection of morphine leads to hyperknesis in response to serotonin and that this interaction is a superadditive or synergistic effect.

Materials & Methods

Animals. Adult male Sprague-Dawley rats (200-300 g) were used according to protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Animals arrived at the university at least one week prior to testing. At least two days prior to testing, animals

underwent light anesthesia with isoflurane (3% in 100% oxygen, <5 min) and their cheeks and necks were shaved.

Intradermal cheek injections. For intradermal cheek injections, awake rats were gently restrained using a transparent flexible plastic cone. One experimenter held an animal within the cone while another experimenter performed the injection. Drugs for intradermal injection included serotonin creatinine sulfate complex (9-180 µg, Sigma), α-methylserotonin maleate salt (α-Me-5HT; 3-30 µg, Sigma), or 0.9% normal saline vehicle. Drugs were injected in a 10 µl volume using a 28-gauge hypodermic needle inserted into the skin on the face below the eye and caudal to the vibrissal pad. Intradermal injection was confirmed by observation of a small bleb at the injection site.

Intracisternal injections. Rats were lightly anesthetized using isoflurane (3% in 100%, <5 min) and intracisternal injections were performed according to Appel and Van Loon (1986). One experimenter held the animal's head between the thumb and forefinger of the left hand behind the animal's ears and suspended the animal with the right hand, maximally opening the foramen magnum at the base of the skull. Another experimenter performed the injection by inserting a 25-gauge 5/8-inch needle attached to a 100 µl Hamilton microsyringe through the shaved skin on the back of the neck until the needle came into perpendicular contact with the occipital bone. The needle was then moved ventrally, depressing muscles in the neck, until it could be inserted under the atlantooccipital membrane through the foramen magnum into the cisterna

magna. Drugs for intracisternal injection included morphine sulfate (0.01-333.0 μg, Sigma) or 0.9% normal saline vehicle. Drugs were injected in a 20 μl volume. After behavioral testing, several animals used in intracisternal injection protocols received an intracisternal injection of methylene blue (2%, 20 μl) prior to being euthanized and perfused with 0.9% normal saline. Intracisternal placement was confirmed by the presence of blue dye under the dura of the caudal medulla and/or rostral cervical spinal cord.

Intraperitoneal (i.p.) or subcutaneous (s.c.) injections of morphine (1 mg/kg, i.p.) or naloxone (1 mg/kg, i.p.; 0.5 mg/kg, s.c.) were administered 10 min prior to subsequent intradermal and/or intracisternal injections. When intradermal serotonin and intracisternal morphine were delivered in combination, intracisternal injections occurred within 5 min prior to intradermal injections.

Behavior protocol. One week before testing began, animals were habituated for 60 min/day for 5 days to an acrylic chamber (15x15x23 cm) located in a lighted room. On the following test days, before intradermal or intracisternal injection, animals were habituated for 15 min before baseline activity was video-recorded for 30 min. Animals were videotaped from above; chambers were surrounded by angled mirrors to enable viewing of each part of the animals' body with a single recording. Two animals were tested at a time in separate side-by-side chambers. The mirrors surrounding the chambers prevented animals from viewing one another during testing. Animals were returned to chambers immediately after completion of intradermal or

intracisternal injections. Experimenters left the room while behavior was videotaped for ≥60 min.

Data analysis. Observers were blinded to treatment(s) during observation of previously recorded video tapes. Approximately one-third of videotapes was scored by >1 observers and a test for the joint-probability of agreement showed a high degree of inter-rater reliability (Yelton et al. 1977). For videos which were scored by >1 observers, scores determined by the observers were averaged. Scratching, wiping, and grooming behaviors were recorded. Scratching was recorded in bouts because previous studies have demonstrated the number of scratch bouts to be the most robust and reliable measure of scratching behavior (Nojima and Carstens 2003; Klein et al. 2011). A scratch bout consisted of scratching an area of the head with the hindlimb; a scratch bout began when the hindlimb was first directed toward the head and ended when the hindpaw was placed on the ground or in the animal's mouth. Individual wipes were recorded, with a wipe beginning when the forelimb was first directed to the face and ending when the forelimb was brought away from the face (often to be placed in or near the mouth). Grooming behavior was defined as bilateral movement of the foreblimbs over the head (including the face), with a bout beginning when the forelimbs were first directed toward the face and ending when the forelimbs were held in or near the mouth or oral grooming behavior was directed toward other body areas. For intradermal injections of serotonin or α-Me-5HT, only scratch bouts or wipes directed toward the site of intradermal injection were counted (Fig.

2,3). For intracisternal injections of morphine and for all data included in the isobolographic analysis, scratch bouts or wipes directed to any area of the head or neck rostral to the shoulders were counted (Fig. 5,6). Data are expressed as mean ± standard error of the mean (SEM). Student's t-test or one-way ANOVAs with Tukey post tests were used to compare effects across treatments, with $p<0.05$ considered significant.

For isobolographic analysis of drug interactions when intradermal injection of serotonin was combined with intracisternal injection of morphine (Fig. 6), the amount of each drug needed to elicit 50% of the maximum number of scratch bouts in 60 min (ED_{50}) is represented by the y-intercept for intradermal injection of serotonin or x-intercept for intracisternal injection of morphine. These points are connected by the theoretical additive line representing the combined amounts of intradermal injection of serotonin and intracisternal injection of morphine expected to yield 50% of the maximum effect. The theoretical additive ED_{50} and its 95% confidence interval are plotted spanning the theoretical additive line. The observed ED_{50} for combined delivery of serotonin and morphine and confidence interval is also plotted for comparison to the theoretical ED_{50}. Isobolographic analyses were performed according to Tallarida (2001).

Results

Intradermal injections of serotonin into the face of rats elicited dose-dependent scratching with the hindlimb and, to a lesser degree, wiping with the

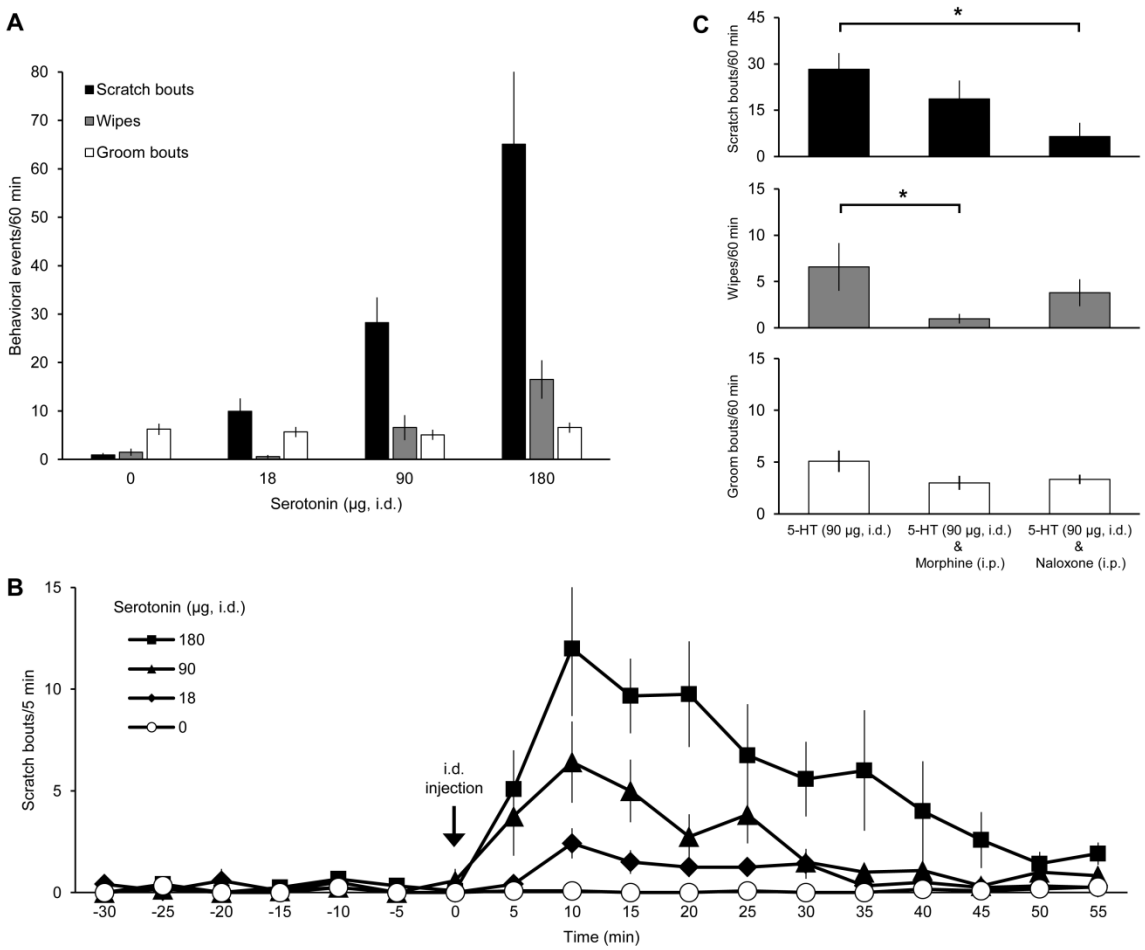

Figure 2. Effects of intradermal injection of serotonin on facial scratching, wiping, and grooming. ***A***, dose-response data for scratch bouts, wipes, and groom bouts elicited by intradermal (i.d.) injection of serotonin in the cheek (n=12). Only scratch bouts and wipes directed to site of i.d. injection are included. Control application of drug vehicle denoted as 0 µg serotonin. ***B***, time course for scratching evoked by different amounts of serotonin (data from *A*). Scratch bouts were averaged in 5 min bins beginning 30 min before i.d. injection ("-30"). ***C***, mean number of each behavioral event elicited by serotonin (5-HT) after intraperitoneal (i.p.) administration of morphine (n=6) or naloxone (n=6). * indicates statistically significant difference between groups denoted by black bar (p=0.015 for scratch bouts elicited by 5-HT (i.d.) vs. 5-HT (i.d.) & Naloxone (i.p.), p=0.049 for wipes elicited by 5-HT (i.d.) vs. 5-HT (i.d.) & Morphine (i.p.); one-way ANOVA with Tukey post test).

forelimb directed toward the site of injection (Fig. 2*A*). The number of grooming bouts did not differ across doses of serotonin. The time course of serotonin-induced scratching is depicted in Figure 2*B*. For each dose of serotonin tested, scratching peaked 10 min after serotonin injection. For the greatest dose tested (180 µg), scratching returned to baseline levels within 50 min. In order to test whether scratching induced by serotonin involves activation of opioid receptors, naloxone (1 mg/kg, i.p.) was administered prior to intradermal injection of serotonin (90 µg). Naloxone significantly decreased the number of scratch bouts induced by serotonin (Fig. 2*C*). When morphine (1 mg/kg, i.p.) was administered prior to serotonin, the number of wipes elicited by serotonin was significantly reduced (Fig. 2*C*); there was no significant effect of intraperitoneal application of morphine on serotonin-induced scratching. Neither morphine nor naloxone affected grooming induced by serotonin.

The 5-$HT_{1/2}$ receptor agonist α-Me-5HT was also administered intradermally in the face to test for its effects on scratching. Unlike serotonin, α-Me-5HT is not enzymatically degraded by monoamine oxidase and therefore has a longer duration of action compared to serotonin (Sourkes et al. 1990). α-Me-5HT produced a dose-dependent increase in scratching and wiping with no significant effect on grooming (Fig. 3*A*). For α-Me-5HT-induced scratching, the time of peak scratching occurred 15 min after injection and scratching had not returned to baseline by the end of the recording period (60 min after injection) (Fig. 3*B*).

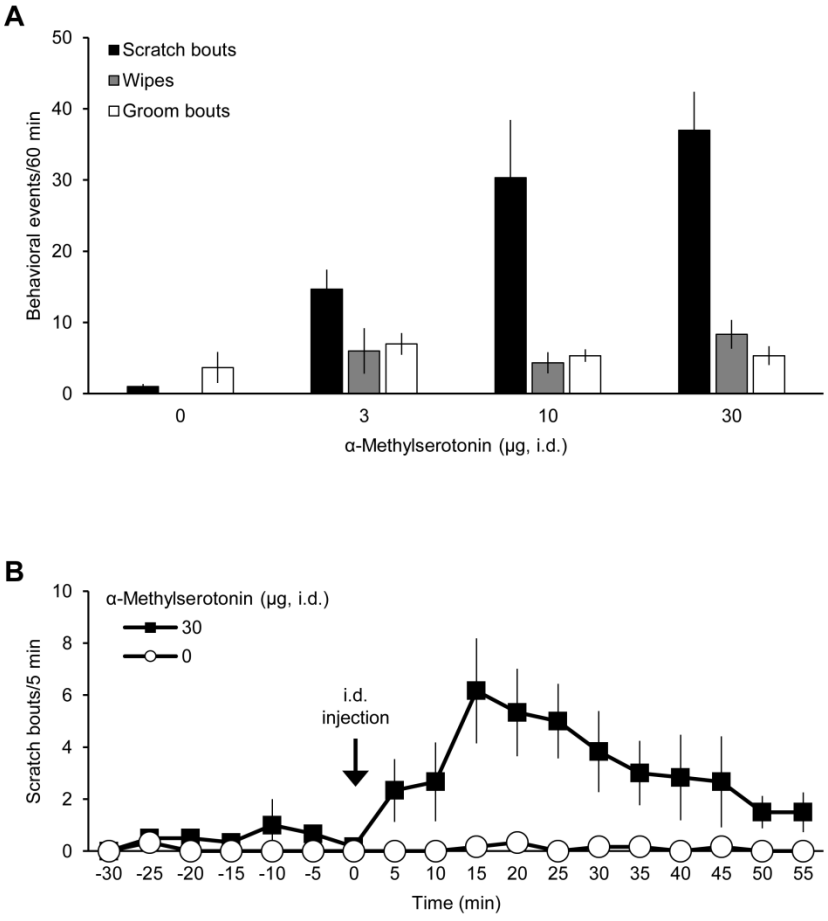

Figure 3. Effects of intradermal injection of α-methylserotonin on facial scratching, wiping, and grooming. ***A***, dose-response data for scratch bouts, wipes, and groom bouts elicited by intradermal (i.d.) injection of α-Me-5HT in the cheek (n=6). Only scratch bouts and wipes directed to site of i.d. injection are included. ***B***, time course for scratching evoked by α-Me-5HT (data from *A*).

To test the effects of opioid receptor activation in the central nervous system on scratching, wiping, and grooming, morphine was administered intracisternally. After anesthetization with isoflurane and intracisternal injections, animals awoke and returned to normal movements within 2 ± 1.1 min. In all of the 15 animals which received intracisternal injection of methylene blue dye, post-mortem analysis indicated that the dye was concentrated under the dura over the caudal medulla and rostral cervical spinal cord (Fig. 4). These results suggest that in the majority of intracisternal injections, the needle punctured the dura and the injected drug was administered to the desired area of the nervous system.

Intracisternal injection of morphine produced a dose-dependent increase in scratching (up to 1 μg) of the head and neck rostral to the shoulders (Fig. 5*A*). The highest dose of morphine tested (3.3 μg) resulted in a decreased number of scratch bouts compared to lower doses (0.33-1 μg). Grooming was also decreased at the highest dose, suggesting a possible sedative effect. Morphine did not affect wiping compared to baseline levels. Scratching began to increase within 5 min after intracisternal injection of morphine and remained elevated up to 60 min afterwards (Fig. 5*B*). When naloxone (0.5 mg/kg, s.c.) was administered prior to morphine injection, scratching and grooming behaviors were each significantly reduced (Fig. 5*C*), demonstrating that these effects of morphine involve opioid receptor activation.

In the last set of experiments, we wished to determine whether combined delivery of intradermal injection of serotonin and intracisternal injection of

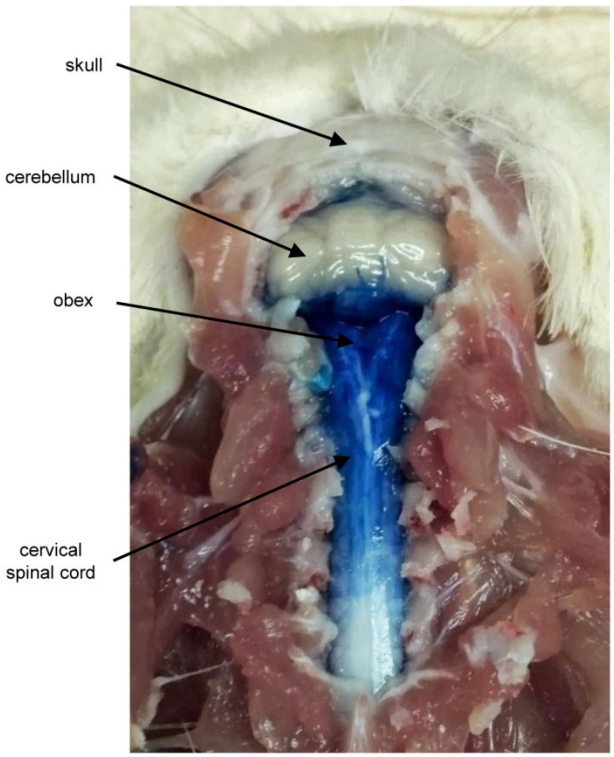

Figure 4. Distribution of methylene blue dye after intracisternal injection.

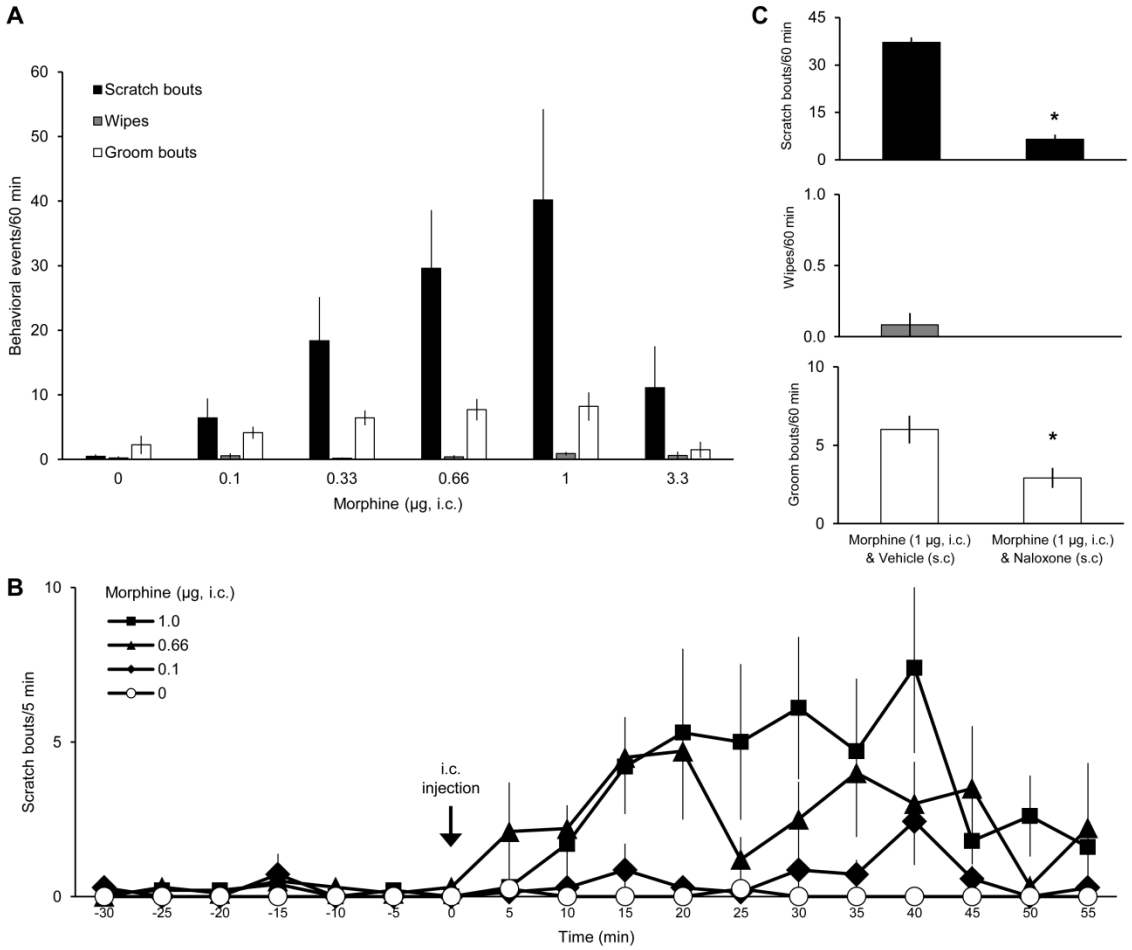

Figure 5. Effects of intracisternal injection of morphine on facial scratching, wiping, and grooming. ***A***, dose-response data for scratch bouts, wipes, and groom bouts elicited by intracisternal (i.c.) injection of morphine (n=7-11). All scratch bouts and wipes directed to the head or neck rostral to the shoulders are included. ***B***, time course for scratching evoked by different amounts of morphine (data from *A*). ***C***, mean number of each behavioral event elicited by morphine after subcutaneous (s.c.) administration of naloxone (n=12). * indicates statistically significant difference from Vehicle (s.c.) ($p<0.0001$ for scratch bouts, $p=0.01$ for wipes; Student's t-test).

morphine results in hyperknesis. For these experiments, all scratching with the hindlimb directed to the head and neck rostral to the shoulders was counted. When morphine was administered intracisternally (0.1 μg) prior to intradermal injection of serotonin (90 μg), the number of scratch bouts was significantly increased compared to either morphine or serotonin alone (Fig. 6*A*). We next tested several combinations of intradermal injection of serotonin and intracisternal injection of morphine to establish a dose-response curve for scratching induced by the drugs administered in combination (Fig. 6*B*). When administered in combination, the amount of either drug necessary to produce a maximal scratching effect was less than the amount of either drug required alone. The increase in scratching caused by combined delivery of serotonin and morphine was attenuated by naloxone (0.5 mg/kg, s.c.).

In order to test whether the scratching induced by combined delivery of intradermal injection of serotonin and intracisternal injection of morphine was simply an additive effect, we performed an isobolographic analysis (Fig. 6*C*). The ED_{50} of combined delivery of intradermal injection of serotonin and intracisternal injection of morphine was significantly less than the theoretical additive ED_{50}. Therefore, it can be concluded that when intradermal injection of serotonin and intracisternal injection of morphine are administered in combination, the resulting increase in scratching is a superadditive effect.

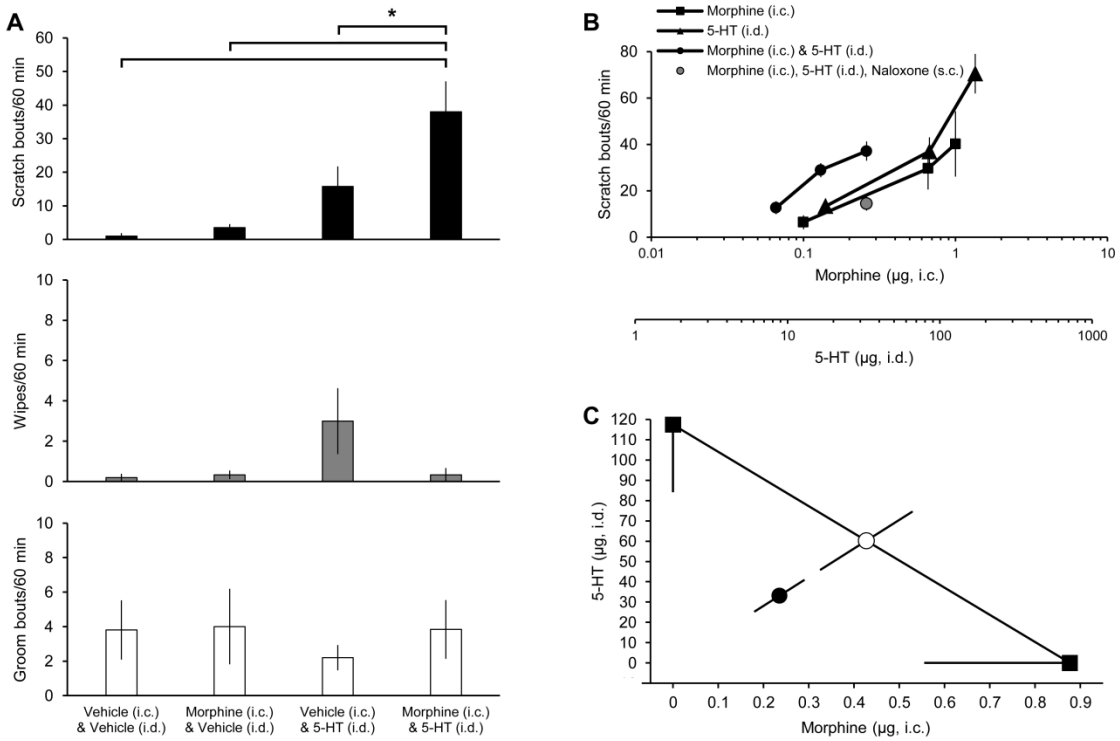

Figure 6. Effects of combined delivery of serotonin and morphine on facial scratching, wiping, and grooming. ***A***, scratch bouts, wipes, and groom bouts elicited by intradermal (i.d.) injection of serotonin (5-HT; 90 µg) (or vehicle) combined with intracisternal (i.c.) injection of morphine (0.1 µg) (or vehicle) (n=6). All scratch bouts and wipes directed to the head or neck rostral to the shoulders are included. * indicates statistically significant difference between groups denoted by black bar (p=0.0005 for scratch bouts elicited by Morphine (i.c.) & 5-HT (i.d.) vs. Vehicle (i.c.) & Vehicle (i.d.), p=0.0006 for Morphine (i.c.) & 5-HT (i.d.) vs. Morphine (i.c.) & Vehicle (i.d.), p=0.02 for Morphine (i.c.) & 5-HT (i.d.) vs. Vehicle (i.c.) & 5-HT (i.d.); one-way ANOVA with Tukey post test). ***B***, dose-response curves for scratch bouts elicited by 5-HT (i.d.) (n=12), morphine (i.c.) (n=7-11), or combined delivery of 5-HT (i.d.) and morphine (i.c.) (n=6-8). X-axes denote dose of i.c. injection of morphine (top) and/or i.d. injection of 5-HT (bottom). ***C***, isobolographic analysis using 50% of the maximum scratching effect from the data in *B*. The y-intercept represents the ED_{50} for 5-HT (i.d.) while the x-intercept represents the ED_{50} for morphine (i.c.). The observed ED_{50} for combined delivery of 5-HT (i.d.) and morphine (i.c.) (●) was significantly lower ($p<0.05$, t-test) than the theoretical additive ED_{50} (○), indicating that the increase in scratching was superadditive or synergistic.

Discussion

We utilized the face model of itch to characterize the behaviors elicited by intradermal injection of serotonin in the face and intracisternal application of morphine in rats. Intradermal injection of serotonin produced dose-dependent itch-related scratching and pain-related wiping. Scratching was reduced by naloxone and wiping was reduced by morphine. Intracisternal morphine produced dose-dependent scratching as well as a slight increase in grooming behavior. Both scratching and grooming were reduced by naloxone. When delivered in combination, intradermal injection of serotonin and intracisternal injection of morphine produced a synergistic increase in scratching which was blocked by naloxone. Together, these data suggest that opioid receptor activation is involved in scratching produced by either serotonin or morphine and that systems which contain serotonin receptors and opioid receptors interact to modulate the sensation of itch.

The dose-response curve established here for scratching induced by intradermal injection of serotonin is nearly identical to that reported by Klein et al. (2011), with the maximum effect (65.1 ± 16.5 scratch bouts/60 min for the current data and 60.1 ± 6.3 scratch bouts/60 min by Klein et al.) elicited by 180 µg/10 µl (47 mM) serotonin. Likewise, the time course for serotonin-induced scratching is the same; Klein et al. report that serotonin produces scratching which peaks 10-15 min after intradermal injection and returns to baseline levels within 60 min. Our finding that scratching induced by α-Me-5HT has a longer duration than

scratching induced by serotonin is consistent with α-Me-5HT having a longer duration of action due to not being degraded by monoamine oxidase (Sourkes et al. 1990). α-Me-5HT may be a suitable experimental pruritogen for use in protocols requiring a longer duration of scratching than that induced by serotonin. In a comparison of eleven various chemical stimuli, only the chemical formalin produced a maximum level of scratching that was greater than that produced by serotonin (Klein et al. 2011). As seen with serotonin, nearly all chemicals tested (except chloroquine) which produced an increase in scratching also produced an increase in wiping. A notable difference was found for wiping produced by intradermal injection of serotonin, with Klein et al. reporting no significant difference from vehicle (4.1 ± 2 wipes/60 min) while our data report a significant increase in wiping (16.4 ± 4 wipes/60 min) at this same dose (180 μg/10 μl or 47 mM). The pain-related wiping behavior in response to pruritogens such as serotonin may be analogous to the increase in noxious sensations such as burning and stinging which often accompanies the application of pruritogens in humans (Schmelz et al. 2003; Sikand et al. 2009). Together with these previous studies, our data further support the use of serotonin as a pruritogen in rat studies.

It has previously been demonstrated that morphine causes significantly increased scratching when delivered intracisternally but not intrathecally (at the lumbar level) or intracerebroventricularly in rats (Lee et al. 2003). The highest dose of morphine used by Lee et al. (0.1 μg) corresponds to the lowest dose

included on the dose-response curve for scratching induced by intracisternal morphine in our study. At the concentrations tested, morphine did not affect grooming in the study by Lee et al. It has also been shown that injection of morphine within the spinal trigeminal nucleus causes a robust increase in facial scratching in rats (Thomas and Hammond 1995). The area of the spinal trigeminal nucleus which was injected by Thomas and Hammond (1995) corresponds to the level of the brainstem/spinal cord targeted by intracisternal injections in our study. Thus, it is possible that the scratching induced by morphine in our study is due to effects localized to the spinal trigeminal nucleus, as suggested by Thomas and Hammond.

Our current findings suggest that endogenous opioid systems may be involved in modulating behavioral responses to application of pruritogens to the face. We show that naloxone reduces the number of scratch bouts elicited by serotonin, a finding which has been previously shown using the opioid receptor antagonist naltrexone (Spradley et al. 2012). μ-Opioid receptor antagonists have been used to reduce itch produced by several pruritogens in a variety of human conditions (Phan et al. 2010). It has even been suggested that in rodent models, reduction by μ-opioid receptor antagonists be used as a defining criterion for itch-related behaviors while reduction by μ-opioid receptor agonists be used as a defining criterion for pain-related behaviors (Nojima and Carstens 2003; Akiyama et al. 2010). In our study morphine was effective at reducing serotonin-induced wiping, though this is not in accordance with previous studies (Spradley et al.

2012). In our study, morphine produced an increase in grooming which was reduced by naloxone. Similarly, treatment with naltrexone results in attenuated grooming behavior (Spradley et al. 2012).

In order to study the combined effects of intradermal injection of serotonin and intracisternal injection of morphine on itch, we performed an isobolographic analysis which showed that when these two drugs are delivered in combination, they have a superadditive effect on scratching. This result suggests that in addition to serotonin and morphine causing scratching separately from one another, the neural circuits containing receptors for either drug interact to modulate scratching behavior. Although in the rat model it has not been established whether serotonin and morphine produce itch via peripheral and/or central actions, it is likely that serotonin is activating peripheral pruriceptors (Berendsen and Broekkamp 1991; Yamaguchi et al. 1999) while morphine causes itch via actions in the central nervous system (Ko et al. 2004; Kuraishi et al. 2008). It is possible that morphine works within the central nervous system (e.g. the spinal trigeminal nucleus) to sensitize input from peripheral pruriceptors such that intradermal injection of serotonin causes enhanced itch when morphine is present.

In summary, we have established dose-response curves for scratching induced by intradermal injection of serotonin and intracisternal injection of morphine and show that when these drugs are delivered in combination, the increase in scratching is a superadditive effect. The rat face model provides an

excellent system for the study of these stimuli, as itch and pain-related behaviors can be readily distinguished. However, the anatomical and physiological substrates of these phenomena in the rat remain poorly understood. Given its involvement in pain processing and modulation by opioids, the spinal trigeminal system is a good candidate for beginning to explore the mechanisms underlying facial itch.

CHAPTER 3

Characterization of pruriceptive trigeminothalamic tract neurons in rats

Introduction

Itch and pain are experienced as distinct sensations. Itch causes the desire to scratch and pain typically results in protective behaviors such as withdrawal from painful stimuli. A substantial body of evidence indicates that neurons which respond to pruritogens comprise a subset of a population of cells which also respond to painful stimuli (Carstens 1997; Drzezga et al. 2001; Jinks and Carstens 2002; Simone et al 2004; Davidson et al. 2007; Johanek et al. 2008). However, several studies have suggested that itch and pain are processed by separate neurons and may even involve separate types of receptors (Sun and Chen 2007; Liu et al. 2009; Sun et al. 2009; Wilson et al. 2011). Recently, it has been demonstrated that although itch-responsive neurons also respond to painful stimuli, selective activation of these neurons, even by a painful stimulus such as capsaicin, results only in itch-related behaviors (Han et al. 2013), indicating that itch and pain information may indeed be processed by separate populations of neurons.

In rodent studies of the neural mechanisms contributing to itch and pain, pruritogens and algogens that have been used are based on corresponding itch and pain sensations evoked in humans. In order to study the underlying mechanisms and relationships between itch and pain, it is important to employ experimental models that elicit distinct behavioral responses to each sensation. In many rodent studies pruritogens have been applied to the skin on the nape of the neck, a body region which can only be accessed by the hindlimb (e.g. for

scratching) and therefore does not allow study of other potentially relevant behaviors. In the rostral back, algogens and pruritogens each elicit scratching and it is not possible to behaviorally discriminate itchy from painful stimuli. In contrast, application of pruritogens or algogens can elicit distinct behavioral responses when applied to the face in mice (Shimada and LaMotte 2008). In this face model of itch, pruritogens applied to the cheek elicited scratching with the hindlimb while algogens elicited wiping with the forelimb. More recently the face model has been used to demonstrate that rats also show distinct behavioral responses to stimuli that produce itch or pain in humans (Klein et al. 2011). When applied to the face, serotonin and chloroquine elicit scratching with the hindlimb while the algogen mustard oil elicits wiping with the forelimb. Interestingly, facial application of histamine or capsaicin causes a mixture of scratching and wiping in rats, depending on the concentrations used. These chemicals can also produce mixed sensations in humans (Sikand et al. 2009).

The differences between the face model and the rostral back model of itch in rodents highlight the usefulness of studying itch using the face model and examining underlying neural mechanisms within the trigeminal sensory system. However, few studies have focused on this approach (Akiyama et al. 2010; Klein et al. 2011). The neurons in the spinal trigeminal nucleus which convey information to the forebrain about itch occurring on the face have not been identified or characterized. Information about itch in body regions below the head and neck is carried to the brain by cells in the spinothalamic tract (STT), in

addition to other possible tracts that have not been examined. In humans, lesions of the venral lateral quadrant of the spinal cord (including STT axons) abolish both itch and pain sensations (White and Sweet 1969). In nonhuman primates, a subset of STT cells responds with an increase in discharge rate to the pruritogens histamine or cowhage (Simone et al. 2004; Davidson et al. 2007, 2012). These responses are decreased during scratching of the receptive field (Davidson et al. 2009), suggesting that these neurons are involved in producing the sensation of itch. In the face, information about pruritic and algogenic stimuli is carried by the trigeminal nerve (cranial nerve V) to several targets in the brainstem, including the spinal trigeminal subnucleus caudalis (Willis et al. 1995). A subset of neurons in this nucleus sends axons to the thalamus via the trigeminothalamic tract (VTT). We propose that, like the STT for lower body receptive fields, the VTT carries information regarding facial itch to the forebrain. Here, we have examined the responses of antidromically identified VTT neurons to the application of several pruritogens applied to facial receptive fields in anesthetized rats. The pruriceptive and nociceptive responses, receptive fields, recording points, and axon projections of these cells were characterized.

Materials & Methods

Animal preparation. Adult male Sprague-Dawley rats (300-450 g) were used according to protocols approved by the University of Minnesota's Institutional Animal Care and Use Committee. Animals were deeply anesthetized

with urethane (1.5 mg/kg, i.p.; Sigma) and tracheostomized. A laminectomy was performed over the first and second cervical segments and a craniotomy was performed over the right thalamus. A low-impedance stainless steel electrode was positioned at stereotaxic coordinates for the caudal pole of the ventroposterior medial (VPM) nucleus in the thalamus. Pulses of electrical current (300-500 μA, 200 μs, 3 Hz) were delivered through the electrode as an initial search stimulus. A recording electrode (stainless steel, 10 MΩ; FHC for most cases or carbon fiber, 4-10 MΩ for some experiments) was lowered through the contralateral spinal trigeminal nucleus extending from the caudal medulla to the second cervical segment of the spinal cord to search for time-locked single unit responses which met the following standard criteria for antidromically-activated VTT neurons: 1) stable antidromic response latency (<0.05 ms variation); 2) ability to follow high frequency (>300 Hz) antidromic stimulus train; 3) collision of mechanically-evoked orthodromic spike with putative antidromic spike. Any single unit response that met these criteria and had a cutaneous facial receptive field was used for further study, although neurons responsive to noxious stimulation of the receptive field were favored over those maximally responsive to low-threshold mechanical stimuli.

Axon projection mapping. The stimulating electrode was positioned at 200 μm intervals throughout dorsal-ventral stimulating tracks separated by medial-lateral and rostral-caudal intervals of 300-500 μm in the brain; the amount of current necessary to elicit an antidromic spike was determined at each position.

The point at which the threshold for antidromic activation was lowest (≤30 μA) was assumed to be the most accurate indication of the position of the axon terminal (Burstein et al. 1991; Dado et al. 1994). If the antidromic latency remained unchanged by ≤0.05 ms at more rostral positions, it was assumed that the axon did not extend rostrally beyond the identified low threshold point.

Characterization of neurons with mechanical and thermal stimuli. Innocuous brushing with a soft-bristled brush and the minimum necessary amount of noxious stimuli were used to identify the mechanical receptive field boundaries for each antidromically-identified VTT neuron. Brushing, and pressure and pinch applied with small clips were used to classify each neuron as low threshold (LT; maximally responsive to innocuous brushing), high threshold (HT; responsive only to noxious pressure and/or pinching), or wide dynamic range (WDR; responsive to innocuous and noxious stimuli, with a higher frequency response to noxious stimuli). Thermal responses were tested by applying a noxious heat stimulus (50° C from a 32° C baseline) to the center of the mechanical receptive field with a feedback controlled Peltier thermode (3 mm^2; Yale Instruments).

Pruritic characterization. After responses to mechanical and thermal stimuli were obtained, sensitivity of each cell to the following chemical stimuli was determined: histamine dihydrochloride (HA, 900 mM; Sigma), serotonin creatinine sulfate complex (5-HT, 47 mM; Sigma), chloroquine diphosphate salt (CQ, 100 mM; Sigma), bovine adrenal medullary 8-22 peptide (BAM8-22, 1 mM;

Tocris Bioscience), capsaicin (CAP, 3.3 mM; Sigma), and/or cowhage (COW, ≥10 spicules). Concentrations were chosen within ranges that elicited scratching in the rat face model (Klein et al. 2011). BAM8-22 causes hindlimb scratching upon facial application in mice at an amount comparable to what was used in the current study (Wilson et al. 2011); to our knowledge, the behavioral effects of application of BAM8-22 to the rat face have not been reported. Each drug (except cowhage) was injected intradermally in a 10 µl volume with a 28-gauge needle at separate sites within the mechanical receptive field for each cell; for cowhage, ≥10 spicules were inserted within a small area of the receptive field using an applicator consisting of spicules attached to the end of a cotton-tipped swab. Vehicles (pH-matched saline for injected drugs and heat-inactivated spicules for cowhage (IA COW)) were always applied before their respective active stimuli. An attempt was made to test each cell with every drug however recording stability did not always allow this. Drugs were applied in random order on each trial, with the exception that capsaicin was always the last drug applied. A cell's ongoing discharge rate was always allowed to return to baseline prior to application of a subsequent stimulus, with a minimum of 5 minutes between drug applications.

Histology. At the end of each experiment, low threshold antidromic stimulation point(s) in the thalamus and recording point(s) in the spinal trigeminal nucleus were marked with electrolytic lesions. Rats were perfused with 0.9% normal saline followed by 10% formalin with 1% ferrocyanide (for Prussian blue

reaction at lesion sites). The brain was removed and cut on a freezing microtome; sections containing thalamus were sectioned by 75 μm and sections containing the spinal trigeminal nucleus were sectioned by 50 μm. Sections were stained with neutral red and a rat brain atlas (Paxinos and Watson 1982) was used to identify thalamic nuclei for axon projection sites.

Data analysis. A cell was considered responsive to a stimulus if it displayed a post-stimulus discharge rate ≥1.5 times the mean discharge rate over 60 sec before stimulus application and the increased discharge rate outlasted any response to vehicle. Individual response histograms are reported in 1 sec bins. Mean responses are reported in 1, 5, or 15 sec bins (specified in text) with errors bars representing standard error of the mean (SEM). For statistical analyses, discharge rates were normalized to 60 sec of baseline activity preceding stimulus application. For measures of variability in spike trains elicited by a pruritogen, spike timing data was analyzed over a 3 min period beginning 30 sec after application of the pruritogen (to avoid activity evoked by insertion of the needle during injection of the pruritogen) or during the entire 5-10 sec application of mechanical or thermal stimuli. The coefficient of variation (CV) of a spike train was calculated as the standard deviation of interspike intervals (ISIs) divided by the mean duration of ISIs within the spike train. For ISI distribution histograms, the number of ISIs contained in each of 20 log-scaled bins was divided by the total number of ISIs in a given spike train, and multiplied by 100 to obtain percentage; mean percent in each bin was calculated for responses to each

stimulus. For all statistical tests, effects across stimuli or cell types were compared using Wilcoxon rank sums or Kruskal-Wallis ANOVA analyses with Dunn's post-test, with $p<0.05$ considered significant.

Results

One hundred four VTT neurons from 76 rats were tested for responses to several pruritogens including serotonin, BAM8-22, chloroquine, histamine, capsaicin, and cowhage. Recording points of VTT neurons were located throughout the spinal trigeminal nucleus in the caudal medulla and first and second cervical segments of the spinal cord, and axons terminated in the contralateral thalamus. Every neuron identified using the antidromic stimulation methods employed in this study responded to mechanical stimulation of its receptive field; each time a mechanically-sensitive cutaneous receptive field could not be identified, a mechanically-sensitive intraoral, intranasal, or corneal receptive field was found to be present. Of the 90 VTT neurons which responded to noxious mechanical stimulation of the facial skin, 54% were classified as WDR and 46% as HT; 31% of the 77 neurons tested with a 50° C stimulus responded to noxious heat. VTT cells which responded to any of the scratch-inducing chemicals tested will be referred to as "pruriceptive" neurons; all pruriceptive VTT neurons also responded to noxious mechanical stimuli. Cells which responded to noxious mechanical or thermal stimulation but did not respond to any tested pruritogen will be referred to as "nociceptive-only" neurons. Fourteen VTT

neurons responded maximally to subnoxious mechanical stimuli and were classified as LT; none of the LT neurons was pruriceptive.

Pruriceptive VTT neurons. Figure 7 shows an example of a pruriceptive VTT neuron which responded to multiple chemicals injected intradermally within its cutaneous receptive field (Fig. 7*A*). The recorded unit was located in the superficial layers of the spinal trigeminal subnucleus caudalis near the level of the pyramidal decussation (Fig. 7*B*), and its axon projected to the posterior triangular (PoT) nucleus of the contralateral thalamus, as evidenced by antidromic activation of the cell by 20 μA pulses applied to the PoT nucleus but not at levels rostral to the PoT (Fig. 7 *C,D*). This cell was classified as HT, as it responded selectively to noxious pressure and pinch (Fig. 7*E*) applied to its receptive field. This cell did not respond to a 50° C heat stimulus (Fig. 7*F*). Each of the six chemical stimuli employed in this study were consecutively tested on this neuron. The cell responded to intradermal injection of vehicle while the needle was in the skin, but there was no sustained response to vehicle after the needle was removed (Fig. 7*G*). Likewise, the placement of the needle into the skin during the injection of capsaicin caused a short discharge, but unlike vehicle, capsaicin produced a response that lasted >10 min following removal of the needle (Fig. 7*H*). Injection of BAM8-22 elicited an increased discharge rate for >40 minutes (Fig. 7*I*). The cell did not respond to serotonin, chloroquine, histamine, or cowhage (Fig. 7*J-M*).

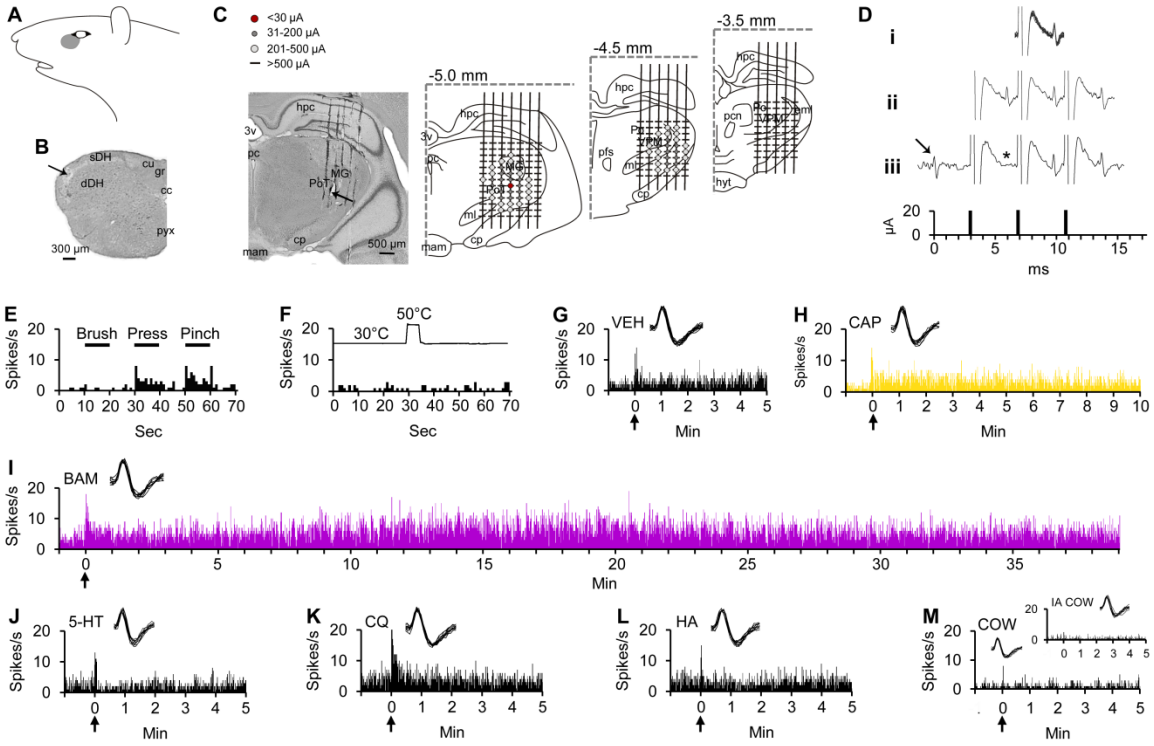

Figure 7. Characterization of a pruriceptive VTT neuron responding to BAM8-22 and capsaicin. ***A***, receptive field. ***B***, lesion (arrow) made at the recording point in the superficial layers of the spinal trigeminal nucleus. ***C***, lesion (arrow) made at the point in the thalamus with the lowest threshold for antidromic activation. Drawings indicate the location of each antidromic test site at three rostral-caudal planes within the thalamus; threshold for antidromic activation at each point is indicated by the color/size of the marker at each point (see legend). Numbers above each drawing indicate distance caudal to Bregma. ***D***, antidromic spikes have a fixed latency from the antidromic stimulus (i), follow a high-frequency stimulus train (ii), and an orthodromic spike (arrow) collides with an antidromic spike (expected location indicated by *) (iii). ***E***, responses to mechanical stimulation of the receptive field (black bar above histogram indicates duration of mechanical stimulus). ***F***, response to thermal stimulation of the receptive field (trace above histogram indicates onset and duration of 50°C thermal stimulus). ***G***, intradermal injection (indicated by arrow) of vehicle into the receptive field produced no sustained response. ***H-I***, the cell responded to capsaicin and to BAM8-22, each injected intradermally into the receptive field. Responses are colored differently according to pruritogen, so that data regarding a single pruritogen can be more easily identified throughout the following figures. ***J-M***, the cell did not respond to intradermal injection of serotonin, chloroquine, or histamine, or to application of cowhage spicules (*M*, inset: response to heat-inactivated cowhage). Insets: 10 randomly selected overlaid spike traces. 3v: third ventricle; cc: central canal; cp: cerebral peduncle; cu: cuneate fasciculus; dDH: deep dorsal horn; eml: external medullary lamina; gr: gracile fasciculus; hpc: hippocampus; hyt: hypothalamus; mam: mammillary n.; MG: medial geniculate n.; ml: medial lemniscus; pc: posterior commissure; pcn: paracentral n.; pfs: parafascicular n.; Po: posterior thalamic n.; PoT: posterior triangular n.; pyx: pyramidal decussation; sDH: superficial dorsal horn; VPM: ventroposterior medial n.

An example of a pruriceptive cell that projected to the VPM nucleus in the contralateral thalamus is illustrated in Figure 8. This neuron had a mechanical receptive field superior to the eye (Fig. 8*A*). It was recorded from superficial layers of the spinal trigeminal subnucleus caudalis at the border between the first and second cervical segments of the spinal cord (Fig. 8*B*). The location from which its axon was antidromically activated is shown in Figure 8*C*. This cell was classified as HT (Fig. 8*D*) and responded to noxious heat (Fig. 8*E*). This neuron exhibited a short discharge during needle insertion and injection of vehicle (Fig. 8*F*); intradermal injection of serotonin, histamine, or capsaicin produced an increase in discharge rate which outlasted the short response to vehicle (Fig. 8*G-I*). The response to serotonin lasted >15 minutes, while responses to histamine and capsaicin returned to baseline within 5 min following injection. None of the other pruritogens elicited a response (data not shown).

An example of a pruriceptive VTT neuron recorded in the deep dorsal horn is provided in Figure 9. Its cutaneous receptive field was located on the cheek (Fig. 9*A*). It was recorded in the first cervical segment (Fig. 9*B*) and projected to the VPM nucleus (Fig. 9*C*). The cell was classified as WDR (Fig. 9*D*) and did not respond to noxious heat (Fig. 9*E*). Histamine, chloroquine, and capsaicin each produced an increase in discharge rate compared to vehicle (Fig. 9*F-I*); the response to each of these 3 chemicals remained elevated above baseline for >5 min. The cell did not respond to serotonin or BAM8-22 (data not shown).

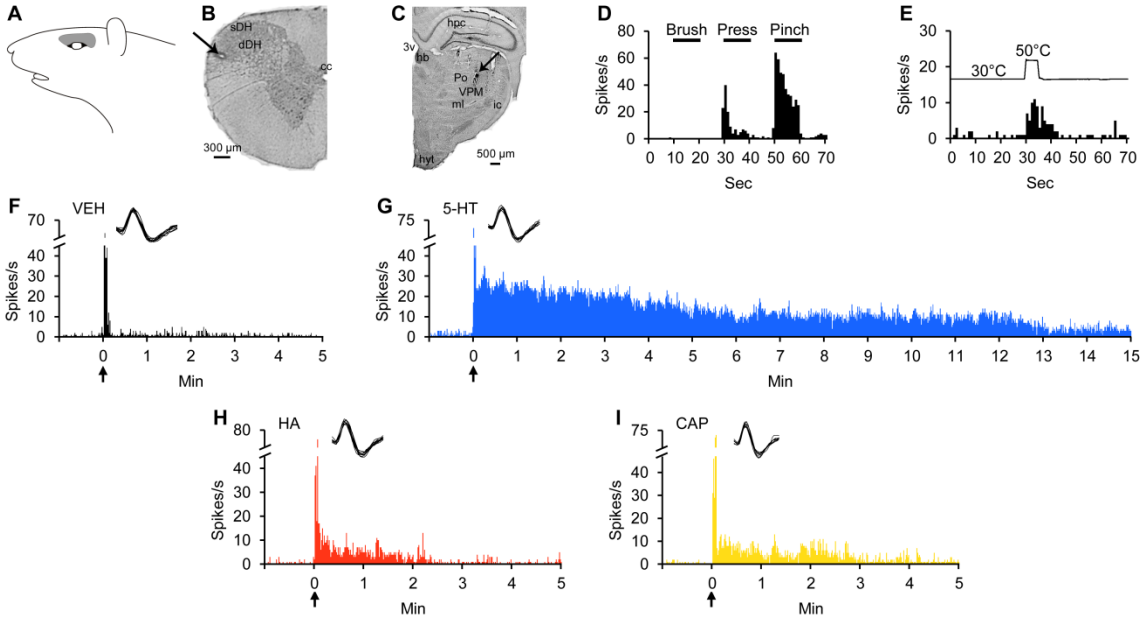

Figure 8. Characterization of a pruriceptive VTT neuron responding to serotonin, histamine, and capsaicin. ***A***, receptive field. ***B***, lesion (arrow) made at the recording point in the superficial layers of the spinal trigeminal nucleus. ***C***, lesion (arrow) made at the point in the thalamus with the lowest threshold for antidromic activation. ***D***, responses to mechanical stimulation of the receptive field. ***E***, response to thermal stimulation of the receptive field. ***F***, the cell did not respond to intradermal injection of vehicle into the receptive field. ***G-I***, the cell responded to serotonin, histamine, and capsaicin, each injected intradermally into the receptive field. hb: habenular n.; ic: internal capsule.

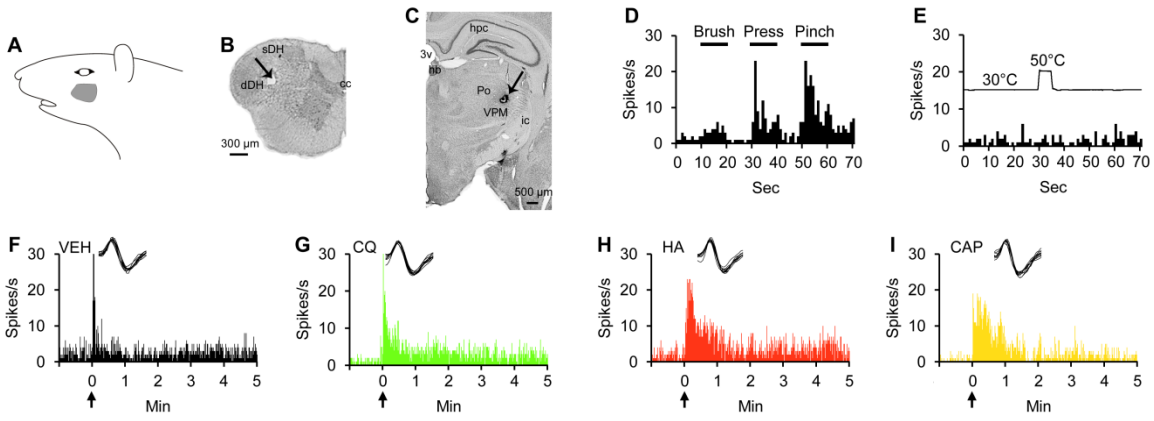

Figure 9. Characterization of a pruriceptive VTT neuron responding to chloroquine, histamine, and capsaicin. ***A***, receptive field. ***B***, lesion (arrow) made at the recording point in the deep layers of the spinal trigeminal nucleus. ***C***, lesion (arrow) made at the point in the thalamus with the lowest threshold for antidromic activation. ***D***, responses to mechanical stimulation of the receptive field. ***E***, response to thermal stimulation of the receptive field. ***F***, the cell did not respond to intradermal injection of vehicle into the receptive field. ***G-I***, the cell responded to chloroquine, histamine, and capsaicin, each injected intradermally into the receptive field.

Nociceptive-only VTT neurons. In contrast to pruriceptive VTT neurons, nociceptive-only VTT neurons did not respond to any tested pruritogens. An example of a nociceptive-only VTT neuron is shown in Figure 10. This cell was recorded from superficial layers of the spinal trigeminal subnucleus caudalis at the level of the pyramidal decussation (Fig. 10*B*) and projected to the VPM nucleus (Fig. 10*C*). The neuron responded to noxious mechanical (Fig. 10*D*) and thermal (Fig. 10*E*) stimulation of its receptive field (Fig. 10*A*) and was classified as HT. None of the six tested pruritogens produced a response in this cell (Fig. 10,*F-L*). It was therefore classified as nociceptive-only.

Characterization of responses to each pruritogen. The mean response to each pruritogen for all cells which met the criteria for being responsive to a pruritogen is shown in Figure 11. The mean response to serotonin (Fig. 11*A*) reached a maximum discharge rate within 5 min after application (Fig. 11*F*) and remained elevated above the pre-serotonin baseline discharge rate for over 20 min (Fig. 11*G*); the mean duration of the response to serotonin was significantly greater than the mean duration of responses to chloroquine, histamine, or capsaicin. In 5 neurons, we were able to obtain stable recordings to demonstrate responses to serotonin which remained elevated above baseline for greater than 30 min. The only pruritogen which produced a response that outlasted the response to serotonin was BAM8-22 (Fig. 11 *B,G*). The mean response to BAM8-22 reached a maximum discharge rate approximately 10 min after application (Fig. 11*F*) and remained elevated above baseline for 35-40 min (Fig.

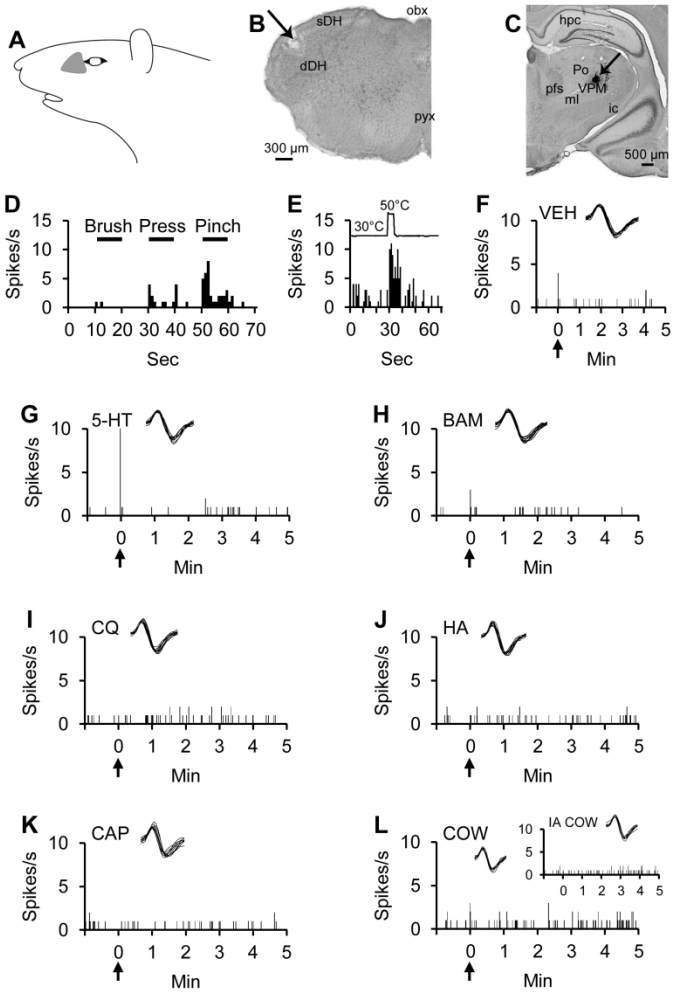

Figure 10. Characterization of a nociceptive-only neuron responding to noxious mechanical and thermal stimulation, but not to any tested pruritogens. ***A***, receptive field. ***B***, lesion (arrow) made at the recording point in the superficial layers of the spinal trigeminal nucleus. ***C***, lesion (arrow) made at the point in the thalamus with the lowest threshold for antidromic activation. ***D***, responses to mechanical stimulation of the receptive field. ***E***, response to thermal stimulation of the receptive field. ***F-L***, the cell did not respond to intradermal injection of vehicle, serotonin, BAM8-22, chloroquine, histamine, or capsaicin, or insertion of cowhage spicules into the receptive field (*L*, inset: response to heat-inactivated cowhage spicules). obx: obex.

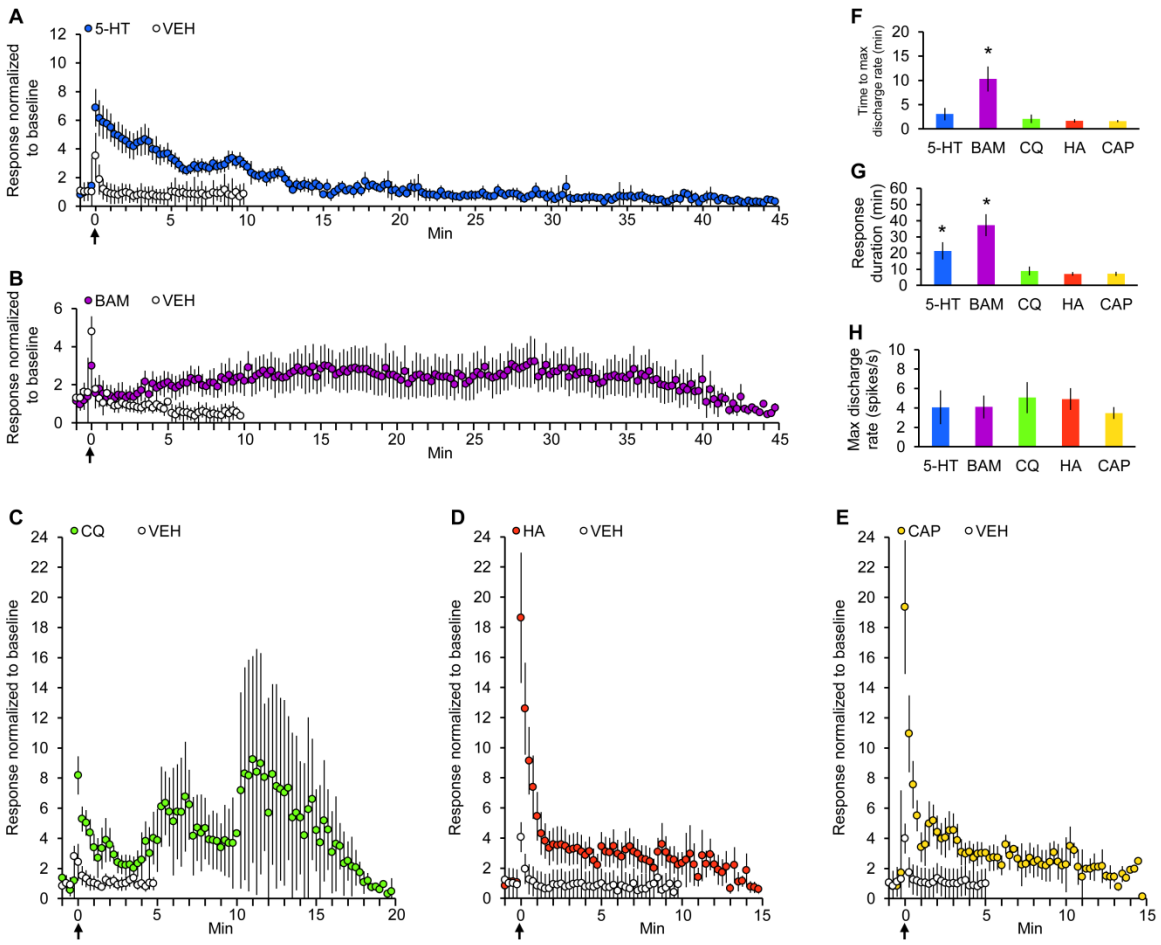

Figure 11. Characterization of the mean response to each pruritogen. ***A***, mean response to serotonin (blue); mean response to vehicle in serotonin-responsive cells (white) (n=22). ***B***, mean response to BAM8-22 (purple); mean response to vehicle in BAM8-22-responsive cells (white) (n=6). ***C***, mean response to chloroquine (green); mean response to vehicle in chloroquine-responsive cells (white) (n=7). ***D***, mean response to histamine (orange); mean response to vehicle in histamine-responsive cells (white) (n=23). ***E***, mean response to capsaicin (yellow); mean response to vehicle in capsaicin-responsive cells (white) (n=13). ***F***, mean time to reach maximum discharge rate, calculated by finding the highest discharge rate in a 60-sec sliding window following stimulus application for each pruritogen. * indicates statistically significant difference from other pruritogens ($p<0.01$ for 5-HT, $p<0.01$ for BAM; Kruskal-Wallis ANOVA with Dunn's post test). ***G***, mean time to return to baseline discharge rate following stimulus application for each pruritogen. * indicates statistically significant difference from other pruritogens ($p<0.01$; Kruskal-Wallis ANOVA with Dunn's post test) ***H***, mean maximum discharge rate for each pruritogen. Data only included from neurons for which the receptive field was not manipulated during the response to a pruritogen. For panels *A-E*: data normalized to 60 sec of baseline activity preceding stimulus application and reported in 15 sec bins.

11*G*); in 4 neurons we were able to obtain stable recordings of responses to BAM8-22 lasting at least 40 min. Mean responses to chloroquine, histamine, and capsaicin peaked within the first minute following application (Fig. 11*F*) and returned to baseline activity levels within 10 min (Fig. 11*G*). The high SEM for the response to chloroquine (Fig. 11*C*) suggests that responses elicited by chloroquine were more variable across individual cells than those elicited by the other pruritogens. The mean maximum discharge rates during responses to the different pruritogens did not significantly differ. The mean maximum discharge rate for each chemical ranged from 3-5 spikes/s (Fig. 11*H*).

To determine whether VTT cells displayed different spike timing dynamics or spiking patterns in response to different stimuli, the CV was calculated during responses to each pruritogen. The CV was also calculated during responses to noxious mechanical (pinch) and thermal stimuli, in order to determine whether there was a difference between pruriceptive and nociceptive responses within pruriceptive VTT neurons responsive to serotonin. The CV is a measure of the variability of ISIs and values greater than 1.0 indicate higher degrees of variability within a spike train. The mean CV did not significantly differ between responses produced by different pruritogens. In neurons responsive to serotonin, the mean CV did not significantly differ between responses to the pruritogen versus the noxious stimuli pinch or heat (Fig. 12*A*). ISI distribution histograms were compared for responses to each of the pruritogens, as well as for responses to

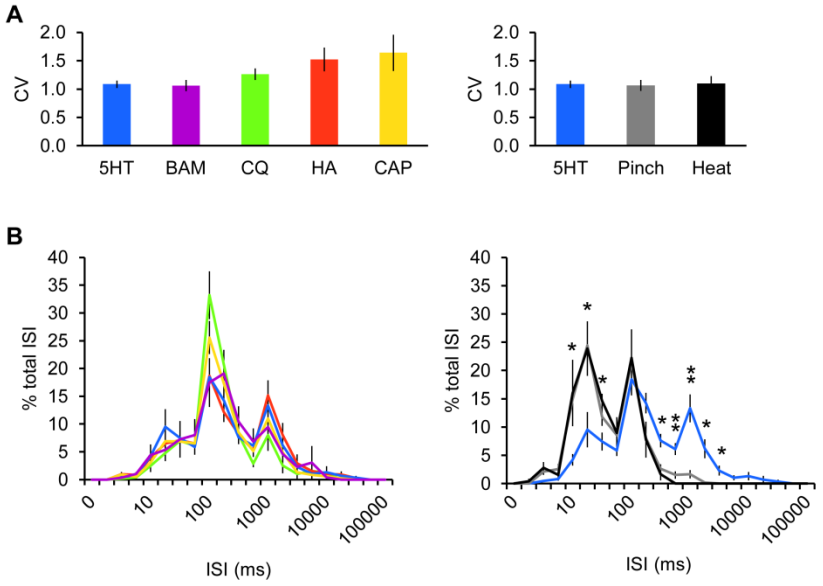

Figure 12. Analysis of spike timing dynamics and spike patterns in pruriceptive and nociceptive-only VTT neurons. ***A***, CV of ISIs for responses to each pruritogen and for responses to noxious stimuli within serotonin-responsive neurons. ***B***, ISI distribution histograms for responses to each pruritogen and for responses to noxious stimuli within serotonin-responsive neurons. Asterisks indicate statistically significant difference between responses to serotonin versus responses to pinch or heat (*p<0.05, **p<0.005; Kruskal-Wallis ANOVA with Dunn's post test). Colors in *B* correspond to colors labeled for each pruritogen in *A*. Data compiled from same set of neurons used in Fig. 11.

pruritic versus noxious stimuli within serotonin-responsive cells (Fig. 12*B*). ISIs produced during pruritic responses were highly variable in distribution, but the ISI distributions of the responses to various pruritogens did not significantly differ from each other. There was a significant difference in ISI distribution between responses to serotonin versus responses by the same cells to noxious pinching or heat. Noxious stimuli displayed a bimodal ISI distribution indicative of bursting, with a greater proportion of shorter ISIs (10-50 ms) compared to serotonin which elicited responses with a greater proportion of longer ISIs (500-5000 ms). Because CV and ISI distributions did not differ between pruritogens, analysis of CV and ISI distributions for responses to noxious stimuli are only shown for serotonin-responsive neurons. The same analyses performed with histamine or capsaicin-responsive neurons provided similar results (data not shown); the number of neurons responsive to BAM8-22 or chloroquine were too small to perform reliable statistical testing. Increased numbers of long ISIs were also noted in responses of primate STT neurons to a pruritogen compared to responses to an algogen (Davidson et al. 2012). It is possible, therefore, that low frequency discharges contribute a distinct signal during pruriceptive processing.

Each pruritogen tested elicited a response in a subset of VTT neurons, except cowhage which did not produce a response in any of the cells in which it was tested. Of the cells in which at least five pruritogens were tested, 55 percent responded to at least one pruritogen (Table 2). Of these pruriceptive cells, 36 percent responded to multiple pruritogens. Twenty-seven percent of neurons

# Pruritogens tested	% Responding to *n* pruritogens					
	0	1	2	3	4	5
≥1 (N=104)	54 (52%)	32 (31%)	10 (10%)	6 (6%)	2 (2%)	0
≥2 (N=92)	53 (58%)	21 (23%)	10 (11%)	6 (6%)	2 (2%)	0
≥3 (N=87)	52 (60%)	17 (20%)	10 (11%)	6 (7%)	2 (2%)	0
≥4 (N=62)	31 (50%)	13 (21%)	10 (16%)	6 (10%)	2 (3%)	0
≥5 (N=47)	21 (45%)	9 (19%)	10 (21%)	5 (11%)	2 (4%)	0

Table 2. *Percentage of VTT neurons tested with and responding to a specific number of pruritogens injected intradermally in the face.* Percentages are calculated by dividing the number of neurons responding to specific number of pruritogens (n) by the total number of neurons tested with the same number of pruritogens (N). Data include neurons tested with serotonin, BAM8-22, chloroquine, histamine, capsaicin, and/or cowhage.

responded to serotonin, 22 percent to BAM8-22, 9 percent to chloroquine, 28 percent to histamine, and 27 percent to capsaicin (Table 3). All cells which responded to a pruritogen also responded to noxious mechanical stimulation; roughly one third of pruriceptive VTT neurons was responsive to noxious heat. For each pruritogen, responsive cells consisted of HT and WDR neurons. No LT cells (n=14) responded to any of the chemical stimuli tested.

VTT neurons were also classified based on their responses to mechanical and thermal stimulation, as well as on the location of their recording points. Of the 90 VTT neurons which responded to noxious mechanical stimulation, 56 percent were pruriceptive (n=27 WDR; n=23 HT) and 44 percent were nociceptive-only (n=22 WDR; n=18 HT) (Fig. 13*A*). Thirty-seven percent of pruriceptive neurons and 23 percent of nociceptive-only neurons responded to a 50° C noxious heat stimulus (Fig. 13*B*). In regard to recording location, 52 percent of either pruriceptive or nociceptive-only VTT neurons were located in superficial layers of the spinal trigeminal nucleus (Fig. 13*C*). The remaining neurons were recorded in the deep dorsal horn. The contribution of each of these classes of VTT neurons (WDR vs. HT; heat-responsive vs. nonresponsive; superficial vs. deep dorsal horn recording location) to the mean response to each pruritogen was determined by comparing the mean discharge rate during a 5 or 10 min period following pruritogen application. Neurons classified as WDR had a significantly greater discharge rate during histamine responses than neurons classified as HT (Fig. 14*A*); no significant difference was seen between WDR

Drug	# Responsive to drug	Mechanical classification			Heat
		HT	WDR	LT	
5-HT (47 mM)	26 of 95 (27%)	11 (42%)	15 (58%)	0	8 of 21 (38%)
BAM (1 mM)	6 of 27 (22%)	5 (83%)	1 (17%)	0	2 of 6 (33%)
CQ (100 mM)	7 of 81 (9%)	2 (29%)	5 (71%)	0	2 of 7 (29%)
HA (900 mM)	26 of 92 (28%)	13 (50%)	13 (50%)	0	9 of 25 (36%)
CAP (3.3 mM)	13 of 49 (27%)	6 (46%)	7 (54%)	0	5 of 12 (42%)
COW (≥10 spicules)	0 of 49	0	0	0	0

Table 3. *Incidence of response of VTT neurons to each pruritogen.* For mechanical classification and heat responsiveness, percentages are calculated by dividing the number of neurons responding to noxious stimulus (denoted by column) by the number of neurons responding to the pruritogen (denoted by row).

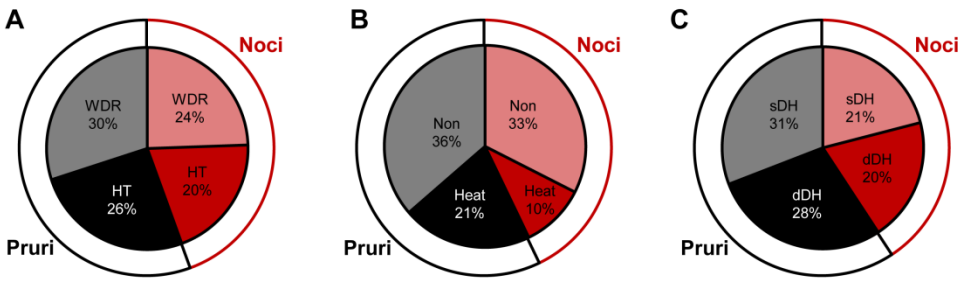

Figure 13. Proportions of pruriceptive and nociceptive-only VTT neurons belonging to various subclasses. ***A***, proportions of pruriceptive (black) and nociceptive-only (red) neurons classified as WDR or HT based on responses to mechanical stimulation (N=90). ***B***, proportions of pruriceptive and nociceptive-only neurons classified as heat responsive or nonresponsive based on responses to thermal (50°C) stimulation (N=77). ***C***, proportions of pruriceptive and nociceptive-only neurons recorded from superficial or deep dorsal horn (N=81).

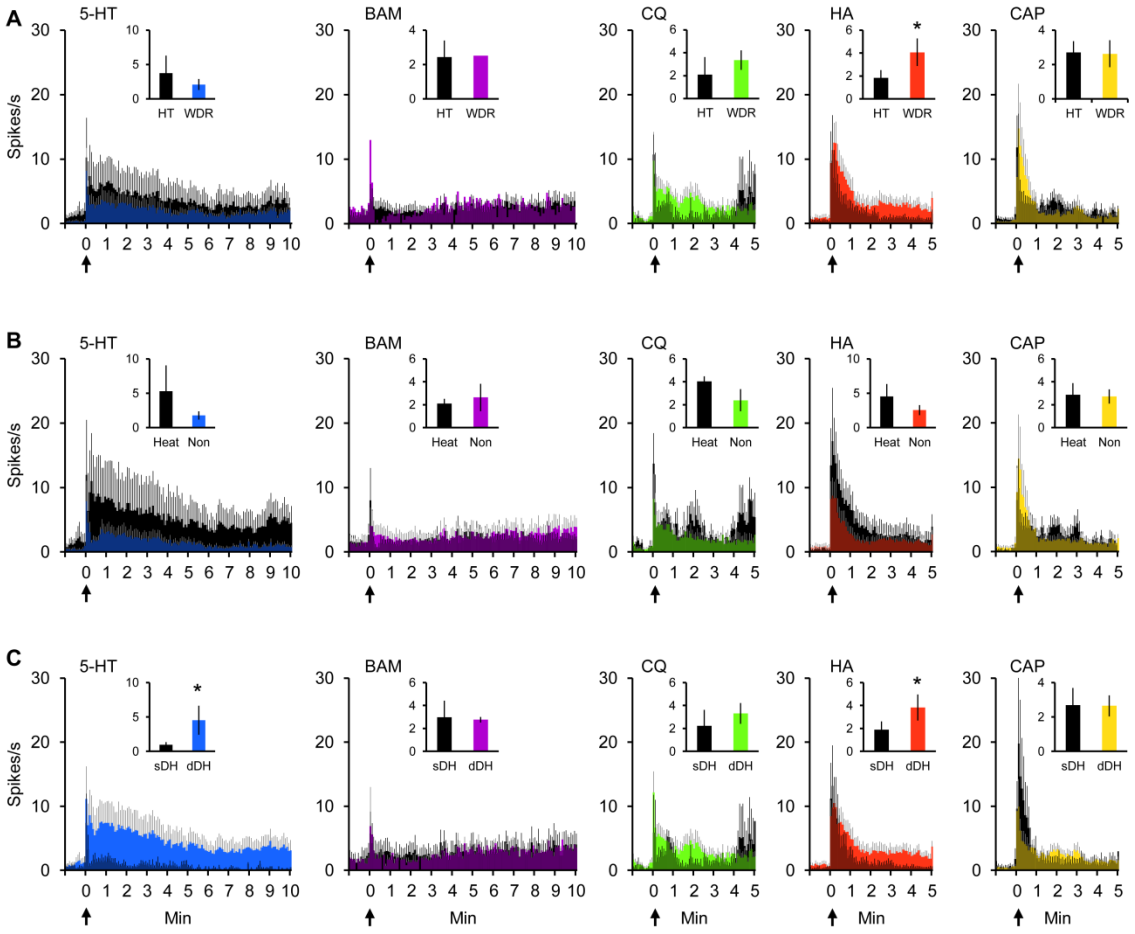

Figure 14. Responses to each pruritogen by various subclasses of pruriceptive VTT neurons. ***A***, mean response to each pruritogen by WDR versus HT neurons. * indicates statistically significant difference between HT vs. WDR (p=0.01 for histamine; Wilcoxon rank sums). ***B***, mean response to each pruritogen by heat responsive (Heat) versus nonresponsive (Non) neurons. ***C***, mean response to each pruritogen by cells recorded from superficial (sDH) versus deep (dDH) dorsal horn. * indicates statistically significant difference between sDH vs. dDH (p=0.03 for serotonin, p=0.03 for histamine; Wilcoxon rank sums). For all panels: histograms represent actual (not normalized) discharge rates and reported in 5 sec bins; insets: mean discharge rate across response period shown in histogram (10 min for serotonin and BAM8-22, 5 min for chloroquine, histamine, and capsaicin).

versus HT neurons for any of the other pruritogens. Nor was there a significant difference in discharge rates during pruritogen application between neurons responsive or nonresponsive to noxious heat (Fig. 14*B*). Neurons in deep layers exhibited a significantly greater discharge rate than those in superficial layers during responses to serotonin or histamine (Fig. 14*C*).

Comparison of responses of pruriceptive and nociceptive-only VTT neurons to mechanical and thermal stimuli. The mean discharge rates of responses of pruriceptive versus nociceptive-only VTT neurons to mechanical and thermal stimulation were compared. Pruriceptive HT neurons responded with a higher discharge rate during pressure and pinch compared to nociceptive-only HT neurons (Fig. 15*A*). There was no difference in responses to brush, pressure, or pinch between pruriceptive and nociceptive-only WDR neurons (Fig. 15*B*). Normalized responses to pressure and pinch by pruriceptive HT neurons were significantly greater than those by nociceptive-only HT neurons (Fig. 15*C*). Additionally, pinch responses in pruriceptive or nociceptive-only WDR neurons were significantly greater than the response to pinch by nociceptive-only HT neurons but significantly less than the response to pinch by pruriceptive HT neurons. The mean response to noxious heat did not vary between pruriceptive and nociceptive-only VTT neurons (Fig. 15 *D,E*).

Recording point and axon projection locations. The recovered recording locations for pruriceptive and nociceptive-only VTT neurons are compiled in Figure 16. Pruriceptive and nociceptive-only VTT neurons were each located

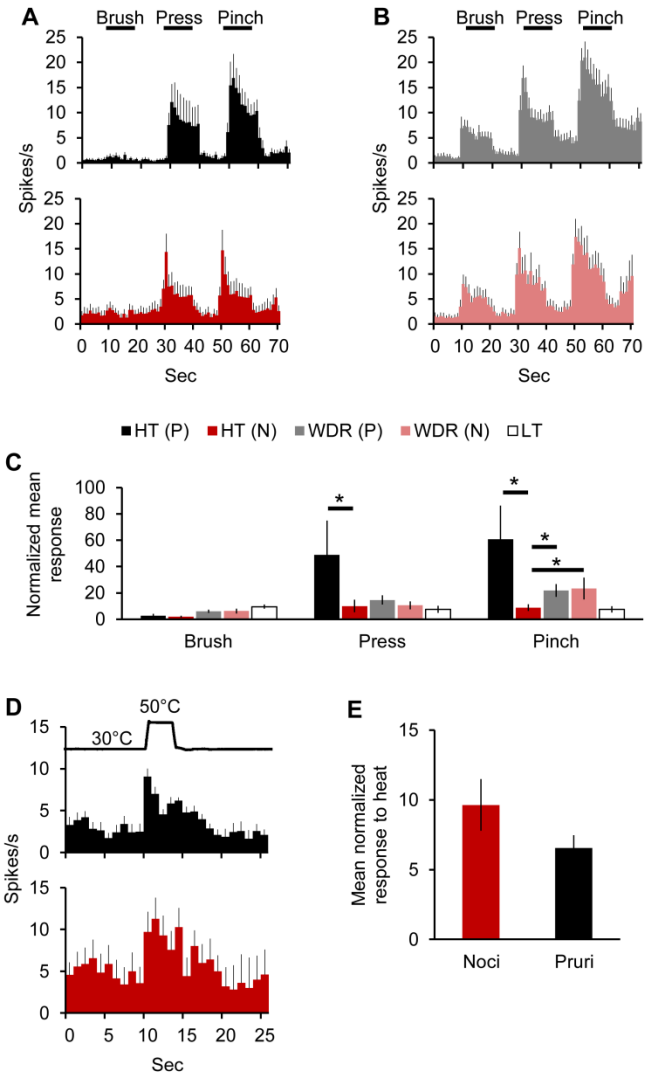

Figure 15. Responses to mechanical and thermal stimulation by pruriceptive versus nociceptive-only VTT neurons. ***A***, mean discharge rate elicited by mechanical stimulation in pruriceptive (black) versus nociceptive-only (red) HT neurons. ***B***, mean discharge rate elicited by mechanical stimulation in pruriceptive versus nociceptive-only WDR neurons. ***C***, mean response (normalized to 60 sec of baseline activity preceding stimulus application) to brush, pressure, or pinch in pruriceptive (P), nociceptive-only (N), or LT neurons. * indicates statistically significant difference between groups denoted by black bar (p=0.02 for HT(P) vs. HT(N) press, p=0.03 for HT(N) vs. WDR(N) pinch, p=0.01 for HT(P) vs. HT(N) pinch, p=0.003 for HT(N) vs. WDR(P) pinch; Kruskal-Wallis ANOVA with Dunn's post test). ***D***, mean discharge rate elicited by 50°C heat stimulus in pruriceptive versus nociceptive-only neurons. ***E***, mean response (normalized to 60 sec of baseline activity preceding stimulus application) to 50°C heat stimulus in pruriceptive (Pruri) versus nociceptive-only (Noci) neurons.

throughout the superficial and deep layers of the spinal trigeminal nucleus, with no apparent relationship between location and responsiveness to pruritogens (indicated by colored wedges in dots that depict recording location), or mechanical classification. The axon locations for all pruriceptive and nociceptive-only VTT neurons for which the low threshold stimulation point lesion was recovered are compiled in Figure 17. Pruriceptive and nociceptive-only VTT axons projected primarily to either the posterior thalamus, often terminating within the PoT, or more rostrally to the VPM nucleus. Axons which were surrounded rostrally by points at which they could not be antidromically activated by ≥300 μA are indicated by a red outline in Figure 17. The locations to which axons project do not appear to be associated with responsiveness to mechanical stimulation or specific pruritogens.

Receptive fields. All examined VTT neurons had a cutaneous receptive field on the face ipsilateral to the recording site. The mechanically-sensitive receptive field for each neuron is depicted in Figure 18. The majority of VTT neurons had mechanically sensitive receptive fields located below the eye and caudal to the vibrissal pad, an area of the face corresponding to the area used in studies utilizing the face model to distinguish between itch and pain-related behaviors in rodents.

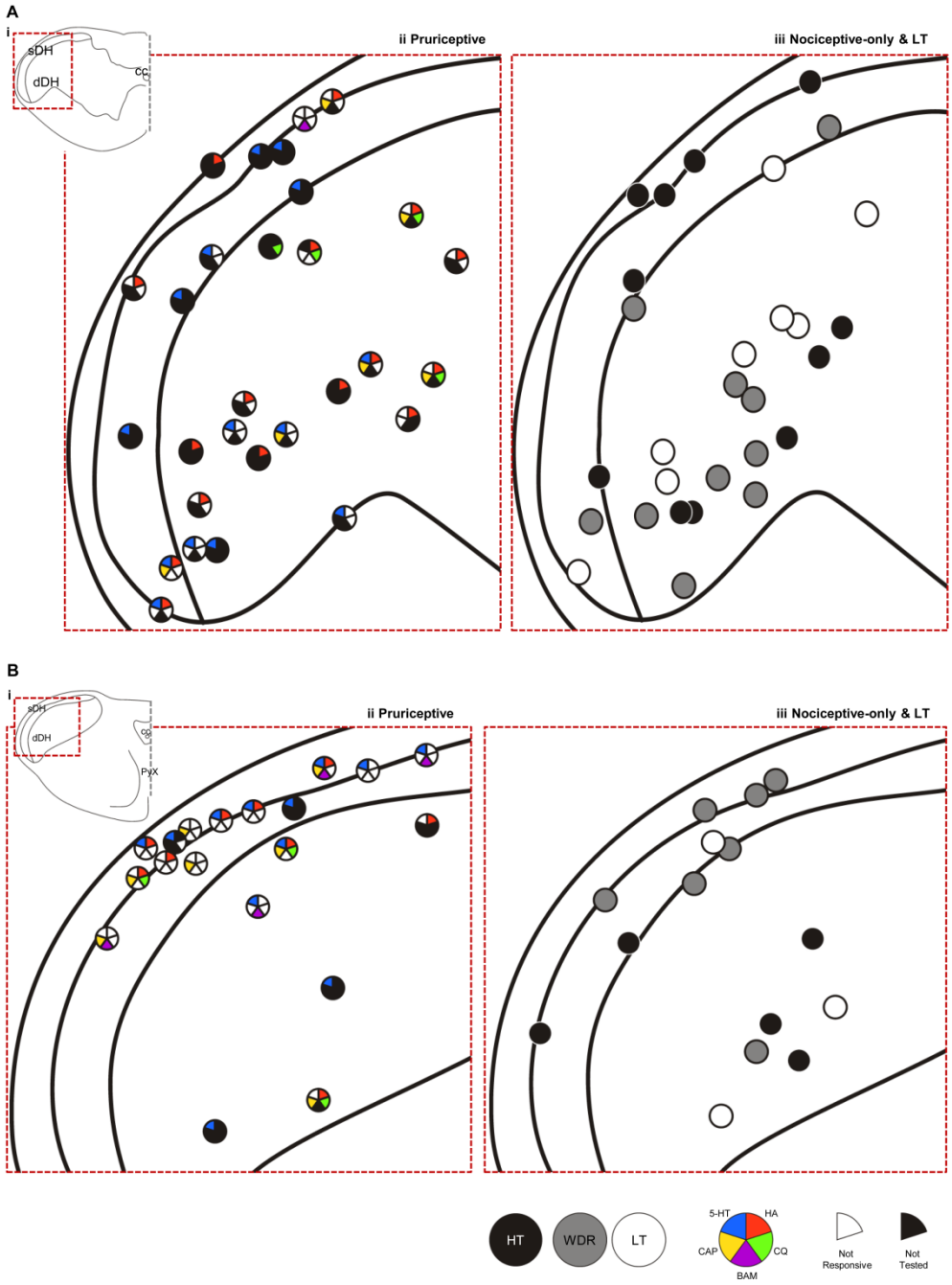

Figure 16. Recording locations of each neuron for which a recording point lesion was recovered. ***A***, recording points located throughout the first and second cervical segments of the spinal cord. ***B***, recording points located in the medulla, at and rostral to the level of the pyramidal decussation. All panels: red dashed line indicates area in (i) which has been expanded in (ii) and (iii). Cells responsive to each pruritogen are indicated by color code; cells tested with a pruritogen but not responding to that pruritogen have a white section in the space corresponding to that pruritogen; cells not tested with a pruritogen have a black section in the space corresponding to that pruritogen (see legend).

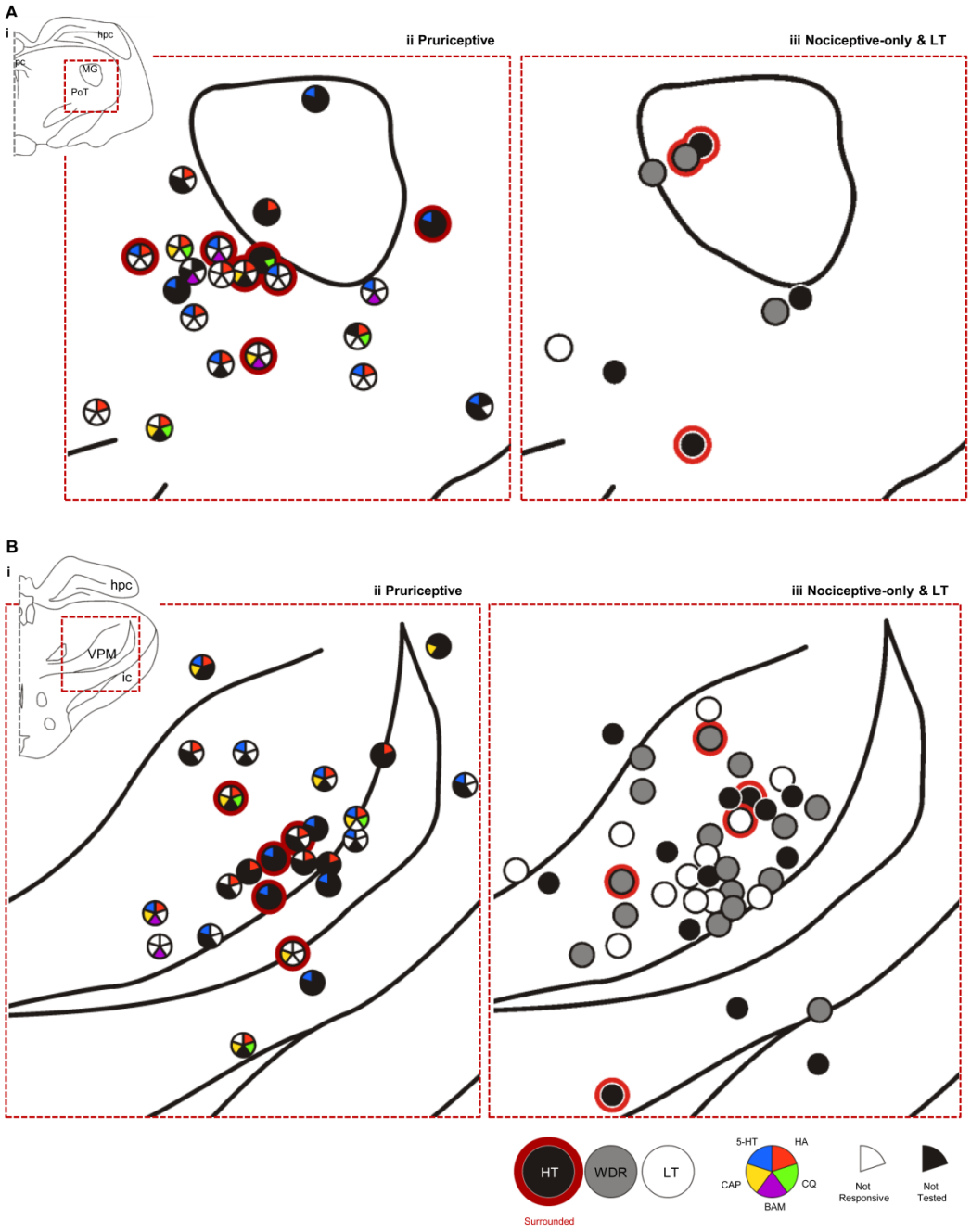

Figure 17. Location of lowest threshold for antidromic activation of each neuron for which a lesion was recovered. ***A***, low threshold points located within the posterior thalamus. ***B***, low threshold points located within the more rostral thalamus, near VPM. All panels: red dashed line indicates area in (i) which has been expanded in (ii) and (iii). Points circled with red indicate points surrounded rostrally by antidromic test sites with a threshold ≥300 µA (see legend).

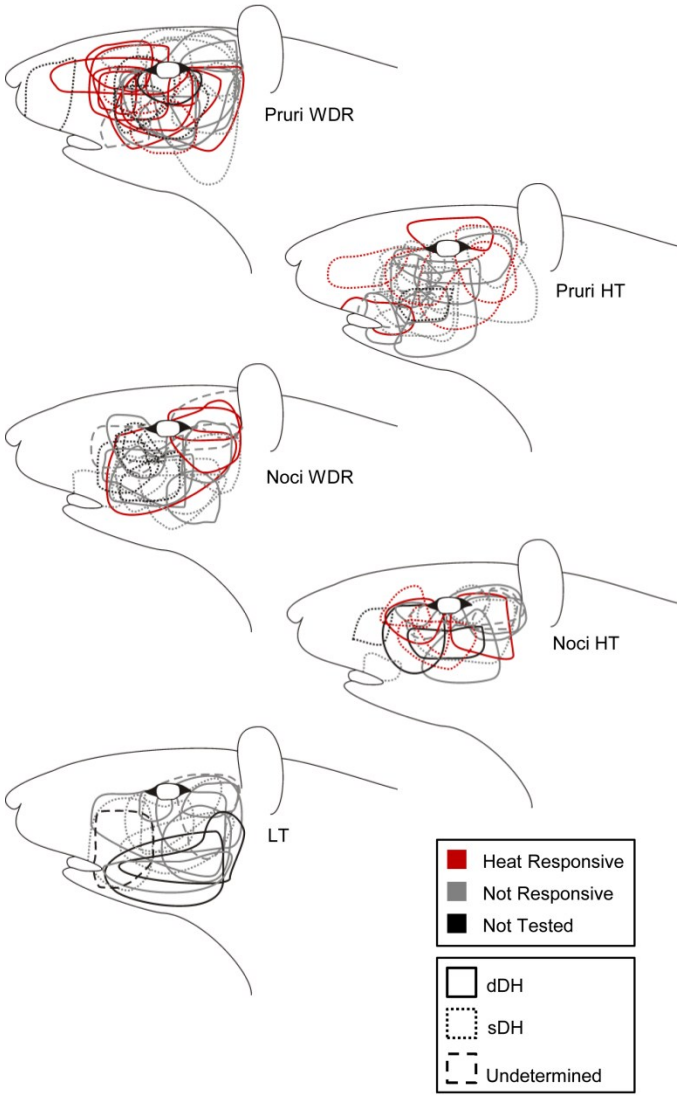

Figure 18. Receptive fields for each neuron included in the current study. Thermal responsiveness and recording location indicated by line color and dash type, respectively (see legend).

Discussion

In this study, we characterized neurons which projected to the thalamus from the spinal trigeminal nucleus and tested their responses to noxious mechanical and thermal stimuli and chemical pruritogens applied to facial cutaneous receptive fields in rats. When tested with all of the six pruritogens used in this study, 55 percent of VTT neurons responded to at least one pruritogen. Each pruriceptive VTT cell also responded to noxious mechanical and/or thermal stimulation; no mechanically-insensitive cells were identified. The majority of pruriceptive neurons (64 percent) responded to only one of the chemicals tested, with the remainder responding to multiple pruritogens. Pruriceptive neurons were located in both superficial and deep layers of the spinal trigeminal nucleus extending from the caudal medulla to the second cervical segment of the spinal cord, and had axons which projected to the VPM, PoT, or other nuclei in the contralateral thalamus.

The current findings describe a population of VTT neurons in which responses to pruritogens paralleled behavioral responses in studies employing the rat face model of itch. When injected intradermally in the face of rats, serotonin causes scratching which peaks within 10 min and subsides within 60 min (Fig. 2; Klein et al. 2011); the mean response to serotonin in VTT neurons has a peak timing and duration which corresponds reasonably well with scratching behavior (Fig. 11). Mean responses to histamine and capsaicin in VTT neurons also match scratching behavior elicited by these chemicals in the cheek,

with behavioral and electrophysiological responses to each peaking within 5 min and returning to baseline within 20 min (Fig. 11; unpublished data). Previously, we have shown that pruriceptive VTT neurons respond to intrathecal application of morphine (Moser and Giesler 2013) with a similar response latency and response duration to scratching induced by morphine applied to the central nervous system (Koenigstein 1948; Frenk et al. 1984; Thomas and Hammond 1995; Lee et al. 2003). Intrathecal application of morphine increased responses in pruriceptive VTT neurons to pruritogens and to innocuous mechanical stimuli, possibly contributing to hyperknesis and alloknesis, sensory phenomena which likely involve opioid receptor activation (Koenigstein 1948; Fjellner and Hagermark 1982; Onigbogi et al. 2000; Heyer et al. 2002). Together, the present and previously published findings suggest that pruriceptive VTT neurons contribute prominently to producing the sensation of itch located on the face.

Our current data demonstrate that neurons which convey information about itch to the thalamus also respond to noxious mechanical stimuli (pressure and/or pinch). Many pruriceptive VTT cells also responded to noxious heat. These data are in accordance with findings that all nonhuman primate pruriceptive STT cells are also responsive to noxious mechanical, thermal, and/or chemical stimuli (Simone et al 2004; Davidson et al. 2007, 2009, 2012). In both monkey STT and rat VTT, pruriceptive and nociceptive-only projection neurons are not readily distinguished based on recording point locations in the dorsal horn or on the locations of their axons within the thalamus (Davidson et al.

2012; Figs. 16,17). Instead, pruriceptive and nociceptive-only populations can be differentiated by their responsiveness to pruritogens as well as responsiveness to drugs which modulate itch and/or pain, such as morphine (Chapter 4; Moser and Giesler 2013). In the periphery, pruriceptive primary afferent neurons can be identified by the presence of cell membrane receptors for various pruritogens. In mice, a population of neurons containing the mas-related gene peptide receptor A3 (MrgprA3) responds to several different pruritogens, including histamine, BAM8-22, and chloroquine. Ablation of this population results in loss of itch-related behaviors while pain-related behaviors remain intact; specific activation of MrgprA3-containing neurons, even via a normally painful stimulus, results in scratching (Han et al. 2013). It is possible that, just as was found for primary afferent neurons by Han et al. (2013), selective activation of pruriceptive VTT neurons, without simultaneous activation of nociceptive-only VTT neurons, by any stimulus will result in the sensation of itch. Accordingly, simultaneous activation of both pruriceptive and nociceptive-only VTT populations would likely produce pain. Future studies to test this possibility should provide further insight into how the nervous system codes itch and pain, as well as possibly point out directions for selective treatments of each.

The pruritogens used in the current study were chosen based on the ability of each to induce scratching with the hindlimb when injected intradermally into the rodent cheek (Akiyama et al. 2010; Klein et al. 2011; Wilson et al. 2011). In addition, the peripheral scratch-inducing actions of each drug have been well

characterized and each drug has been implicated in producing itch in humans. It is well-demonstrated that serotonin elicits scratching in rodents, likely via activation of the 5-HT_{1D} and/or 5-HT_2 receptors (Berendsen and Broekkamp 1991; Yamaguchi et al. 1999; Thomsen et al. 2001; Jinks and Carstens 2002; Nojima and Carstens 2003); serotonin produces itch when applied to the skin in humans (Fjellner and Hägermark 1979; Weisshaar et al. 1997; Thomsen et al. 2002; Hosogi et al. 2006; Rasul et al. 2012) and is found at increased levels in human patients experiencing itch, including patients with allergic contact dermatitis (Lundeberg et al. 1999) and atopic dermatitis (Soga et al. 2007). Serotonin can also cause pain in human volunteers (Schmelz et al. 2003); accordingly, it can elicit pain-related wiping when applied to the face in rats (Chapter 2). Application of BAM8-22 to the face of mice causes scratching with the hindlimb (Wilson et al. 2011) via activation of MrgprC11 (Liu et al. 2009) and downstream opening of TRPA1 channels (Wilson et al. 2011); in humans, heat-inactivated cowhage spicules soaked in BAM8-22 evoke itch accompanied by pricking/stinging and burning sensations (Sikand et al. 2011). Chloroquine produces scratching in rodents via activation of MrgprA3 and downstream opening of TRPA1 channels (Liu et al. 2009; Wilson et al. 2011); in humans, oral administration of chloroquine as a treatment for malaria causes intense itch as a side effect (Mnyika and Kihamia 1991; Sowunmi et al. 2000), though there is little evidence that direct application of chloroquine to the skin causes itch in humans (Abila et al. 1994). Like serotonin, histamine and capsaicin each produce a

combination of scratching and wiping when applied to the face in rats (Klein et al. 2011). In the present study, many cells which responded to serotonin, histamine, or capsaicin also responded to other pruritogens, suggesting that these cells may be conveying information about the pruritogenic aspects of the stimulus, and that the pain responses associated with pruritogens are mediated by a separate population of neurons. Another possibility is that the same cells may be contributing to both itch and pain, depending on the specific spike pattern elicited by a given stimulus. Although our spike train analyses did not uncover any differences in spike timing dynamics between responses to the different pruritogens (Fig. 12), the ISI distributions differed between responses to noxious mechanical or thermal stimuli and chemical pruritogens. These differences may be due to differences in signaling pain versus itch, or could be due to differences in the type of stimulus (mechanical/thermal versus chemical).

The finding that cowhage did not produce a response in any of the VTT cells tested is consistent with findings that cowhage spicules applied to the rat face do not elicit any behavioral response (Klein et al. 2011). In humans, cowhage likely produces itch via activation of protease-activated receptor (PAR) subtypes 2 and/or 4 by mucunain, the itch-causing protease present in cowhage spicules (Reddy et al. 2008). The short peptide SLIGRL-NH_2 has commonly been used as a PAR-2 agonist to induce scratching in mice (Akiyama et al. 2009b) and study itch responses in spinal neurons (Akiyama et al. 2009a, 2009b, 2011). However, it has been shown that in mice, SLIGRL-NH_2-induced scratching does

not require activation of PAR-2, but instead requires activation of MrgprC11, the same receptor underlying itch induced by BAM8-22 (Liu et al. 2011). Neither the PAR-2/MrgprC11 agonist SLIGRL-NH_2 nor the PAR-4 agonist AYPGKF-NH_2 induces scratching when applied to the rat face (Klein et al. 2011); SLIGRL-NH_2 does cause pain-related wiping, an effect not seen with cowhage spicules or the PAR-4 agonist. Accordingly, in future studies it will be important to maintain a distinction between the target receptors for cowhage spicules versus injections of SLIGRL-NH_2.

In the central nervous system, less is known about the specific receptors involved in conveying information about pruritogens to the brain. The gastrin-releasing peptide receptor (GRPR) is implicated in itch processing in the spinal cord, as both the receptor and spinal neurons containing the receptor are necessary for scratching responses induced by a variety of histaminergic and nonhistaminergic pruritogens (Sun and Chen 2007; Sun et al. 2009). The GRPR agonist gastrin-releasing peptide (GRP) is likely released from spinal neurons receiving primary afferent input, rather than from primary afferent terminals themselves (Mishra and Hoon 2013). GRPR is likely located on tertiary spinal neurons receiving input from GRP-containing secondary spinal neurons. Therefore, it is possible that GRPR is located on spinal projection neurons such as pruriceptive VTT neurons, although this has not yet been directly demonstrated via immunohistochemistry or other anatomical methods.

Axons of pruriceptive VTT neurons appear to terminate primarily in either the posterior thalamus or more rostrally in the VPM nucleus (Fig. 17). The posterior thalamus, including PoT and medial geniculate nuclei, also receives input from a large portion of rat STT neurons in the cervical enlargement (Zhang and Giesler 2005). This region of the thalamus sends somatosensory input, both directly (Ottersen and Ben-Ari 1979; Bordi and LeDoux 1994; LeDoux et al. 1985) and indirectly via cortical loops (LeDoux et al. 1985; Kurokawa et al. 1990; Linke 1999; Linke and Schwegler 2000; Gauriau and Bernard 2004), to the amygdala and has been implicated in fear conditioning (LeDoux et al. 1986a,b; Shi and Davis 1999). It is possible that affective responses to itch are mediated via these projections.

Our study highlights several interesting differences between itch responses in spinal projection neurons in rats versus other species. Previously, a small subset of STT neurons in cats was found to be mechanically-insensitive yet respond to histamine. It was suggested that these cells may be part of a labeled line for itch information, although the number of histamine-responsive neurons which did not also respond to the algogen mustard oil, when tested, was low (n=2). In addition, other algogens, such as capsaicin which activates a large portion of STT neurons, were not tested (Andrew and Craig 2001). In contrast, in monkey STT neurons as well as in rat VTT neurons, all pruriceptive cells were responsive to noxious mechanical stimulation (Simone et al. 2004; Davidson et al. 2007, 2009, 2012; Moser and Giesler 2013). Pruriceptive monkey STT

neurons were found to respond either to histamine or to cowhage (Davidson et al. 2007), suggesting a separation between histaminergic and nonhistaminergic forms of itch. Our current data suggest that this separation of histaminergic and nonhistaminergic responses in spinal projection neurons does not exist in the rat trigeminal system. In fact, the only predictive relationship identified in rat VTT neurons is that all neurons which responded to chloroquine (a pruritogen generally referred to as nonhistaminergic) also, when tested, responded to histamine. It is possible that, as more pruritogens are tested in monkey STT neurons, the separation of populations responding to histamingeric and nonhistaminergic stimuli will become less distinct. In addition, it is likely that the previous finding that approximately one-third of monkey STT neurons is pruriceptive (versus approximately half of rat VTT neurons) will change when monkey STT neurons are characterized with a greater number of pruritogens. Another major difference between species was found for the contribution of superficial versus deep dorsal horn units to itch responses. Monkey STT neurons located within the superficial dorsal horn exhibited a greater discharge rate than cells located within the deep dorsal horn in response to histamine, cowhage, or capsaicin (Davidson et al. 2012). In contrast, in rat VTT neurons, deep dorsal horn units exhibited a greater discharge rate compared to superficial dorsal horn units in response to the pruritogens histamine and serotonin, highlighting the importance of including both superficial and deep dorsal horn neurons in studies of itch. It should also be noted that observed differences between previous

studies of STT neurons in monkeys and the current studies of VTT neurons in rats may be due in part, or in full, to differences between the characteristics of skin and/or underlying nervous system of the body versus face.

Application of pruritogens and algogens to the rodent cheek provides a reliable approach to distinguish between itch-evoked and pain-evoked behaviors and thus is valuable for examining how the nervous system distinguishes between these distinct sensations. VTT neurons in rats respond to a variety of pruritogens which evoke scratching with the hindlimb when applied to the face. The time course of pruritic responses in VTT neurons is similar to the time course of facial scratching for corresponding pruritogens. Therefore, the VTT appears to be a highly promising system for future studies of processing and transmission of pruriceptive and nociceptive information within the central nervous system.

CHAPTER 4

Itch and analgesia resulting from intrathecal application of morphine: contrasting effects on different populations of trigeminothalamic tract neurons

Introduction

Morphine remains one of the most commonly prescribed drugs for treatment of severe chronic pain, and intrathecal application of morphine is one of the most powerful treatments available for patients. However side-effects, including itch, can limit the maximum tolerable dose, and thus the effectiveness of morphine for producing analgesia. The incidence of opioid-induced pruritus is especially high (20-100%) following intrathecal administration (Baraka et al. 1982; Bromage et al. 1982; Ballantyne et al. 1988; Szarvas et al. 2003; Ganesh and Maxwell 2007). Itch can be accompanied by debilitating phenomena such as hyperknesis, increased itch caused by pruritogens, and alloknesis, itch caused by innocuous mechanical stimuli that normally do not cause itch. Opioids likely play a role in producing both; morphine administration causes hyperknesis (Fjellner and Hägermark 1982; Onigbogi et al. 2000) and alloknesis (Koenigstein 1948), and opioid receptor antagonists reduce alloknesis (Heyer et al. 2002). Endogenous opioids are likely involved in producing pruritus associated with atopic dermatitis, chronic urticaria, or cholestasis, as itch accompanying these conditions is treated with opioid receptor antagonists (Phan et al. 2010).

Surprisingly, pruritus caused by intrathecal application of morphine is often localized to facial regions of patients (Scott et al. 1980; Baraka et al. 1981; Collier 1981; Bromage et al. 1982), suggesting the value of using animal models of facial itch to study this phenomenon. Pruritogens and algogens produce distinct behavioral responses when applied to the face of mice (Shimada and LaMotte

2008) or rats (Klein et al. 2011), indicating that sensory neurons receiving input from rodent facial skin are valuable for investigating mechanisms of itch and pain. Spradley et al. (2012) showed that itch-related facial scratching is reduced by μ-opioid receptor antagonists while pain-related wiping is reduced by morphine, suggesting that μ-opioid receptor activation has opposite effects on itch versus pain signaling related to the face. Our own data from Chapter 2 support this idea. In addition, intracisternal injection of morphine causes robust body and facial scratching in rats (Koenigstein 1948; Lee et al. 2003) as does injection of morphine within the spinal trigeminal nucleus (Thomas and Hammond 1995). Thus, the rat trigeminal system appears to be valuable for studies of the mechanisms underlying morphine-induced itch.

We examined the responses of trigeminothalamic tract (VTT) neurons of rats to facial application of algogens and pruritogens (Chapter 3; Moser and Giesler 2011). More than half of such neurons were powerfully activated by intradermal injections of pruritogens such as chloroquine, histamine, and serotonin into the face. Serotonin evokes robust itch responses in rats (Berendsen and Broekkamp 1991; Thomsen et al. 2001; Klein et al. 2011) and humans (Fjellner and Hägermark 1979; Weisshaar et al. 1997; Thomsen et al. 2002; Hosogi et al. 2006; Rasul et al. 2012) and it is elevated within the skin in various human dermatologic diseases that produce itch (Lundeberg et al. 1999; Soga et al. 2007).

Here, we examined the effects of intrathecal application of morphine on rat

VTT neurons. Our findings indicate that morphine often inhibits nociceptive-only VTT neurons but activates pruriceptive VTT neurons and increases their responses to pruriceptive and innocuous mechanical inputs. The activation and increased evoked responses of pruriceptive VTT neurons likely contribute to itch, hyperknesis, and alloknesis.

Materials & Methods

Animals. Adult male Sprague-Dawley rats (300-450 g) were used according to protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Animals were deeply anesthetized with urethane (1.5 mg/kg, i.p., Sigma) and tracheostomized. An intravenous catheter was placed in the left jugular vein. A laminectomy was performed over the first cervical segment (C1) to allow recording of neurons with receptive fields on the face below the eye and caudal to the vibrissal pad, an area corresponding to that in which pruritogens were applied in behavioral studies (Shimada and LaMotte 2008; Klein et al. 2011). A craniotomy was performed over the right thalamus. The dura was removed from brain and spinal cord.

Antidromic identification of VTT neurons. A low-impedance stainless steel electrode was positioned at stereotaxic coordinates for the ventroposterior medial (VPM) nucleus in the thalamus. Pulses of electrical current (300-500 μA, 200 μs, 3 Hz) were delivered through the electrode as an initial search stimulus. A stainless steel recording electrode (10 MΩ, FHC Inc.) was lowered through the

dorsal horn of the contralateral caudal medulla and the first cervical segment of the spinal cord to search for time-locked single unit responses which met the following criteria for antidromically-activated VTT neurons: 1) stable response latency (<0.05 ms variation); 2) ability to follow stimulus trains of ≥300 Hz; and 3) collision of orthodromic spikes with putative antidromic responses. Single unit responses that met these criteria and had cutaneous facial receptive fields located caudal to the vibrissal pad were used for further study. Action potentials were amplified, filtered, and digitized and wave-form discriminated using DAPSYS data acquisition processor system software (www.dapsys.net).

Axon projection mapping. The amount of current required to elicit an antidromic spike was determined at 200 μm intervals throughout vertical stimulating tracks separated by medial-lateral and rostral-caudal intervals of 300-500 μm. The point at which the threshold for antidromic activation was lowest (≤30 μA) was assumed to be the most accurate indication of the position of the axon terminal (Burstein et al. 1991; Dado et al. 1994). If the antidromic latency did not change at any more rostral position, it was assumed that the axon did not extend rostrally beyond the identified low threshold point.

Characterization of neurons with mechanical & chemical stimuli. Innocuous brushing with a soft-bristled brush and the minimum necessary amount of noxious stimuli were used to identify the mechanical receptive field boundaries for each antidromically-identified VTT neuron. Brushing, and pressure and pinch applied with small clips were used to classify each neuron as

high-threshold (HT; responsive only to noxious pinching), wide dynamic range (WDR; responsive to brushing with higher discharge rate in response to pressure and pinching), or low-threshold (maximally responsive to innocuous brushing).

Each cell was characterized using the following established pruritogens in rodents (Klein et al. 2011; Wilson et al. 2011): histamine dihydrochloride (600 mM, Sigma), serotonin creatinine sulfate complex (47 mM, Sigma), chloroquine diphosphate salt (100 mM, Sigma), and/or bovine adrenal medullary 8-22 peptide (BAM8-22, 1 mM, Tocris). Each drug was injected intradermally in a 10 µl volume within the mechanical receptive field using a 28-gauge hypodermic needle; every effort was made to use sites separated by >5 mm whenever possible. The effects of injection of pH-matched 0.9% normal saline vehicle were always determined before an active stimulus. Drugs were injected in random order on each trial, with a minimum of 5 minutes between drug injections. If a cell responded to a pruritogen, no other pruritogen was applied and the effects of morphine were determined.

Morphine sulfate (100 ng in 500 µl, Sigma) or vehicle was applied to the surface of the spinal cord using a syringe and polyethylene tubing. Agar was used to build a dam enclosing the caudal medulla and the first and second cervical spinal cord segments. When testing effects of morphine on subsequently applied stimuli, the second stimulus (intradermal injection of serotonin or brush/pinch) was applied ≥ 15 min after intrathecal application of vehicle or morphine. Naloxone hydrochloride (1 mg/kg, Tocris) was delivered via intravenous catheter.

Histology. At the end of each experiment, low threshold antidromic stimulation site(s) in the thalamus and recording site(s) were marked with electrolytic lesions. Rats were perfused with 0.9% normal saline followed by 10% formalin with 1% ferrocyanide (for Prussian blue reaction at lesion sites). The brain, including thalamus, medulla, and rostral cervical spinal cord, were removed and sectioned (50-75 μm) on a freezing microtome and stained with neutral red. A rat brain atlas (Paxinos and Watson 1982) was used to identify thalamic nuclei.

Data analysis. A cell was considered responsive to a stimulus if it exhibited a change in mean ongoing discharge rate of ±50% after stimulus application, with a longer duration than any change due to vehicle. For all analyses, discharge rates were normalized to 60 sec of baseline activity before treatment. For effects on ongoing activity, mean responses to vehicle, morphine, or naloxone were measured during a 3 min period beginning 1 min after stimulus application. For effects on subsequent applications of pruritogen, the mean response to serotonin was measured over the 5 min period following injection of the pruritogen. To examine effects of morphine on responses to mechanical stimuli, mean responses to brush or pinch were calculated over the 10 seconds of brush or pinch application. Histograms are reported in 1 sec bins, unless otherwise noted. All error bars represent standard error of the mean (SEM). Wilcoxon rank sums or Kruskal-Wallis ANOVA analyses with Dunn's post test were used to compare effects across treatments, with $p<0.05$ considered significant.

Results

The effects of morphine were tested on two populations of antidromically-identified nociceptive VTT neurons located within the caudal medulla and first cervical spinal segment: pruriceptive cells and nociceptive-only cells. Pruriceptive cells responded to noxious stimulation and to at least one pruritogen. Nociceptive-only cells responded to noxious stimulation but not to any tested pruritogens.

Effects of intrathecal application of morphine on ongoing activity. Intrathecal application of morphine excited pruriceptive cells and inhibited nociceptive-only cells. An example of the effects of intrathecal application of morphine on a pruriceptive VTT neuron is shown in Figure 19. This cell was recorded in the superficial spinal trigeminal subnucleus caudalis (Fig. 19*A*) and had a receptive field located below the eye and caudal to the vibrissal pad (Fig. 19*B*).The cell was activated by high-frequency stimulus trains with stable antidromic latencies, and orthodromic action potentials collided with antidromic spikes (Fig. 19*C*). Its axon terminated in the contralateral VPM nucleus in the thalamus (Fig. 19*D*). This VTT neuron responded to innocuous and noxious mechanical stimuli (Fig. 19*E*) as well as to intradermal injection of serotonin (Fig. 19*F*), and was therefore classified as a pruriceptive VTT neuron. Vehicle applied to the cord had no effect on the ongoing response of the cell to serotonin. However, intrathecal application of morphine resulted in a pronounced increase in ongoing discharge, which lasted more than 5 min. The mean discharge rate

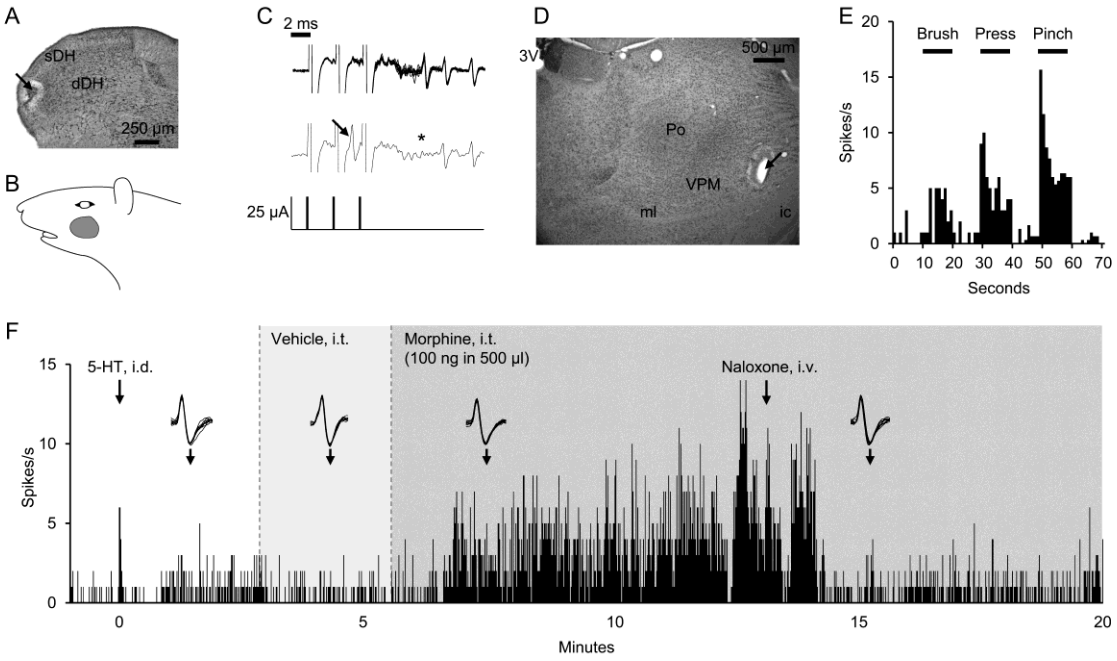

Figure19. Example of activation of a pruriceptive VTT neuron by intrathecal application of morphine. ***A***, lesion at the recording point (arrow) in sDH of C1 segment. Abbreviations: sDH, superficial dorsal horn; dDH, deep dorsal horn. ***B***, receptive field. ***C***, ten overlaid spike traces exhibit ability to follow high frequency stimulus train with a fixed latency (11.4 ms) between antidromic stimulation (25 µA) and recorded spike (top), and collision of orthodromic spike (arrow) with antidromic spike (asterisk at expected latency) (bottom). ***D***, lesion at the stimulation point (arrow) in the thalamus. Abbreviations: 3V, 3rd ventricle; Po, posterior nucleus; ml, medial lemniscus; ic, internal capsule. ***E***, responses to brush, pressure, and pinch. ***F***, response to intradermal (i.d.) injection of serotonin (5-HT) into the receptive field, intrathecal (i.t.) application of vehicle and morphine, and intravenous (i.v.) injection of naloxone. Onset of i.d. and i.v. injections denoted by arrows; onset of i.t. application denoted by dotted line, with duration denoted by shading. Insets: 10 overlaid consecutive spike traces, beginning at arrow.

during morphine application reached a level that exceeded its response to the pruritogen by greater than four-fold. Intravenous injection of naloxone reduced activity to the level seen before morphine application (Fig. 19*F*), indicating that the effect was likely mediated via opioid receptors.

An example of the contrasting responses of nociceptive-only VTT neurons to intrathecal application of morphine is illustrated in Figure 20. This cell was recorded within the sDH of the caudal medulla (Fig. 20*A*) and projected to the contralateral VPM nucleus in the thalamus (Fig. 20*C*). This neuron responded to innocuous and noxious mechanical stimulation of the receptive field (Fig. 20 *B,D*) but not to any of the four tested pruritogens (Fig. 20*E*). Application of vehicle to the spinal cord had no effect, whereas the same dose of intrathecally applied morphine resulted in a reduction in discharge rate which was reversed by naloxone (Fig. 20*F*); administration of naloxone often resulted in an increase in activity to a higher level than observed before morphine application, a phenomenon which has been noted in several past studies (Le Bars et al. 1975; 1976; Henry 1979; Lombard and Besson 1989; Jones et al. 1990).

The mean responses of pruriceptive and nociceptive-only VTT neurons to intrathecal application of morphine are illustrated in Figure 21. In pruriceptive VTT neurons, including cells responsive to serotonin, histamine, and chloroquine, morphine significantly increased the mean discharge rate in response to the pruritogen by more than two-fold (Fig. 21*A*,B). Seven of the eleven tested pruriceptive VTT neurons met our criteria for activation by intrathecal application

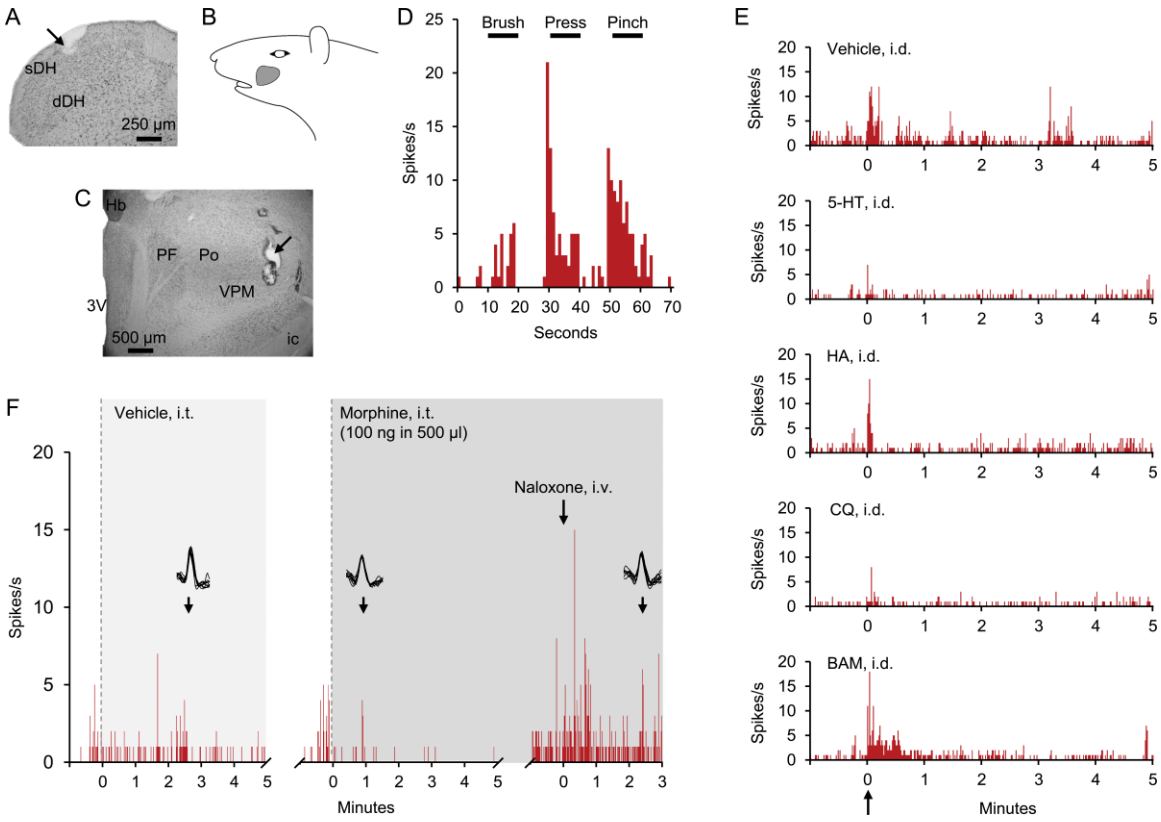

Figure 20. Example of inhibition of a nociceptive-only VTT neuron by intrathecal application of morphine. ***A***, lesion at the recording point (arrow) in the sDH of caudal medulla. ***B***, receptive field. ***C***, lesion at the stimulation point (arrow) in thalamus. Abbreviations: Hb; habenular nucleus; PF, parafascicular nucleus, others are as in Fig.19. ***D***, responses to brush, pressure, and pinch. ***E***, discharge rates following intradermal (i.d.) injections of vehicle, serotonin, histamine (HA), chloroquine (CQ), and BAM8-22 (BAM) into the receptive field. ***F***, responses during intrathecal (i.t.) application of vehicle and morphine, and intravenous (i.v.) injection of naloxone.

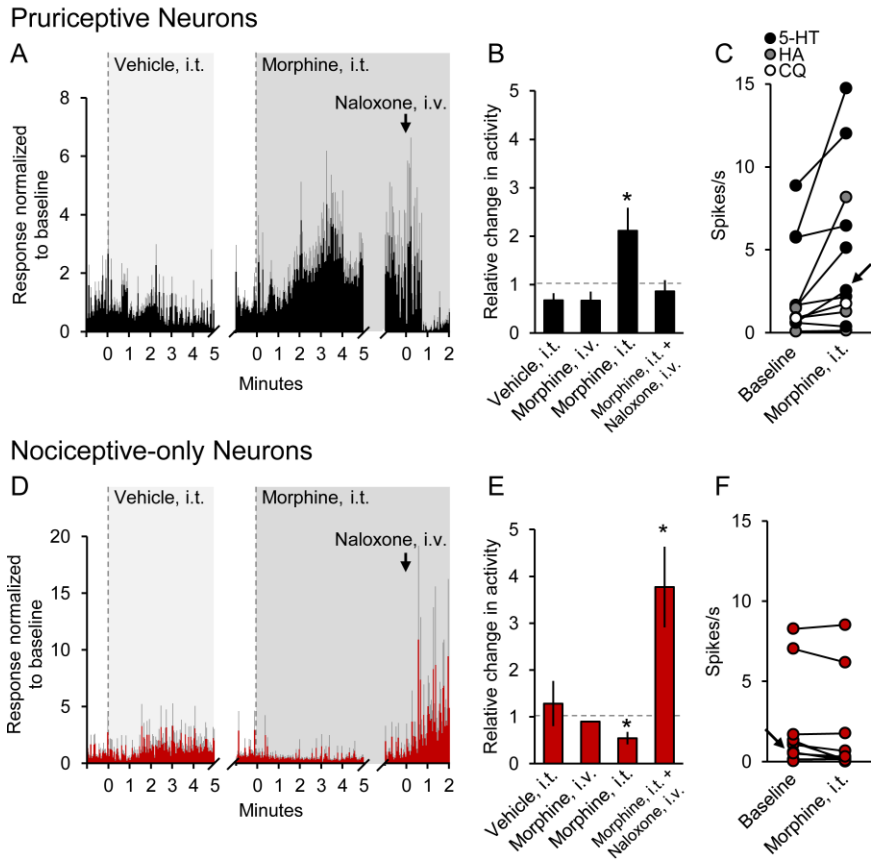

Figure 21. Mean responses of VTT neurons to intrathecal application of morphine. ***A***, mean histogram (3-s bins) of response of pruriceptive cells (n=11) to intrathecal (i.t.) application of vehicle and morphine, and intravenous (i.v.) injection of naloxone during an ongoing response to a pruritogen. Gray bars represent SEM. ***B***, mean changes in discharge rate for pruriceptive cells during four conditions. Dotted line indicates level of no change. * indicates statistically significant difference from vehicle (p=0.008; Kruskal-Wallis ANOVA with Dunn's post test). ***C***, discharge rates for each pruriceptive cell, including cells responsive to serotonin (black; n=7), histamine (gray; n=3), and chloroquine (white; n=1), during baseline and after i.t. application of morphine. Arrow indicates cell used in Figure 19. ***D***, mean histogram (3-s bins) of response of nociceptive-only cells (n=11) to i.t. application of vehicle and morphine, and i.v. injection of naloxone during ongoing activity. ***E***, mean changes in firing rates for nociceptive-only cells during four conditions. * indicates statistically significant difference from vehicle (p=0.02 for Morphine, i.t., p=0.009 for Morphine, i.t. + Naloxone, i.v.; Kruskal-Wallis ANOVA with Dunn's post test). Error bar for Morphine, i.v. condition too small to visualize (SEM=0.0098). ***F***, discharge rates for each nociceptive-only cell during baseline and after i.t. application of morphine. Arrow indicates cell used in Figure 20.

of morphine. None of the pruriceptive neurons were inhibited by morphine (Fig. 21*C*). Activity in none of the pruriceptive VTT neurons was significantly affected by intravenous injection of 100 ng of morphine (Fig. 21*B*), indicating that the same dose given intrathecally has a spinal site of action. In contrast, intrathecal application of morphine significantly decreased ongoing discharge rate in nociceptive-only VTT neurons (Fig. 21 *D,E*). None of the nociceptive-only neurons were excited by morphine (Fig. 21*F*). As was the case with pruriceptive VTT neurons, intravenous injection of 100 ng of morphine had no significant effect on the firing of nociceptive-only VTT neurons (Fig. 21*D*). In all cases, the effect of morphine was reversed with intravenous injection of naloxone.

Four pruriceptive VTT neurons were classified as HT and seven as WDR. Five nociceptive-only VTT neurons were classified as HT and six as WDR. Seven pruriceptive VTT neurons were recorded in the sDH and 4 in the deep dorsal horn (dDH). Six nociceptive-only VTT neurons were recorded in the sDH and 5 in the dDH. Six pruriceptive VTT neurons projected to the VPM nucleus, two to the posterior thalamus, and two to the ventral lateral geniculate nucleus; the axon location was not recovered for one pruriceptive VTT neuron. Seven nociceptive-only VTT neurons projected to the VPM nucleus, two to the posterior thalamus, and one to the medial geniculate nucleus; the axon location was not recovered for one nociceptive-only VTT neuron (data not shown). In summary, there were no distinct differences noted in response classification (HT vs. WDR), cell body position, or thalamic axon location for pruriceptive and nociceptive-only VTT neurons.

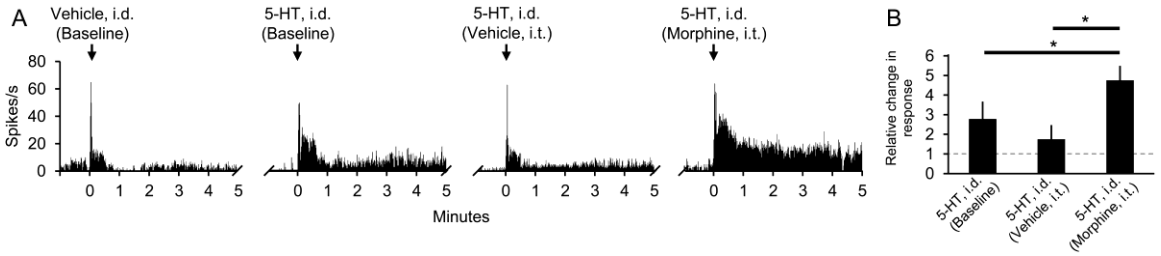

Figure 22. Effects of intrathecal application of morphine on responses to the pruritogen serotonin. ***A***, example of responses of a single cell to intradermal (i.d.) injections of vehicle and serotonin into the receptive field under baseline conditions, to a second i.d. injection of serotonin 15 min after intrathecal (i.t.) application of vehicle, and to a third i.d. injection of serotonin 15 min after i.t. application of morphine. ***B***, effects of i.t. application of vehicle or morphine on mean response to subsequent i.d. injection of serotonin (n=4). * indicates statistically significant difference between groups denoted by black bar (p=0.04 for 5-HT, i.d. (Baseline) vs. 5-HT, i.d. (Morphine, i.t.), p=0.02 for 5-HT, i.d. (Vehicle, i.t.) vs. 5-HT, i.d. (Morphine, i.t.); Kruskal-Wallis ANOVA with Dunn's post test).

Effects of intrathecal application of morphine on subsequent responses to pruritogens. Morphine can cause hyperknesis in humans (Fjellner and Hägermark 1982) and rats (Onigbogi et al. 2000). Figure 22*A* illustrates an example of a neuron in which the pruritogen serotonin was injected intradermally before and then repeated a second time after intrathecal application of vehicle, and a third time after intrathecal application of morphine. In each of the 4 neurons tested, the response to intradermal injection of serotonin was reduced after intrathecal application of vehicle, although the mean response was not significantly different from baseline. Intrathecal application of morphine increased the response to intradermal injection of serotonin in each of the 4 neurons. The magnitude of the response to intradermal injection of serotonin was significantly increased after intrathecal application of morphine compared to either the baseline response or to the response after intrathecal application of vehicle (Fig. 22*B*). These results suggest that morphine may cause hyperknesis by increasing responses of pruriceptive VTT neurons to pruritogens.

In a small number of neurons (n=3), responses to serotonin were compared before and after intravenous injection of naloxone (data not shown). After naloxone, responses to serotonin were slightly reduced, though the effect was not significant and it was not possible to distinguish from normal tachyphylaxis seen after repeated applications of serotonin. Naloxone also slightly reduced the discharge rate when it was administered during ongoing responses to serotonin (n=2) and histamine (n=2); again, these effects were not statistically significant.

Effects of intrathecal application of morphine on responses to innocuous versus noxious mechanical stimuli. Opioids have also been implicated in the production of alloknesis (Koenigstein 1948; Heyer et al. 2002). We tested the effects of intrathecal application of morphine on responses evoked by innocuous brushing in pruriceptive and nociceptive-only VTT neurons and found that responses to brushing were increased by morphine in 8 of 10 pruriceptive VTT neurons. An example of a dramatically increased response of a pruriceptive VTT neuron to brushing during intrathecal application of morphine is illustrated in Figure 23*A*. Mean responses of pruriceptive cells to brushing were increased five-fold in the presence of morphine (Fig. 23 *B,E*). These increases were reversed by naloxone (Fig. 23*E*). Neither intrathecal application of vehicle nor intravenous injection of morphine (100 ng) altered responses to brushing (Fig. 23*E*).

Unlike in pruriceptive cells, the mean response of nociceptive-only VTT neurons to brushing was not significantly affected by intrathecal application of morphine (Fig. 23 *C,D,F*); the response to brushing was increased by morphine in only 1 of 10 nociceptive-only cells. In addition, intrathecal application of morphine had no effect on responses to brushing in non-pruriceptive cells that respond only to low-threshold stimuli (Fig. 23*F*). These results suggest that intrathecal application of morphine likely contributes to alloknesis by increasing responses to innocuous mechanical stimuli only in pruriceptive cells, that the actions are likely mediated by opioid receptors, and that the site of action is within the spinal cord.

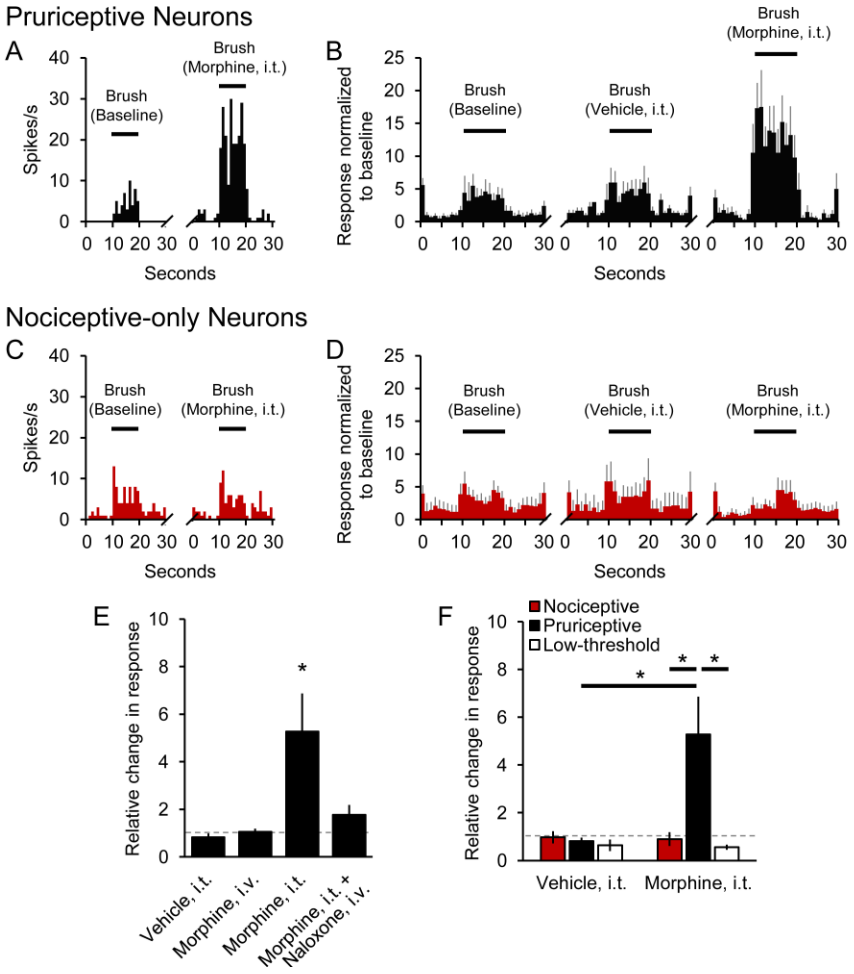

Figure 23. Differing effects of intrathecal application of morphine on responses to innocuous brushing. ***A***, example of a pruriceptive VTT neuron's baseline response to brushing and greatly increased response in the presence of intrathecal (i.t.) morphine. ***B***, mean histograms of response of pruriceptive cells (n=10) to brushing under three conditions. ***C***, example of a nociceptive-only VTT neuron's baseline response to brushing and similar response in the presence of i.t. morphine. ***D***, mean histograms of response of nociceptive-only cells (n=10) to brushing under three conditions. ***E***, normalized mean discharge rates during brushing for pruriceptive cells during four conditions. * indicates statistically significant difference from Vehicle, i.t. (p=0.02; Kruskal-Wallis ANOVA with Dunn's post test). ***F***, effects of i.t. application of vehicle and morphine on normalized mean responses of nociceptive-only, pruriceptive, and low-threshold (n=5) VTT neurons to brushing. * indicates statistically significant difference between groups denoted by black bar (p=0.02 for Pruriceptive/Vehicle, i.t. vs. Pruriceptive/Morphine i.t., p=0.01 for Pruriceptive/Morphine i.t. vs. Nociceptive-only/Morphine, i.t., p=0.01 for Pruriceptive/Morphine i.t. vs. Low-threshold/Morphine, i.t.; Kruskal-Wallis ANOVA with Dunn's post test).

We also examined the effects of intrathecal application of morphine on responses to noxious pinching in both pruriceptive and nociceptive-only VTT neurons. Morphine did not alter pinch-evoked responses in pruriceptive cells; an example is shown in Figure 24*A*. The mean responses of all tested pruriceptive VTT neurons to pinching at baseline, in the presence of intrathecal vehicle and intrathecal morphine are illustrated in Figure 24*B*; only 2 of 10 pruriceptive cells exhibited decreased responses to pinching during intrathecal application of morphine. In nociceptive-only VTT neurons, intrathecal application of morphine reduced responses to pinching by nearly 80% (Fig. 24 *C,D*); 9 of 10 nociceptive-only cells exhibited decreased responses to pinching during intrathecal application of morphine. This reduction was reversed by naloxone, and intravenous injection of morphine (100 ng) had no effect (Fig. 24*E*). Figure 24*F* summarizes and compares the effects of morphine on pinch-evoked responses of pruriceptive and nociceptive-only cells. These results suggest that, although both types of VTT neurons are clearly activated by noxious pinching, intrathecal application of morphine at the dose used inhibits pinching responses only in nociceptive-only neurons. Reduced ongoing activity (Fig. 21 *C,D*) and responses to noxious stimuli by nociceptive-only VTT neurons may contribute to analgesia resulting from intrathecal application of morphine.

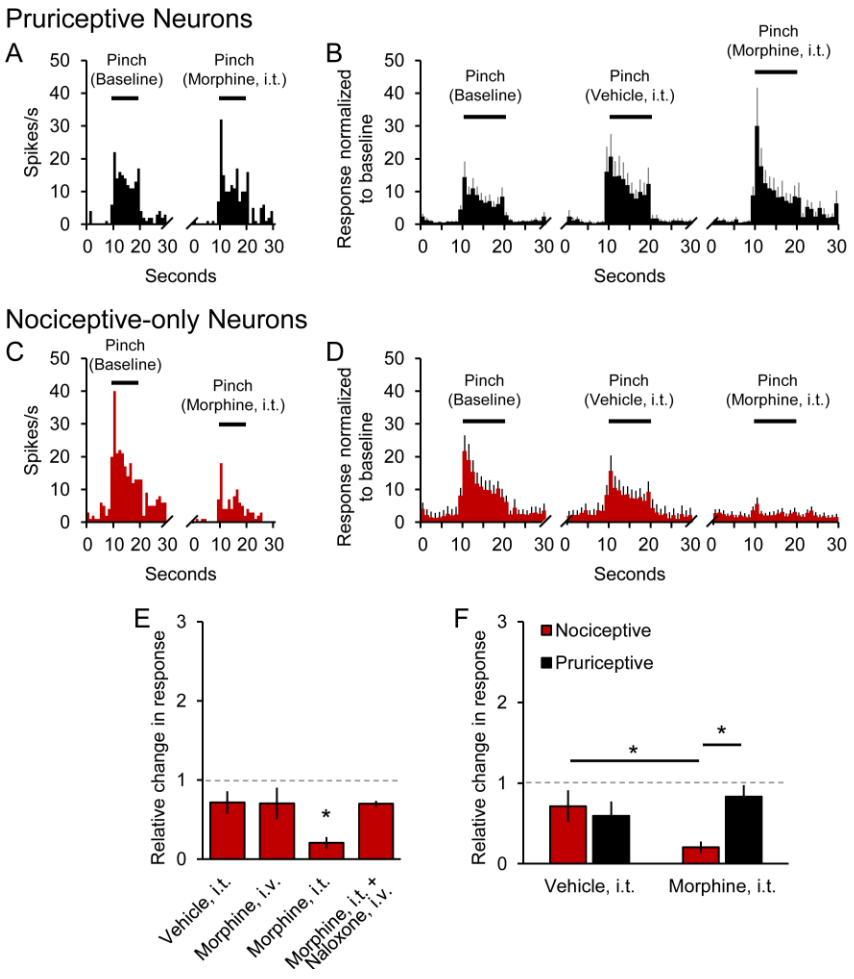

Figure 24. Differing effects of intrathecal application of morphine on responses to noxious pinching. ***A***, example of a pruriceptive VTT neuron's baseline response to pinching and similar response in the presence of intrathecal (i.t.) morphine. ***B***, mean histograms of responses of pruriceptive cells (n=10) to pinching under three conditions. ***C***, example of a nociceptive-only VTT neuron's baseline response to pinching and greatly reduced response in the presence of i.t. morphine. ***D***, mean histogram of responses of nociceptive-only cells (n=10) to pinching under three conditions. ***E***, normalized mean discharge rates during pinching for nociceptive-only cells during four conditions. * indicates statistically significant difference from Vehicle, i.t. (p=0.04; Kruskal-Wallis ANOVA with Dunn's post test). ***F***, effects of i.t. application of vehicle and morphine on normalized mean responses of nociceptive-only and pruriceptive VTT neurons to pinching. * indicates statistically significant difference between groups denoted by black bars (p=0.04 for Nociceptive-only/Vehicle i.t. vs. Nociceptive-only/Morphine, i.t., p=0.03 for Nociceptive-only/Morphine, i.t. vs. Pruriceptive/Morphine, i.t.; Kruskal-Wallis ANOVA with Dunn's post test).

Discussion

We have found that intrathecal application of morphine has dramatically different effects on pruriceptive and nociceptive-only VTT neurons. Pruriceptive signaling was increased by morphine. In some cases, intrathecal application of morphine increased by up to four-fold the ongoing discharge rates produced by intradermal injection of a pruritogen. If increased discharge rates in pruriceptive VTT neurons signal itch, then it would seem that the dramatically increased discharges caused by morphine should greatly increase the intensity of itch. In addition, responses to intradermal injection of the pruritogen serotonin following intrathecal application of morphine were increased, possibly contributing to hyperknesis, and responses to innocuous brushing were greatly increased, possibly contributing to alloknesis. None of the pruriceptive VTT neurons were inhibited by morphine. In contrast, intrathecal application of morphine inhibited ongoing activity and pinch-evoked responses in nociceptive-only VTT neurons without affecting responses to brushing. None were excited by morphine. The consistent nature of these effects would suggest that excitation or inhibition resulting from intrathecal application of morphine alone could be sufficient to identify these two cell types in future studies. Together, these changes to discharge in both pruriceptive and nociceptive-only VTT neurons produced by intrathecal application of morphine would appear to act to increase itch and reduce pain sensations (Fig. 25).

There is a great deal of evidence supporting an inhibitory role for

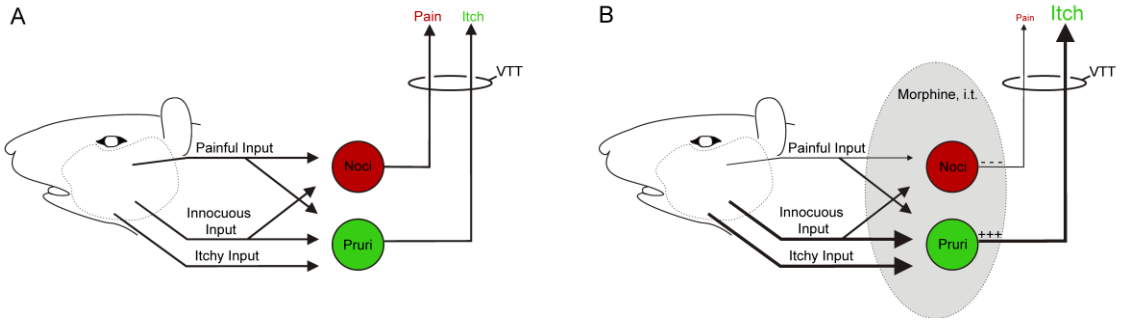

Figure 25. Schematic diagram illustrating potential changes to inputs and outputs of VTT neurons caused by intrathecal application of morphine. ***A***, under normal conditions, nociceptive-only VTT neurons (red) receive painful and innocuous input while pruriceptive VTT neurons (green) receive painful, innocuous, and itchy input. ***B***, during intrathecal (i.t.) application of morphine, painful input is decreased to nociceptive-only VTT neurons and innocuous and itchy input is increased to pruriceptive neurons, resulting in reduced pain and increased itch sensations. Although inputs are denoted with a single arrow, these inputs could be either mono- or polysynaptic. Changes in relative strength of inputs and outputs in presence of morphine are indicated by changes in thickness of lines.

morphine on nociceptive spinal neurons, including projection neurons; supporting data have come from a variety of species, experimental preparations, and routes of administration (Satoh et al. 1971; Kitihata et al. 1974; Le Bars et al. 1975; 1976; Yoshimura and North 1983; Hylden and Wilcox 1986; Willcockson et al. 1986; Craig and Serrano 1994; Chen and Pan 2002). However, morphine can also cause excitation of ongoing activity in some cells in the dorsal horn (Jones et al. 1990; Craig and Hunsley 1991). Willcockson et al. (1986) showed that a minority (~25%) of nociceptive spinothalamic (STT) neurons was excited, rather than inhibited, by iontophoretic application of morphine in the monkey dorsal horn. No clear differences were detected between excited and inhibited STT neurons that might explain the dramatically differing responses to morphine. The effects of pruritogens on the neurons examined in this previous study were not determined and thus neurons could not be categorized as pruriceptive or not. We have found that a similar fraction (~30%) of primate STT neurons is pruriceptive (Davidson et al. 2012), suggesting that the STT neurons activated by morphine in the study by Willcockson et al. (1986) may well have been pruriceptive. If this conclusion is correct, it would suggest that morphine may activate pruriceptive and inhibit nociceptive STT neurons in primates as it does VTT neurons in rats.

Morphine has been implicated in the excitation of pruriceptive spinal neurons via activation of the opioid receptor subtype MOR1D. Upon activation by morphine, MOR1D has been shown to create a heterodimer with the gastrin-releasing peptide receptor (GRPR), leading to phospholipase C/inositol 1,4,5-

trisphosphate-mediated increase in intracellular calcium in heterologous cells (Liu et al. 2011). GRPR-knockout mice as well as mice in which GRPR-containing neurons have been ablated exhibit reduced scratching in response to several pruritogens, without exhibiting behavioral abnormalities in pain assays (Sun and Chen 2007; Sun et al. 2009). Therefore, spinal neurons which contain GRPR likely play a major role in the production of itch, and may produce opioid-induced pruritus via activation of MOR1D by morphine. However, the presence of MOR1D in the rat spinal cord has been questioned (Oldfield et al. 2008), and it is as yet unknown whether spinal projection neurons or interneurons, or both, contain GRPR. The current data do not necessarily implicate the involvement of excitatory μ-opioid receptors; excitation of pruriceptive VTT neurons by morphine could also be a consequence of disinhibition via activation of inhibitory μ-opioid receptors on inhibitory interneurons.

In addition to causing itch, morphine has been implicated in the production of hyperknesis and alloknesis. In humans, itch produced by intradermal injection of histamine was significantly increased by co-administration of morphine as well as β-endorphin or the methionine-enkephalin analogue FK 33-824 (Fjellner and Hägermark 1982). Similarly in rats, morphine increased the amount of scratching caused by intradermal injection of chloroquine (Onigbogi et al. 2000). In addition, intrathecal application of morphine increased responses to injection of histamine into the skin by dorsal horn neurons for which axonal projections had not been determined in rats (Jinks and Carstens 2000). In our study, morphine increased

responses of pruriceptive VTT neurons to intradermal injection of serotonin in the face of the rat, possibly a contributing mechanism for hyperknesis demonstrated in Chapter 2. Morphine can also cause alloknesis, as intracisternal injection of morphine in cats results in a state in which touching the skin of the ear can elicit scratching (Koenigstein 1948). The endogenous opioid system likely plays a role in the production of alloknesis, since the opioid receptor antagonist naltrexone can block experimentally-induced alloknesis (Heyer et al. 2002). We show that morphine may contribute to alloknesis by greatly increasing the excitability of pruriceptive VTT neurons to innocuous mechanical stimuli.

Although responses to pinch were greatly reduced in nociceptive-only VTT neurons by intrathecal application of morphine, such responses were unaffected in pruriceptive VTT neurons. This result might suggest that responses in pruriceptive VTT neurons to noxious stimuli could contribute to any remaining pain sensation present after intrathecal morphine administration. Alternatively, it could suggest that responses to noxious stimuli in pruriceptive VTT neurons do not contribute to nociception. A similar conclusion has been reached recently regarding the responses to noxious stimuli of pruriceptive mouse MrgprA3 receptor-containing DRG neurons (Han et al. 2013). Thus, our results and those of Han et al. (2013) suggest the existence of peripheral and spinal projection neurons that, although responsive to nociceptive and pruriceptive stimuli, only contribute to itch.

In human patients, intrathecal delivery of morphine for pain relief is most

often administered caudal to the spinal cord, at the level of the lumbar vertebrae. Following intrathecal application of morphine at this level, patients experience itch (often on the face) 1-3 hours after intrathecal injection (Szarvas et al. 2003). This corresponds to the time period in which drugs reach peak concentrations in cerebrospinal fluid at the level of the rostral cervical spinal cord following intrathecal delivery at lumber vertebral levels (Rieselbach et al. 1962; Max et al. 1985; Payne and Inturrisi 1985), suggesting that the latency of pruritus following intrathecal lumbar injection of morphine may depend on the time-course of rostral spread of the drug. In rodent studies, scratching induced by intrathecal application of morphine is reported to occur with a shorter latency. In mice, intrathecal application of morphine at the lumbar vertebral level produces an increase in scratching within 5 minutes of injection which peaks at 10 minutes post-injection (Liu et al. 2011). Thomas and Hammond (1995) observed that facial scratching begins to increase within 10 minutes, reaching a statistically significant level 20 minutes after microinjection of morphine into the spinal trigeminal nucleus in rats. Likewise, Lee et al. (2003) reported that intracisternal application of morphine in rats causes significantly increased scratching 20 minutes after administration; scratching at earlier time points was not reported. Frenk et al. (1984) observed vigorous scratch-like behaviors within 1.9±0.9 min of intrathecal application of morphine in rats. In Chapter 2, we show that scratching due to morphine increases within the first 15 minutes following intracisternal injection (Fig. 5). In the present study, morphine increased ongoing

activity in pruriceptive VTT neurons approximately 2 minutes following intrathecal application (Fig. 21*A*). Therefore, the results of the previous behavioral studies in rodents correspond reasonably well to the results reported here.

It has been suggested that morphine may also produce itch by actions in the peripheral nervous system. There is evidence that morphine can activate receptors on mast cells in the skin, causing the release of histamine (Hermens et al. 1985). However, opioid-induced itch is generally not treatable with antihistamines and can be produced by opioid drugs, such as the selective μ-opioid receptor agonist fentanyl, which do not cause histamine release (Kjellberg and Tramer 2001; Szarvas et al. 2003). Clinically, itch as a side effect is much more common following spinal versus systemic application of morphine (Ganesh and Maxwell 2007). In primates, scratching induced by intrathecal application of morphine is blocked by systemic administration of μ-opioid receptor antagonists, but not by forms of antagonists which do not readily cross the blood brain barrier (Thomas et al. 1993; Ko et al. 2004). We found that morphine excites pruriceptive VTT neurons only during intrathecal application; the same amount of morphine delivered systemically had no effect. Together, these results strongly suggest that pruritus induced by intrathecal application of morphine is mediated by actions at μ-opioid receptors in the central nervous system.

There are several potential mechanism(s) underlying morphine's ability to simultaneously inhibit and excite functionally different populations of VTT neurons. Intrathecally applied morphine could conceivably act on opioid

receptors located either directly on VTT neurons, or on primary afferent terminals and/or spinal interneurons that provide input to VTT neurons. If the opioid receptors underlying morphine's inhibitory effects on nociceptive-only cells were located on the cell bodies or dendrites of VTT neurons, we would expect responses to all inputs to be decreased. If the same were true for pruriceptive cells, then we would expect responses to all inputs to be increased. The presence of input-specific effects on responses of both types of neurons suggests that morphine is not likely generating postsynaptic excitatory or inhibitory effects in VTT neurons. It appears more likely that morphine exerts its effects by activating receptors on primary afferent terminals or spinal interneurons, leading to indirect effects in VTT neurons. Identification of the excitatory or inhibitory opioid receptor subtypes and cellular circuitry involved in morphine's distinctly different effects on pruriceptive versus nociceptive-only VTT neurons remains an important area for future research and the development of better treatment options for both itch and pain. The rat VTT appears to be a promising system for such studies.

CHAPTER 5

Concluding discussion

The experiments described in the previous chapters address three important aims:

1. Determine the effects of combined delivery of intradermal injection of serotonin and intracisternal injection of morphine on itch-related behaviors using the face model in rats.

2. Identify the VTT neurons in rats which are likely involved in producing the itch sensation, including characterization of pruriceptive and nociceptive responses, facial receptive fields, recording points in the spinal trigeminal nucleus, and axon projections to the thalamus.

3. Establish the effects of intrathecal application of morphine on pruriceptive versus nociceptive-only VTT neurons in rats.

The rodent face model of itch provides a valid animal model of human itch by allowing the distinction of differential behaviors in response to itch- versus pain-evoking stimuli applied to the face. The experiments in Chapter 2 employ the face model of itch to establish dose-response curves for scratching induced by intradermal injection of serotonin, intracisternal injection of morphine, and combined delivery of the two pruritogens in rats. The data are in accordance with

data from other groups employing the same animal model (Klein et al. 2011; Spradley et al. 2013). Together with data from similar experiments in mice (Shimada and LaMotte 2008; Akiyama et al. 2010; Wilson et al. 2011), the face model of itch has been established as a highly replicable model of human itch. The face model is particularly relevant to certain human pruritic conditions in which itch is often localized to the face, including opioid-induced pruritus (Scott et al. 1980; Baraka et al. 1981; Collier 1981; Bromage et al. 1982), allergic itch (Friedlaender 2011; Turner and Kemp 2012), and certain forms of brain cancer (Adreev and Petkov 1975; Summers and MacDonald 1988)

There is a plethora of stimuli which can cause itch in humans. Most of these stimuli are not pure pruritogens, but instead produce itch accompanied by other noxious sensations such as burning or stinging (Sikand et al. 2009, 2011). Serotonin, the primary pruritogen used in the experiments included here, produces both itch and pain in humans (Schmelz et al. 2003). Accordingly, serotonin produces both itch-related scratching and pain-related wiping when applied to the face in rats (Fig. 2). Serotonin-induced scratching in rodents is reduced by μ-opioid receptor antagonists (Fig. 2; Spradley et al. 2012), a characteristic shared with itch induced by histamine in human subjects (Bernstein et al. 1982). Together, the past and present data suggest that when applied to the face in rats, serotonin is a moderately powerful pruritogen which produces a small but significant increase in pain-related behaviors, responses analogous to data from human psychophysics studies.

Like the STT for lower body receptive fields in primates, the VTT likely plays a critical role in producing the sensation of itch in the face of rodents. Responses to the pruritogen serotonin in VTT neurons (Figs. 8,11) correspond reasonably well with the time-course of scratching induced by serotonin (Fig. 2; Klein et al. 2011). Likewise, histamine and capsaicin produce responses in pruriceptive VTT neurons that exhibit similar peak timing and duration to scratching induced by these chemicals (Fig. 11; unpublished data). Pruriceptive VTT neurons are excited by intrathecal application of morphine (Figs. 19,21), a treatment which often causes severe itch as a side effect. Intrathecal application of morphine also increases responses to the pruritogen serotonin in VTT neurons, a likely mechanism for morphine-induced hyperknesis, a phenomenon established in humans (Fjellner and Hägermark 1982) and rats (Onigbogi et al. 2000). Similarly, morphine increases responses to innocuous mechanical stimulation in pruriceptive VTT neurons, a finding which could explain the established role of receptors in producing alloknesis (Koenigstein 1948; Heyer et al. 2002). Together, these data strongly suggest that VTT neurons play a role in producing the sensation of itch.

The study of itch requires the simultaneous study of pain. The two sensations, while perceived as distinct experiences in the human mind, appear to be produced by similar if not identical populations of neurons within the mammalian nervous system. While the behaviors related to itch and pain are distinct, most chemicals which cause itch-related behaviors also cause pain-

related behaviors. VTT neurons which respond to pruritogens also respond to noxious mechanical and/or thermal stimulation. The analyses used in this dissertation revealed a significant difference in ISI distribution for responses to noxious versus pruritogenic stimuli within the same neurons (Fig. 12), suggesting that the brain may use differences in spike timing dynamics to decode responses to itchy stimuli from responses to painful stimuli within the same neurons. However, the differences noted here could also simply be due to the difference in the types of stimuli compared (chemical for itch versus mechanical/thermal for pain). It is also possible that the brain decodes itch from pain via a population code. Indeed, recent evidence suggests that activation of itch-selective primary afferent neurons, even by a normally painful stimulus such as capsaicin, will result in itch-related behaviors (Han et al. 2013). In our experiments, morphine increased pruritogenic activity while having no effect on nociceptive responses in pruriceptive VTT neurons; nociceptive responses in nociceptive-only VTT neurons were reduced by morphine (Figs. 21,24). Together, these findings suggest that when an itch-selective subpopulation of nociceptive neurons (e.g. pruriceptive VTT neurons) is activated, the brain interprets the incoming signal as “itch”. However, when the whole population of nociceptive neurons (e.g. pruriceptive and nociceptive-only VTT neurons) is activated, the brain interprets the incoming signal as “pain”.

This dissertation addresses salient questions regarding the study of facial itch and characterizes a valuable system for the future study of the mechanisms

in the central nervous system underlying the sensations of itch and pain. The face model will allow the future study of behaviors elicited by putative pruritogens applied to the skin on the face or to the central nervous system via intracisternal injection; receptor antagonists can also be applied intracisternally to study the effects of receptors in the central nervous system (e.g. GRPR, Npra, NK-1). Due to the unique qualities of facial receptive fields innervated by the trigeminal nerve, the VTT provides a promising system for the study of allergic itch and other pruritic conditions affecting the mucosal membranes of the face. Finally, the face model of itch can be used in combination with other animal models such as dry-skin and post-burn models to study the effects of these pruritic conditions on pruriceptive and nociceptive processing in VTT neurons underlying facial itch.

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