Trigeminal Nociception in the African Naked Mole-Rat: The Acid Test

BY

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THESIS

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This thesis is dedicated to my family and especially my parents, John and Pam LaVinka, without whom this thesis would NEVER have been finished. They kept me going, sometimes even dragging me, towards the goals I wanted to accomplish. Dad, Mom, Jillian, Shannon, we'll always be a fivesome.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES cont.

FIGURE PAGE

LIST OF ABBREVIATIONS

AM	Aδ Mechanoreceptor Fibers
ASIC	Acid-sensing Ion Channel
ABC	Avidin-Biotin Complex
cAMP	Cyclic AMP
CGRP	Calcitonin Gene-Related Peptide
CH	C fiber Heat sensitive
CIP	Congenital Insensitivity to Pain
CMH	C fiber Mechanical Heat sensitive
CMHi	C fiber Mechanical Sensitive Heat Insensitive
CMiHi	C fiber Mechanical Insensitive Heat Insensitive
CRLR	Calcitonin Receptor Like-Receptor
DEG/ENaC	Degenerin/Epithelial Na Channel
DMR	Damaraland Mole-rat
GDNF	Glial Derived Neurotrophic Factor
GPCR	G Protein-coupled Receptors
HTM	High Threshold Mechanical
IP	Intraperitioneal Injection
K2P	Two-pore domain potassium channel
nACHR	Nicotinic Acetylcholine Receptor
Nav	Voltage-gated Sodium Channel
NGF	Nerve Growth Factor
NK1	Neurokinin 1 Receptor

LIST OF ABBREVIATIONS cont.

NMR	Naked mole-rat
NTS	Nucleus Tractus Solitarius
PBS	Phosphate-buffered Saline
PEPD	paroxysmal extreme pain disorder
PPK	Pickpocket Gene
PPT-A	Preprotachykinin-A gene
RAMP	Receptor Activity Modifying Protein
RCP	Receptor Component Protein
SP	Substance P
Sp5	Spinal Trigeminal Nucleus
Sp5o	Spinal Trigeminal Nucleus – oral
Sp5i	Spinal Trigeminal Nucleus – interpolar
Sp5c	Spinal Trigeminal Nucleus – caudal
TRP	Transient Receptor Potential channel
TRPA	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPN	Transient Receptor Potential NOMPC-like
TRPP	Transient Receptor Potential Polycystin
TRPV	Transient Receptor Potential Vanilloid
TTX	Tetrodotoxin

LIST OF ABBREVIATIONS cont.

TTX-R	Tetrodotoxin Resistant
WDR	Wide Dynamic Range Neurons

SUMMARY

Naked mole-rats live in underground burrows where carbon dioxide levels are unusually high. High levels of carbon dioxide cause tissue acidosis in the upper respiratory tract and pain via activation of the trigeminal nerve. That naked mole-rats live under these conditions in the wild suggests that they have an unusual tolerance for pain from acid. The goal of my project was to assess the naked mole-rat response to acidic stimulation of the trigeminal nerve as well as examine physical properties of the trigeminal nerve.

I first examined naked mole-rats' response to acidic fumes. It was previously shown that in the skin of the naked mole-rat, acidic saline could not activate the saphenous nerve. To test the trigeminal response to acid in naked mole-rats, I used acetic acid fumes, a known noxious irritant of the trigeminal nerve. I tested naked mole-rats' response to acetic acid both behaviourally and physiologically. Behaviourally, naked mole-rats showed a much higher tolerance to acidic fumes than other species tested, including a close relative, the Damaraland mole-rat. Physiologically, naked mole-rats showed no increase in activity in the spinal trigeminal nucleus, the area post-synaptically stimulated by pain signals from the trigeminal nerve. In fact, upon high enough stimulation with acid, the naked mole-rats showed a decrease in background activity in the spinal trigeminal nucleus. Hence, for the highest concentration of acidic fumes tested naked mole-rats showed avoidance behaviour but with no corresponding increase in activity of the trigeminal nerve.

I next looked at the nucleus tractus solitarius. This region receives chemical information from the vagus nerve, the nerve innervating the respiratory system. Naked

mole-rats showed increased activity within the nucleus tractus solitarius upon stimulation with the highest concentration of acetic acid. Therefore, naked mole-rats' avoidance of high concentrations of acidic fumes corresponds with activation of sensory cells in the respiratory system and not activation of the trigeminal nerve.

The transmission of pain signals from the trigeminal nerve to the brain is reliant on the release of neurotransmitters and neuropeptides, such as Substance P (SP) and calcitonin gene-related peptide (CGRP). Naked mole-rats lack SP and CGRP from their trigeminal ganglion. I previously showed that naked mole-rats have an increased tolerance to painful stimulation with ammonia fumes because this species lacks SP and CGRP from their trigeminal nerve. I wanted to further examine the role of SP in trigeminal pain processing. I tested SP knockout mice behaviourally and physiologically with ammonia and acetic acid. SP knockout mice avoided the noxious irritants but did not show an increase in activity in their spinal trigeminal nucleus or nucleus solitarius. These results were unexpected and show that the role of SP within nociception is not clear cut.

My last aim was to look at the anatomy of the trigeminal nerve in the naked mole-rat compared to mouse. Naked mole-rats' tolerance of painful irritants is not due to lack of unmyelinated fibers within their trigeminal nerve. They also have comparable number of pain fibers to those found in mice. I also stained for Substance P within the naked mole-rat brain and showed that lack of SP was specific to the spinal trigeminal nucleus.

All my findings support the hypothesis that naked mole-rats have adapted their trigeminal pain pathway to withstand their extreme living conditions.

I. INTRODUCTION

A. Pain

The International Association for the Study of Pain defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Pain is a protective mechanism. If pain is absent, an organism could walk around damaged without knowing it, as seen in conditions such as congenital insensitivity to pain (CIP) (Cox et al, 2006). So pain is a necessity but when the pain system becomes aberrant and overactive with no corresponding wound to protect, life can become excruciating. These conditions such as neuropathy or paroxysmal extreme pain disorder (PEPD) are not warning a person of being hurt, this is pain that occurs under circumstances when no pain signal is needed (Fertleman et al, 2006). By learning how to manipulate and control the pain resulting from different causes, we can help improve the quality of life of people who suffer from such disorders.

The nociceptive system can produce several types of pain sensation including pressure, hot, cold, itching, stinging and throbbing. Furthermore, these sensations can be acute or long-lasting. Long-lasting pain states include hyperalgesia, when the pain threshold is lowered and sensitized, and allodynia, when previously innocuous touch is now painful. The pain pathway has an unusual ability to be activated by different natural stimulus modalities (e.g. mechanical, thermal, and chemical) (Basbaum et al, 2001). Other sensory systems such as vision are triggered by one type of physical stimulus.

In mammals, light is detected by rods which activate an enzyme that degrades cGMP and causing cation channels to close. The closing of the cGMP-gated cation channels causes a membrane hyperpolarization (Luo et al, 2008). One stimulus is

activating the pathway. In comparison, within the nociceptive system, heat, capsaicin and low pH can all activate the same pain fibers.

B. Pain Fibers

Pain receptor cells are called nociceptors, from the latin word *nocere* which means 'to hurt.' The nociceptors fall into two classes, Aδ fibers which are myelinated and C-fibers which are unmyelinated. Aδ-fibers have medium-sized cell bodies and moderately thick axons while C-fibers have small cell bodies and thin axons (McHugh et al, 2000).

A painful stimulus usually induces two types of pain signaling: the first or 'fast' pain is attributed to Aδ fibers which, due to their myelination, conduct signals at 2 to 20 m/s. The second or 'slow' pain signal is carried by the C-fibers which conduct signals at less than 2 m/s. The slower conduction velocity of C fibers is a result of their lack of myelin sheaths (Purves et al, 2001). Aδ and C-fiber nociceptors are polymodal, able to react to thermal, mechanical and chemical stimuli but with different thresholds and different molecular receptors.

In anatomical terms, the nociceptor is pseudounipolar, with an axon extending to the periphery as well as to the central nervous system. Nociceptor cell bodies are found in the dorsal root ganglon and the trigeminal ganglion (Basbaum et al, 2009). Under normal circumstances, nociceptors only receive pain signals from the peripheral axon. However, if the neuron is damaged, ectopic firing from the cell body or central axon may result and that leads to pain states such as neuropathy, pain without any pain stimuli (Woolf and Ma, 2007).

Pain fibers can be subtyped into groups depending on what stimulus activates them. Aδ fibers can be divided into two subclasses. Type I Aδ fibers can be activated by chemical and mechanical stimulation and are also called high-threshold mechanical (HTM) fibers. These fibers can be activated by heat but only at temperatures greater than 50°C. Type II Aδ fibers respond to heat but at a much lower threshold in comparison to Type I Aδ fibers. Type II Aδ fibers also have very high mechanical thresholds (Basbaum et al, 2009). C fibers can also be divided into a variety of subclasses. In human peroneal nerves, 6% of C fibers respond to heat only (CH). Half of C fibers respond to heat and mechanical stimuli (CMH), 13% of C fibers respond to just mechanical stimuli and are insensitive to heat (CMHi). The other 25% of C fibers are insensitive to both mechanical and heat stimuli (CMiHi). Each of these classes included C fibers that were responsive to chemical stimuli. Also, after application of mustard oil (a common chemical stimulus in pain studies), some C fibers that weren't responsive to certain types of stimuli became sensitive to them. These were called 'silent' nociceptors, pain fibers that may only be active in inflammatory states. (Schmidt et al, 1995).

C. Receptors on Pain Fibers

Nociceptors are nerve cells that transduce various noxious stimuli into action potentials. They are not triggered by innocuous levels of sensation; it is only when the stimulation reaches high, dangerous levels that might result in tissue damage that nociceptors are activated. Nociceptors are located in the peripheral tissues as free nerve endings near small blood vessels (McHugh et al, 2000). Once triggered, the action potential is conducted down an axonal process to the cell soma in the dorsal root ganglia

or the trigeminal ganglion and several other cranial nerve nuclei. From the cell body, the action potential continues on to the spinal cord or brainstem to the primary synapse. There are three broad types of pain sensation: mechanical, thermal and chemical, and there are different molecular receptors activated by different pain types as described below.

1. Mechanical

Mechanical stimulation of various intensities activates different types of afferent sensory cell types. Light touch will activate Aδ D hair fibers, which respond to innocuous touch, not pain. These fibers contact hairs in the skin. Moderate touch will activate Aβ mechanoreceptors. Painfully intense stimulation will activate high threshold C fibers and slowly adapting Aδ mechanoreceptor fibers (AM) which have free nerve endings in the skin (Basbaum et al, 2009). The specific molecular receptors that are responsible for transducing mechanical pain are not known. There is evidence for the involvement of certain types of receptors from knock out studies but a direct correlation between stimulus and transduction of painful mechanical signals has been elusive (Bausbaum et al, 2009). Three likely candidates are described below.

a. DEG/ENaC channels

Degenerin/epithelial Na channels (DEG/ENaC) are proteins forming a nonvoltage gated, amiloride-sensitive cation channel (Bianchi and Driscoll, 2002; Garty and Palmer, 1997). These channels consist of subunits in multiples of three with the subunit consisting of two transmembrane domains, two short intracellular domains and a large extracellular loop (Bianchi and Driscoll, 2002). It is theorized that mechanical stimulation depresses the large extracellular loop which leads to an opening of the cation

channel but this has not been proven (Ben-Shahar, 2011). It has been shown that the pickpocket (*ppk*) gene, which encodes for a subunit of DEG/ENaC channels is involved in mechanosensory neurons. Mutations in *ppk* have been shown to be involved in the sensation of harsh mechanical stimuli (Hwang et al, 2007). Drosphila larvae mutants for *ppk* have shown reduced nociception to harsh mechanical stimuli (Zhong et al, 2010).

b. K+ channels

Potassium channels may act as receptors for mechanical pain. Potassium channels help to modulate neuron excitability and membrane potential. Two-pore domain potassium (K2P) channels have been shown to play a role in mechanosensation. K2Ps have two pores between four transmembrane domains (Hwang and Oh, 2007). K2P channels TREK-1, TREK-2, and TRAAK have been located in sensory neurons (Kang et al, 2005; Maingret et al., 2000). TRAAK and TREKs are activated by mechanical stretch (Lesage et al, 2000; Honore et al, 2006). TREK-1 aids in controlling membrane sensitivity; disrupting the TREK-1 gene resulted in lower mechanical pain thresholds and increased mechanical hyperalgesia (Alloui et al, 2006).

c. TRP Channels

The transient receptor potential (TRP) cation channels have been shown to be involved in mechanosensation in general and possible painful mechanosensation. There are seven subtypes of TRP channels: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPN (NOMPC-like). TRPN channels are found only in fish and invertebrates (Nilius and Owsianik, 2011). TRPV2 has been found in medium and large-diameter Aδ fibers that react to mechanical stimuli like osmotic pressure and suction (Muraki et al, 2003).

TRPC1 has been shown to be mechanically gated (Maroto et al, 2005) and TRPV4 has been implicated with bladder distension as knockout mice show problems with bladder voiding (Gevaert et al, 2007). TRPV4 knockout mice show reduced sensitivity to noxious mechanical stimulation via pinch to their tails (Suzuki et al, 2003). TRPA1 is also thought to possibly play a mechanosensitive role. TRPA1 mRNA has been found in cochlear hair cells, which are mechanosensitive, and coincides with the appearance of mechanosensitivity in zebrafish and mice (Corey et al, 2004). This is controversial as TRPA1 knockout mice have been shown to have normal auditory responses and mechanical thresholds (Bautista et al, 2006). So it is thought that perhaps TRPA1 plays a modulatory role in mechanosensation but is not a direct transducer. A lessened sensitivity to noxious punctuate, cutaneous mechanical stimuli has been shown in TRPA1 knockout mice. They also have higher mechanical thresholds compared to wildtype mice and reduced responses to suprathreshold mechanical stimulation by von Frey hairs (Kwan et al, 2006). A Drosphila gene called *painless* encodes for a protein in a TRP channel subunit that has been shown to play a role in mechanical nociception. When this gene is disrupted, Drosphila larvae showed ablated responses to harsh mechanical stimulation while response to innocuous touch remained intact (Tracey et al, 2003).

2. Thermal

There are two types of thermal nociception: hot and cold. All thermal pain receptors that have been characterized belong to the TRP family of cation channels.

a. Heat

Low rates of heating in the skin activate C-fibers and trigger discomfort while high rates of heating trigger intense pain via Aδ-fibers with the psychophysical threshold

of Aδ-fibers being 2.5-3 degrees/second higher (Yeomans et al. 1996, Plaghki et al. 2010). At temperatures greater than 43°C, C and type II Aδ nociceptors respond. If temperatures continue to climb, type I Aδ nociceptors become active at 52°C (Schepers and Ringkamp, 2010).

i. TRPV1

TRPV1 has been shown to be a noxious heat transducer. TRPV1 is a calcium channel that is activated at temperatures higher than 43°C (Caterina et al, 1997). TRPV1 was found to be highly expressed in medium and small diameter sensory fibers (Caterina et al., 1997; Tominaga et al, 1998). This suggests that TRPV1 is the thermal nociceptor that is activated on C fibers. TRPV1 knockout mice have altered thermal nociception, having trouble detecting noxious heat as well as decreased formation of thermal hyperalgesia (Caterina et al, 2000). However it was shown that the TRPV1 knockout mice only had trouble detecting noxious heat over 50°C. This suggests that TRPV1 is not the only thermal receptor and omitting its involvement does not disconnect the thermal nociceptive system.

ii. TRPV2

TRPV2 is activated by temperatures higher than 52°C (Caterina et al, 1999). TRPV2 receptors are localized heavily in medium to large diameter neurons, suggesting the TRPV2 receptor is responsible for the type I Aδ fiber heat signal (Caterina et al, 1999). However, it was shown using TRPV2 knockout mice, that TRPV2 is not essential to detect noxious heat in adult mice. Knockout mice were able to respond behaviourally to a large range of noxious temperatures and electrophysiologically, the C fibers still

reacted like those of wildtype mice to thermal stimulation. There was an effect on perinatal viability but TRPV2 is not critical for thermal nociception (Park et al, 2011).

iii. TRPV3 and TRPV4

Also, there are two related TRP family receptors involved in non-painful heat transduction. TRPV3 and TRPV4 are activated by warm temperatures in the ranges of 34-38°C and 27-35°C respectively (Smith et al, 2002; Peier et al, 2002a;Guler et al, 2002). However, in reference to noxious heat sensing for temperatures above 43°C, these channels do not seem to play an important role. Using TRPV3 and TRPV4 knockout mice, no deficits in thermal nociception were seen compared to wildtype mice. Preference on a thermal gradient was also comparable to wildtype mice (Huang et al, 2011). However, one research group did find TRPV3 knockout mice to have lessened responses to noxious heat (Moqrich et al, 2005). So these channels may still prove to play a role in noxious thermosensation in some way.

b. Cold

Temperatures below 15°C are considered noxious and cause nociceptors to fire (Basbaum et al, 2009).

i. TRPM8

TRPM8 is a non-selective cation channel belonging to the melastatin subfamily of the TRP receptors. TRPM8 has a temperature threshold of 25°C. TRPM8 is expressed in small-diameter sensory neurons but ones that do not contain TRPV1, indicating a distinct class of sensory fibers (Peier et al, 2002b; McKemy et al, 2002). TRPM8 knockout mice showed altered cold nociception compared to wildtype mice. Sensory fibers that were shown to lack the receptor were activated less by cold temperatures (18°C), and TRPM8

null mice have decreased behavioural responses to cold stimulation (Colburn et al, 2007). Calcium imaging of trigeminal neurons taken from TRPM8 knockout mice showed a significant decrease in response to noxious cold temperatures (9°C) when compared to wildtype mice (Knowlton et al, 2010). Decreased Fos activity within the spinal cord dorsal horn upon stimulation with 0°C was shown in TRPM8 knockout mice compared to wildtype mice (Knowlton et al, 2010). When tested in temperature preference, TRPM8 knockout mice showed decreased cold aversion but had normal nociceptive responses to subzero centigrade temperatures (Dhaka et al, 2007). This suggests that TRPM8 is not the only cold thermoreceptor.

ii. TRPA1

TRPA1 is also thought to contribute to noxious cold sensation. However, there is conflicting evidence on whether or not this is true. Two different TRPA1 knockout mice were developed. One strain showed no difference in response to cold compared to wildtype mouse neurons (Bautista et al, 2006). Another line of TRPA1 knockout mice however did show a decrease in neuronal response to cold when compared to wildtype mice (Karashima et al, 2009). In vivo testing has also shown mixed results. In one lab, TRPA1-/- mice were shown to have reduced paw lifting from a 0°C coldplate compared to wildtype mice (Kwan et al, 2006). On the opposite side, another TRPA1 knockout mouse experiment showed no difference in paw lifting from a coldplate compared to wildtype mice. Also, both wildtype and TRPA1 knockout mice showed a preference for the room-temperature side in a two-place preference test (Bautistia et al, 2006).

3. Chemical

Avoidance of irritating and damaging environmental chemicals is another aspect of nociception. Painful irritation can also result from chemicals produced during physiologically stressful events, such as chemicals released upon tissue damage (Basbaum et al, 2009).

a. TRPV1

TRPV1 is activated by capsaicin (Caterina et al, 1997). Capsaicin acts by binding intracellularly to TRPV1, near the second and third transmembrane domains (Jordt and Julius, 2002). TRPV1 knockout mice show decreased sensitivity to capsaicin. When capsaicin was injected into the paw of TRPV1 knockout mice, they showed little to no licking of the paw compared to wildtype. TRPV1 knockout mice also drank capsaicin-spiked water at the same rate as normal water. This was not true for wildtype mice (Caterina et al, 2000). TRPV1 has also been shown to be activated by inflammatory mediators that are released upon tissue injury (Gerhold and Bautista, 2009). TRPV1 can be activated by low pH (Kress et al, 2006; Bevan and Geppetti, 1994). A large drop in pH, pH 5.5, is needed though to activate TRPV1 receptors (Hwang and Oh, 2007).

b. TRPA1

TRPA1 is expressed in many of the same fibers as TRPV1 (Bautista et al, 2005; Kobayashi et al, 2005). TRPA1 has been shown to be activated by a variety of chemical irritants. Activation occurs through covalent bonding with TRPA1's cysteine residues (Macpherson et al, 2007a). TRPA1 has been shown to be activated by mustard oil, cinnamon, formalin and other chemical agents (Bandell et al, 2004; Jordt et al, 2004; Macpherson et al, 2007b; Bautista et al, 2006; McNamara et al, 2007; Namer et al, 2005).

TRPA1 can also be activated by 15dPGJ(2), a chemical released upon tissue injury (Taylor-Clark et al, 2008). Acrolein, an irritant found in car exhaust, causes irritation and inflammation. TRPA1 knockout mice have reduced sensitivity to acrolein among other environment irritants (Caceres et al, 2009). This suggests TRPA1 plays an important role in sensing environmental irritants.

c. ASICs

Acid-sensing ion channels (ASICs) are members of the DEG/ENaC receptor family (Basbaum et al, 2009). Tissue damage resulting in inflammation can cause tissue acidification. Acidification can activate receptors, such as ASICs (Reeh and Steen, 1996). ASICs are activated by a smaller drop in pH compared to TRPV1 receptors. ASICs are activated around pH 6.9 (Hwang and Oh, 2007). There are different subtypes of ASIC channels, in particular ASIC3 is found in nociceptors (Basbaum et al, 2009). ASIC3 is associated with fibers innervating cardiac and skeletal muscle. These muscles can activate ASIC3 channels when anaerobic metabolism leads to an increase in lactic acid and protons. This activation can lead to cardiac or skeletal pain (Immke and McCleskey, 2001).

4. Polymodal

Pain receptors can be polymodal, activated by multiple types of stimuli. I will discuss one example, TRPV1.

The first TRP channel found in mammalian sensory nerves was TRPV1. In 1997, TRPV1 was cloned and found to be the receptor activated by capsaicin (Caterina et al, 1997). The finding of TRPV1 opened up a new field searching for molecular pain

receptors. Since then, multiple stimuli have been found to activate TRPV1. TRPV1 has been shown to be involved in mechanosensitivity as it is needed to detect bladder distension (Birder et al, 2002). TRPV1 is activated by acidic pH (Tominaga et al, 1998). TRPV1 also works as a thermosensitive pain receptor. It is activated by temperatures greater than 43°C (Caterina et al, 1997). So TRPV1 has been implicated in each type of pain: mechanical, thermal, and chemical.

D. Sodium Channels

The creation and propagation of an action potential in sensory afferents is reliant on four events: 1. stimulation of a receptor that creates a depolarizing voltage change; 2. activation of voltage-gated sodium channels which causes the upswing of an action potential; 3. inactivation of voltage-gated sodium channels which starts the repolarization phase of an action potential; and 4. activation of potassium channels that returns the nerve back to resting potential (Kress and Mennerick, 2009). Stimulation of the molecular receptors is essential to begin the process, but voltage-gated sodium channels are necessary for initiation and propagation of a full-blown action potential (Hodgkin and Huxley, 1952). The action potential propagates down the axon and continues on to the central nervous system where it affects post-synaptic targets.

Voltage-gated sodium (Nav) channels are constructed of a large α subunit (~260 kDa) linked with auxiliary β subunits (~30 kDa). The α subunits are organized into homologous domains (I-IV) that each contain six transmembrane α helices (S1-S6) with an additional pore loop between S5 and S6. The S5 and S6 segments line the inner, wider exit of the pore. The other pore loops line the outer, narrow entry. The kinetics

and voltage dependence of a Nav channel comes from the β subunits (Leterrier et al, 2011). A dense cluster of voltage-gated sodium channels is found on the axon initial segment of myelinated fibers and properties of neuronal firing can be altered, depending on the sodium channel subunit composition (**figure 1)**. Spike initiation as described above is found in both myelinated and unmyelinated fibers (Kress and Mennerick, 2009).

Voltage-gated sodium channels are not only found at the axon initial segment of myelinated neurons. For example, unmyelinated, intrahippocampal mossy fiber axons have a dense number of sodium channels, 2000 channels per bouton. This high density of channels is thought to help regenerate the action potential in a location that, due to impedence mismatch with the axon, could impede propagation of the signal (Engel and Jonas, 2005). So having customized placements of sodium channels has functional consequences (Kress and Mennericak, 2009). Other locations where Nav channels are found include: Nodes of Ranvier of myelinated nerve fibers and along the entire axons of unmyelinated fibers. Unmyelinated C fibers show a uniform, low-level expression of sodium channels along their entire axon (Waxman and Ritchie, 1985). This includes the peripheral portion of the axons of somatosensory afferents in general, including pain afferents in particular. Thus voltage-gated sodium channels are an effective target to try and block or manipulate pain signaling.

Voltage-gated sodium channels are potential therapeutic targets for the treatment of pain for such a reason. But we do not want to block all electrical signals in the body, which would be catastrophic. But luckily, voltgage-gated sodium channels have various subtypes that are specifically involved in different types of sensory nerves (pain vs.

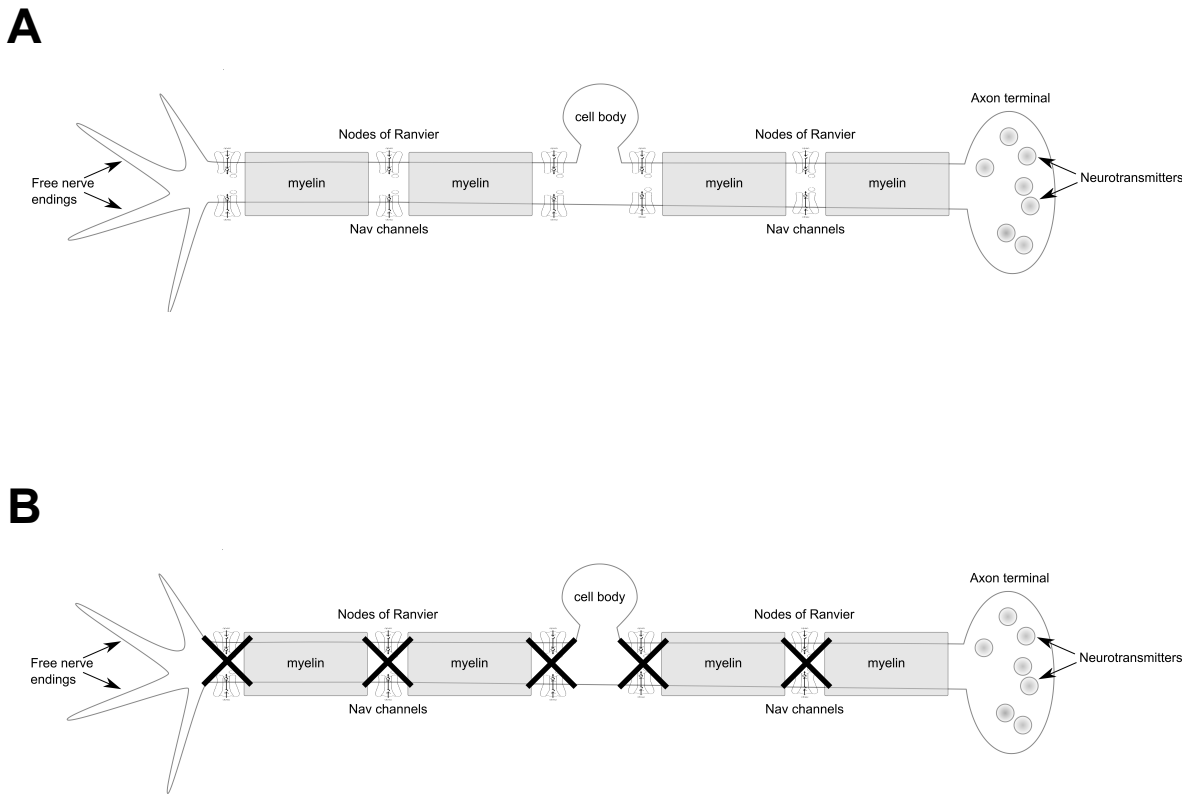


Figure 1. Sodium channels and propagation of action potentials
A. Voltage-gated sodium channels are found densely packed in the Nodes of Ranvier along the entire length of the axon. Activation of these channels propagates the action potential down the axon towards the cell body and then to the nerve terminal. The action potential results in release of neurotransmitters from the nerve terminal.
B. Blocking these channels can stop the propagation of action potentials down the axon.

touch). There are nine subtypes of voltage gated sodium channels, Nav 1.1 through 1.9 whose alpha-subunits vary in expression and association with beta-subunits that shape the properties of the sodium channel in various ways (Catterall, 2000). With concerns to nociception, four subtypes of Nav channels are of particular interest, Nav 1.3, 1.7, 1.8 and 1.9. Within these four subtypes, we can further separate the sodium channels based on their response to tetrodotoxin (TTX). Nav 1.3 and 1.7 are sensitive to TTX and their activity can be blocked by the drug while Nav 1.8 and 1.9 are insensitive to TTX (TTX-R) and in large part, are not affected by exposure to the drug (Priestly, 2004). This is thought to be because of differences in receptor kinetics and binding, aspects that come into effect as to when specific types of sodium channels are upregulated and used in certain states. Interestingly, TTX-R currents are largely associated with small diameter neurons (Aδ and C nociceptors; Arbuckle and Docherty, 1995; Elliott and Elliott, 1993). Experiments conducted in the peripheral nerve endings showed that much higher levels of TTX were needed to suppress electrical activity in the C fibers and slow Aδ nerve endings than in the fast Aδ nerves. This indicates that there is a higher expression of TTX-R channels in the C and slow Aδ nerves than in the fast Aδ nerves. Because this is found in the peripheral nerve endings, this larger expression of TTX-R channels might be shaping the sensitivity and kinetics of action potential initiation opposed to the conductive qualities (Strassman and Raymond, 1999).

1. Nav 1.3

Voltage-gated sodium channel 1.3 became a potential nociceptive target when it was observed that in normal conditions, it is largely absent from the peripheral nervous system. If however, an axotomy is performed or nerve damage occurs, an upregulation

of Nav 1.3 is shown (Hains et al, 2003, Waxman et al, 1994**,** Craner et al 2002.) So Nav 1.3 plays a role in the development of neuropathy, the pain state that occurs after nerve damage. Nav 1.3 accumulates in neuromas in patients with neuropathic pain (Black et al, 2008). Interestingly, this upregulation of Nav 1.3 can be reversed with application of glial derived neurotrophic factor (GDNF) and nerve growth factor (NGF) (Leffler et al 2002). This upregulation of Nav 1.3 channels is thought to contribute to hypersensitivity of the nerve which is found in neuropathic states. However, Nav 1.3 knockout mice can still develop neuropathy (Nassar et al, 2006). Injured nerves still had ectopic firing even in the absence of Nav 1.3 (Nassar et al, 2006). These results suggest that Nav 1.3 is not responsible for the abnormal spontaneous activity that can result from injured nerves (Wang et al, 2011).

2. Nav 1.7

Nav 1.7 is another TTX sensitive subtype that is involved with nociception. It is found in sensory and sympathetic nerves (Wood et al, 2004). Focus on Nav 1.7 and its role in nociception increased when it was found that mutations in this channel lead to pain disorders in humans (Cox et al. 2006, Dib-Hajj et al. 2008). Nav 1.7 is shown to be located prominently in small-diameter neurons within the dorsal root ganglion (Black et al, 1996; Black et al, 2004). When Nav 1.7 is removed only from nociceptive neurons, noxious mechanosensation and inflammatory pain is abolished regardless of stimulation (Nassar et al, 2004). Nav 1.7 shows unique gating kinetics; these channels have slowed closed-gate inactivation (Cummins et al, 1998). The slow closed-gate inactivation of Nav 1.7 means that it will not react to high-frequency stimulation. It will respond to small depolarizing stimuli close to resting membrane potential (Cummins et al, 1998).

However this research has come under fire as it was done only on the α subunit of Nav1.7 and sodium channel β subunits might influence the α subunit properties (Preistley et al, 2004; Scherbatko et al, 1999; Vijayaragavan et al, 2001). Nav 1.7 has been implicated in inflammatory pain. When the paws of mice were injected with inflammatory caraggeenan, Nav 1.7 mRNA levels were upregulated (Black et al, 2004).

3. Nav 1.8

Nav 1.8 channels are found highly expressed in C fibers, only in dorsal root, trigeminal, and nodose ganglia (Sangameswaran et al, 1996; Akopian et al, 1996). The voltage needed for activation and inactivation of Nav 1.8 channels is much more positive than other sodium channels. This means that Nav 1.8 can function at voltages that other sodium channels would not (Akopian et al, 1996; Rabert et al ,1998). Also, Nav 1.8 channels do not inactivate at low temperatures (unlike other sodium channels), this results in Nav 1.8 channels being the action potential producer for noxious cold signals. Nav 1.8 knockout mice are shown to be insensitive to cold temperatures over a wide range (Zimmerman et al, 2007). Nav 1.8 knockout mice were shown to have lost their ability to feel cold pain or mechanical pressure (Akopian et al, 1999) or have attenuated responses (Abrahamsen et al, 2008). Nav 1.8 has faster repriming from its inactivation state than other channels. This combined with Nav 1.8's higher inactivation voltage suggests that Nav 1.8 channels could keep responding to high frequency stimulation, such as seen from damaged nerves (Priestley, 2004). Nav 1.8 also has been shown to play a role in the development of hyperalgesia. Nav 1.8 knockout mice showed absence of visceral sensitization to intraperitoneal injection of capsaicin (Laird et al, 2002). When compared to wildtype mice, Nav 1.8 null mice showed ablated ectopic firing in fibers

collected from neuromas. The Nav 1.8 null mice also did not show the mechanical hypersensitivity that the wildtype mice did (Roza et al, 2003).

4. Nav 1.9

Nav 1.9 channels are found in small-diameter neurons within the dorsal root ganglion and over 62% of the channels were found within non-peptidergic fibers (Fukuoka et al, 2008). Nav 1.9 is called the persistant sodium current. Nav 1.9 has too slow of channel kinetics to contribute to the upswing of an action potential but is thought to contribute to setting membrane potential and activation thresholds. Nav 1.9 shows slow and incomplete inactivation of its channel over its activation range, which is much more negative than other sodium channels (Baker et al, 2003; Herzog et al, 2001). In Nav 1.9 knockout mice, inflammatory agents such as bradykinin, were not able to sensitize the sensory neurons (Ritter et al, 2009; Maingret et al, 2008). These findings suggest that Nav 1.9 channels may be responsible for the hyperexcitability of nociceptors during inflammatory pain (Liu and Wood, 2011).

E. Pain Pathways

Once an action potential is created by stimulation of receptors and propagated by sodium channels, it travels from the periphery to the central nervous system. Pain information travels in one of two pathways, pain from the body traveling up the spinothalamic tract while pain from the face and head travels via the trigeminothalamic tract.

1. Spinothalamic Tract

The axons of primary afferent nociceptors in the body enter the spinal cord by the dorsal root. When the axons reach the dorsal horn, they branch and travel anterior and/or posterior one or two spinal segments, entering the gray matter of the dorsal horn. Dorsal horn neurons are innervated by both Aδ- and C-fibers, with the fibers synapsing within specific laminae in the dorsal horn. The Aδ fibers enter the spinal cord dorsal horn and project to lamina I and V while the C fibers project to lamina I and II (Basbaum and Jessell, 2000). Lamina V also receives input from Aβ fibers that are activated from touch. These inputs converge with the nociceptive inputs onto wide dynamic range (WDR) neurons (Willis and Westland, 1985). These neurons are thought to be the T neurons talked about in Melzack and Wall's Gate Theory. These WDR neurons act as a gate to the brain, controlling the signal being sent on. The large diameter fibers' touch signal competes with the small diameter fibers' nociceptive signal (Melzack and Wall, 1965). This is thought to be the reason why rubbing an injured area can help ease the pain. The second order neurons project through the laminae of the dorsal horn, and cross the midline, and ascend to the brainstem and thalamus. This pathway is called the spinothalamic tract and is the major pathway for information concerning pain and temperature from the body (Purves et al, 2001).

2. Trigeminothalamic tract

When nociception concerns the face, information travels via a different pathway to the thalamus. The face, including the nasal cavity, is innervated by the trigeminal nerve (cranial nerve V). The Oxford English dictionary tells us the name trigeminal originated from the Latin *trigeminus*, meaning "triplets". This is appropriate as the nerve is divided into three branches: opthalamic, maxillary, and mandibular (Purves et al. 2001). Each branch innervates a specific facial region before coming together in the trigeminal ganglion **(Figure 2)**. The mandibular branch of the trigeminal nerve is the only mixed sensory/motor branch. The opthalmic and maxillary branches are purely sensory. It is these two branches that innervate the nasal cavity. The nasal cavity consists of the nasal bone anteriorally and the maxilla and ethmoid bones laterally. It is divided medially by the septum and upon entering, air readily contacts the nasal epithelium (Purves et al. 2001). The nasal cavity is innervated by the free nerve endings of Aδ- and C-fibers in the nasopalatine and ethmoid branches of the trigeminal nerve. The free nerve endings terminate within a few micrometers of the tissue surface (Finger et al. 1990). These trigeminal nerve fibers convey information about pain in the nasal cavity and are anatomically distinct from fibers in the olfactory epithelium that respond to odors. Thus the chemesthetic trigeminal fibers of the nasal cavity are well positioned to play an important role in alerting an animal to pain-causing airborne chemical irritants.

Noxious chemical irritants can stimulate trigeminal nerve endings, producing chemogenic pain (stinging, burning) which triggers protective reflexes of withdrawal and rejection (Silver, 1992). These chemesthetic nerve fibers are polymodal, capsaicin-sensitive C-fiber nociceptors (Silver et al., 1991). As discussed previously, C fibers have

many different types of ligand-gated receptors including ASICs and the capsaicin receptor, TRPV1 (Ichikawa and Sugimoto 2002, Alimohammadi and Silver, 2000; Julius and Basbaum, 2001). Trigeminal pain information is transmitted via the nociceptors

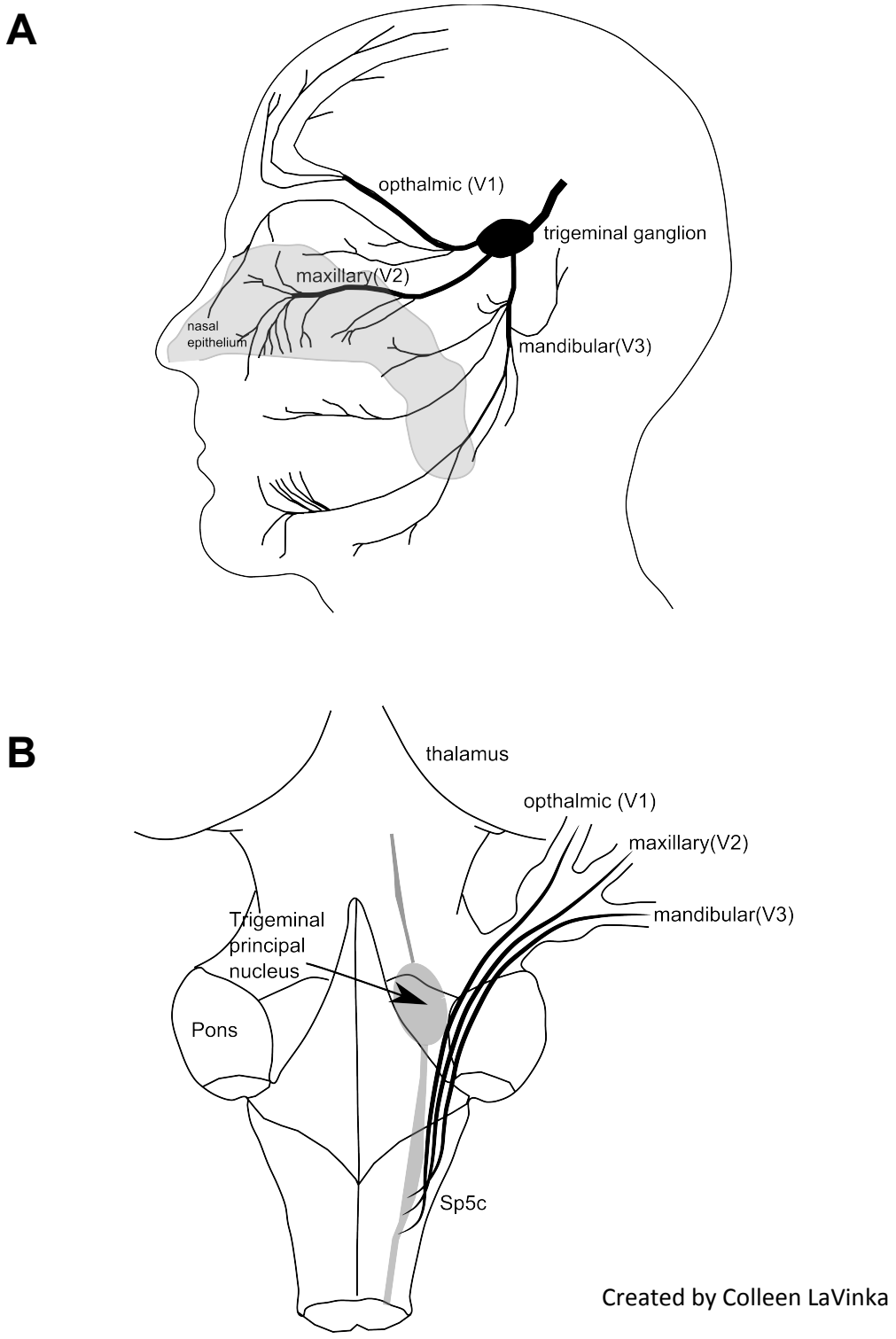


Figure 2. Anatomy of the trigeminal nerve

A. The trigeminal nerve has three branches that innervate the head and face: mandibular, maxillary, and ophthalmic which come together in the trigeminal ganglion

B. The three branches of the trigeminal nerve enter the brainstem at the level of the pons and pain fibers descend and synapse onto the spinal trigeminal nucleus caudal region (Sp5c).

central axons, which enter through the pons and descend to the medulla, synapsing in the spinal trigeminal nucleus. The spinal trigeminal nucleus is divided into three parts, the oral (Sp5O), interpolar (Sp5I) and caudal (Sp5C) **(figure 2)**. The Sp5C is considered the trigeminal equivalent of the dorsal horn pain laminae (Sessle, 2000). The Sp5C receives general sensory afferents from the facial nerve, glossopharyngeal and vagus nerve (Haines, 2008). Ethmoidal primary afferents of the trigeminal nerve project to laminae I and II of the Sp5C and parts of the Sp5I (Anton et al. 1991). When excited, chemesthetic trigeminal C fibers release a variety of neurotransmitters onto Sp5C neurons. These neurotransmitters include the excitatory amino acid glutamate and the neuropeptides Substance P and Calcitonin-Gene Related Peptide (CGRP) (Mitsikostas et al. 1999). **Figure 3** presents a schematic of this release. In contrast, Aδ-fibers primarily release only glutamate onto their post-synaptic targets. This raises the question of what role do the neuropeptides play in chemogenic pain information processing?

F. Neuropeptides

Experiments have shown that high frequency stimulation of afferent pain fibers can lead to the release of neuropeptides. The stimulation needed to release a neuropeptide such as Substance P is much higher than what is needed to release glutamate (Duggan et al, 1995; Marvizon et al, 1997). This suggests that neuropeptides help signal higher levels of pain (Cao et al, 1998). Peptidergic release is described as volume transmission (Agnati et al, 2006). Removal of peptides from the synaptic cleft is

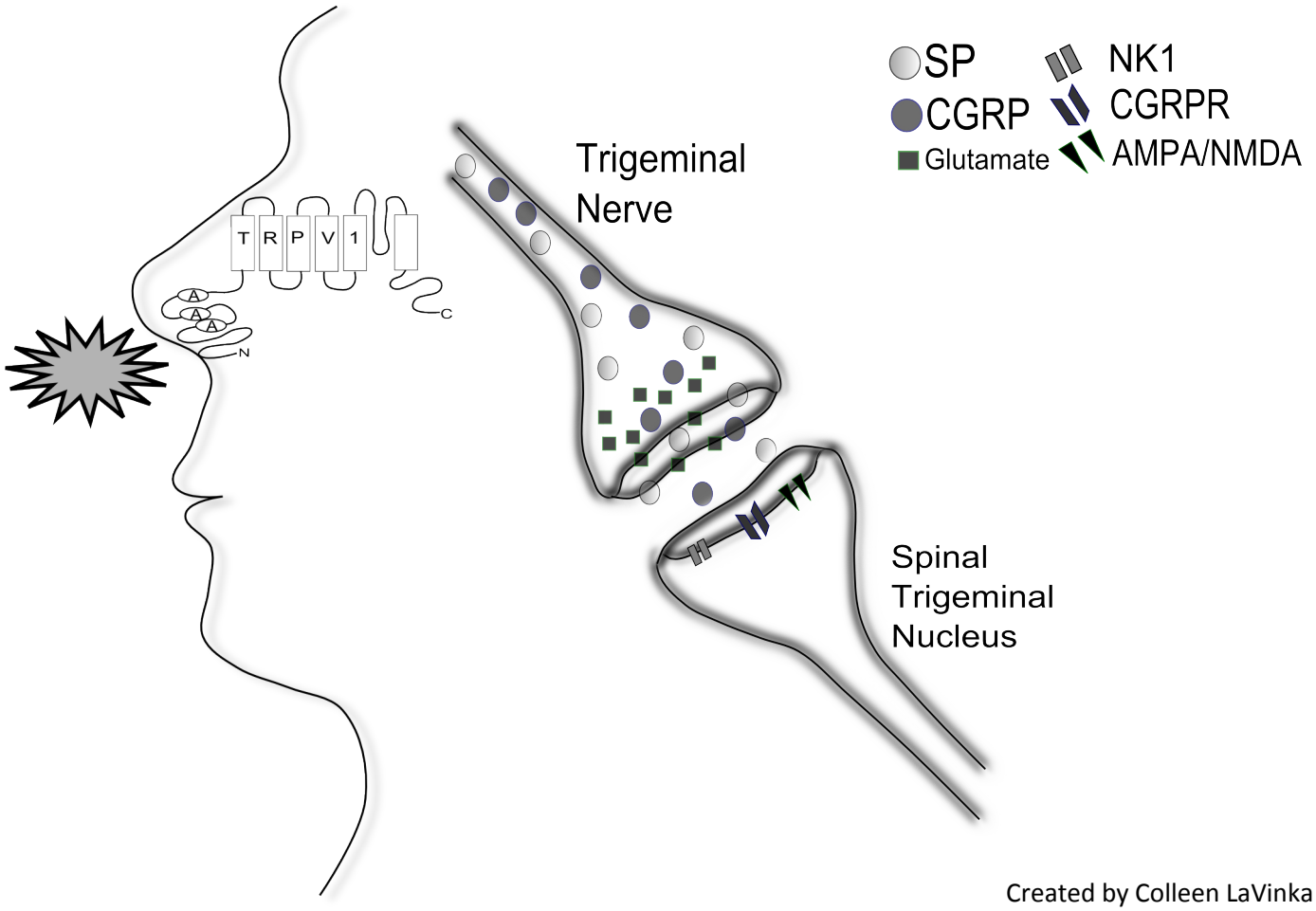


Figure 3. Neuropeptide release upon noxious trigeminal stimulation

Noxious fumes stimulate receptors in the nasal epithelium, such as TRPV1, which activates the trigeminal nerve which releases glutamate, SP, and CGRP onto the spinal trigeminal nucleus.

done by degradation by peptidases. If the volume of the peptide release is large, the peptidases will be overwhelmed, allowing peptides such as Substance P to travel long distances and affect a relatively large number of post-synaptic cells (Seybold, 2009). And since peptide receptors have a high affinity for their ligand, only a small amount of peptide is needed to activate the receptors. This results in a small amount of peptide being able to affect a large volume of nerves. The release of peptides activates G protein-coupled receptors (GPCRs) which results in kinases which can phosphorylate proteins (Seybold, 2009). The phosphorylation of proteins can change the reaction of the receptor to its ligand, resulting in increased efficacy of signal. The expression of receptors postsynaptically can also change in response to the neuropeptides. All these changes can result in a process called central sensitization. Central sensitization is when there is a leftward shift in the stimulus-response relationship in reaction to painful stimuli (Seybold, 2009). I will be discussing two neuropeptides that are released by nociceptive C fibers: Substance P and Calcitonin gene-related peptide (CGRP).

1. Substance P

Substance P comes from a family of neuropeptides called tachkynins. It is encoded by the PPTA gene (Seybold, 2009). Substance P has been found to localize to laminae I, II and V within the dorsal horn of the spinal cord. These are the same laminae where nociceptors terminate (Hokfelt et al, 1997; Seybold and Elde, 1980; Light and Perl, 1979; Sugiura et al, 1986). Substance P activates the neurokinin 1 (NK1) receptor upon noxious stimulation (Trafton et al, 2001). Intrathecal injection of Substance P causes

biting and scratching in rats (Seybold et al, 1982). Substance P causes a slow, excitatory postsynaptic potential that makes it easier for painful stimuli to activate the post-synaptic cells (Randic and Miletic, 1977; Henry, 1976). Experiments with Substance P knockout mice (PPTA-/-) showed significantly reduced responses to noxious mechanical, thermal, and chemical stimulation when the skin of the foot was tested. Thermal hypersensitivity from application of mustard oil is lost in Substance P knockout mice (Mazario and Basbaum, 2007; King and Barr, 2003). In peripheral inflammation, an increase in NK1 receptor expression and binding of Substance P is observed (Honore et al, 1999; Stucky et al, 1993). When primary afferent neuron terminals are examined, Substance P and CGRP are found to coexist about 70% of the time (Tuchscherer and Seybold, 1989). These two neuropeptides are found to be released upon noxious stimulation (Duggan et al 1998; Morton and Hutchison, 1989).

2. CGRP

CGRP is found in the dorsal root ganglion of the spinal cord (Tuchscherer and Seybold, 1989). The neuropeptide is expressed in medium and small-diameter neurons (Noguchi et al, 1990). There are two forms of CGRP, α and β, which only differ by one amino acid (Amara et al, 1985). The CGRP receptor shows a unique physiology. While most receptors have one protein, CGRP receptors are composed of three different proteins. These three proteins are the calcitonin receptor like-receptor (CRLR), receptor activity modifying protein (RAMP), and a receptor component protein (RCP) (Oliver et al, 2001; McLatchie et al, 1998; Evans et al, 2000). The different combinations of these three proteins produce receptors with different affinities for CGRP (Galeazza et al, 1991).

CGRP causes production of adenyl cyclase, which leads to increased levels of cyclic AMP (cAMP) (Seybold et al, 2003; Parsons and Seybold, 1997). CGRP is not thought to cause direct nociceptive activation. Unlike SP, CGRP does not induce a behavioural reaction upon injection (Gamse and Saria, 1986; Wiesenfeld-Hallin et al, 1984). When CGRP is absent or knocked down, mice do not show altered acute nociceptive responses (Zhang et al, 2001; Salmon et al, 1999; Tzabazis et al, 2007). However, injection of CGRP does cause mechanical hyperalgesia (Sun et al, 2003). CGRP postsynaptically increases the excitation of a neuron. Treatment with CGRP resulted in larger postsynaptic currents in lamina II of spinal neurons (Murase et al, 1989; Bird et al, 2006). CGRP is also a potent vasodilator (which is true of Substance P as well). In the trigeminal nerve, CGRP is released from sensory nerves during migraines (Uddman et al, 1986). CGRP helps modulate pain within the trigeminovasculature (Brain, 2004). For this reason, CGRP blockers have been of great interest to pharmaceutical companies. CGRP also helps increase the affect of Substance P by competing for degradation by the peptidases (Mao et al, 1992; Le Greves et al, 1985). Release of CGRP causes increased levels of SP on the contralateral side of the synapse (Schaible et al, 1992).

G. The African naked mole-rat model system

The African naked mole-rat is an exciting new model system for studying neural adaptations for blunting pain. As explained below, this species has evolved in an extremely acidic environment. Recent studies on the skin of naked mole-rats have shown

an inability to drive action potentials in C fibers with acidic saline (Park et al, 2008). It was this interesting observation that drove me to further examine naked mole-rats' responses to acidic fumes.

Naked mole-rats (Rodentia; Bathyergidae, *Heterocephalus glaber*) originate from central east Africa. They are one of only two known eusocial mammals in the world (the other is also an African mole-rat species). Within a given colony of naked mole-rats, there is only one active female breeder, the queen (Jarvis, 1981). The active female breeder obtains this position through fighting and aggression (Clarke and Faulkes, 1997; Faulkes and Abbott, 1997). Once dominance is established, the breeding female will grow larger and remain the breeding queen until she dies or is challenged and beaten by another female. The non-breeding females of the colony are hormonally suppressed by the breeding queen and remain sexually immature (Faulkes et al, 1990; Faulkes and Abbott, 1997). Non-breeding colony members have a division of labor. Some are housekeepers, some are soldiers. The non-breeders work together cooperatively to dig tunnels and find food.

Naked mole-rats are poikilothermic, unable to physiologically control their body temperature (Buffenstein and Yahov, 1991). This means that naked mole-rats take on the temperature of their environments. The temperature in their tunnels is hot and stable, with high levels of humidity. The high humidity prevents evaporation of sweat so naked mole-rats would not be able to cool down. By losing their ability to control their body temperature, naked mole-rats are able to conserve energy that would be lost trying to thermoregulate. They live in extensive underground burrow systems which they dig with

their incisor teeth. Their teeth are extra buccal, protruding through the skin above and below their lips. This allows naked mole-rats to dig with their teeth and not ingest dirt.

Like other subterranean animals, naked mole-rats show adaptations to underground living such as poor vision and hearing (Hetling et al. 2005; Xiao et al. 2006; Crish et al. 2006). However, naked mole-rats also display several species-specific characteristics. For example, they have an unusual pattern of body hairs. Naked mole-rats are not completely naked; they are covered with sensitive sensory hairs in a grid-like pattern. Upon stimulation of these fine hairs, naked mole-rats show a strong topographic orientating behavior (Crish et al. 2003). These sensitive hairs might help compensate for their diminished eyesight to aid in location of objects and sense of direction. Another unusual feature of naked mole-rats is that they are the longest living rodent known, living up to 30 years in captivity with no decrease in activity or reproductive potential (Buffenstein, 2008).

Naked mole-rats dig for food, a costly energetic endeavor. To balance this large energy expense, when naked mole-rats find food such as tubers or corms, they eat the nutrient rich inner parts and leave the outside. Interestingly, they pack the partially consumed tuber with dirt so that it can re-grow for later visits. This allows the tuber to continue to be a certain food supply, helping to sustain the colony thru various seasons (Lovegrove and Wissel, 1988).

Colonies contain large numbers of members, as many as three hundred or more. This many animals in underground tunnels lead to an environment high in carbon dioxide and low in oxygen (Bennett and Faulkes, 2000). They have adapted by having a slow metabolism, so that they have reduced oxygen consumption (Buffenstein and Yahov,

1991). Their hemoglobin also shows a higher affinity to oxygen, so that what oxygen is in the tunnel is extracted and used (Johansen et al, 1976). Since high levels of carbon dioxide leads to tissue acidosis, naked mole-rats show better buffering capabilities in their blood so they can neutralize the carbonic acid made from carbon dioxide (Johansen et al, 1976).

Our laboratory reported that African naked mole-rats (*Heterocephalus* glaber) naturally lack not only Substance P, but also CGRP from the C fibers of their trigeminal and dorsal root ganglion (Park et al. 2003). This finding suggested to us that naked mole-rats might be less sensitive than other mammals to stimuli that act on dorsal root and trigeminal C fiber nociceptors. Indeed, naked mole-rats have been shown to lack hypersensitivity to heat after capsaicin application to their skin (Park et al., 2008), a phenomenon known to involve C fibers. This is similar to the reduced sensitivity to mustard oil and capsaicin in Substance P knockout mice (Mazario and Basbaum, 2007; Simons et al., 2001).

Regarding trigeminal pain, I previously showed that naked mole-rats do show tolerance to the noxious C fiber stimulant, ammonia. Naked mole-rats showed no avoidance to ammonia but did show an increase in postsynaptic activity in the Sp5c (LaVinka et al, 2009). I wanted to examine if naked mole-rats would show a similar tolerance to acidic fumes. If true, this would be a useful attribute for a subterranean species that lives in colonies where levels of CO_2 are extreme (Bennett and Faulkes, 2000) because high levels of CO_2 excite trigeminal C fiber nociceptors (Silver et al, 1991). I expected that naked mole-rats would show an increased tolerance to acetic acid but would not show the same postsynaptic response as to ammonia stimulation. Since it

was shown that in the skin, acidic saline could not drive action potentials in naked mole-rats while capsaicin could (Park et al, 2008), I hypothesized that acetic acid would not drive postsynaptic activity within the trigeminal nerve.

I examined post-synaptic activity by staining the proto-oncogene protein Fos. The Fos protein is a nuclear protein that helps to control the transcription rate of target genes. It is induced rapidly and has a half-life of two hours (Morgan and Curran, 1989). Previous studies have shown that stimulating the nasal epithelium with noxious chemicals causes an increase in the number of labeled cells in the spinal trigeminal nucleus of laboratory rats (Ter Horst et al. 2001; Anton et al. 1991a; Takeda et al. 1999). By comparing Fos expression in naked mole-rats to comparison species of rats, mice and Substance P knockout mice, we can see whether or not cells in the naked mole-rat's spinal trigeminal nucleus are activated by acidic fumes.

I also wanted to further examine the role of Substance P in trigeminal nociception. I used Substance P knockout mice to test if absence of the neuropeptide would affect behaviour and Fos expression in response to noxious trigeminal stimulation with ammonia and acetic acid. I also stained for Substance P within the naked mole-rat brain to demonstrate the selective absence of Substance P from the trigeminal area only. Finally, to see if the morphological characteristics of the trigeminal nerve of naked mole-rats are different from mice, I used transmission electron microscopy to quantify the A and C fibers within the nerve.

With these aims in mind, I endeavored to show the altered nociceptive processing of the trigeminal nerve in the African naked mole-rat, adaptations that we propose have evolved in response to these unique animals' extreme environments.

II. Behavioural Avoidance of Noxious Fumes

A. Introduction

My first aim was to examine the behaviour of the naked mole-rat upon exposure to noxious fumes in comparison to other species, including mice, rats, and a close relative, the Damaraland mole-rat (*Fukomys damarensis*).

The Damaraland mole-rat is the other known eusocial mammal in the world, meaning that only one male and female do the breeding while non-breeding members of the colony help raise the young (Bennett and Faulkes, 2000). The number of individuals in a typical Damaraland colony however is much smaller than in a typical naked mole-rat colony and they only live around 15 years. When captured and examined in African, the mean number of animals per colony was 6.3, with a range from one animal up to 24 (Young and Bennett, 2010). There is still a division of labor with the subordinate Damaralands responsible for looking for food and defending the colony in their underground burrows (Bennett and Faulkes, 2000). The Damaraland mole-rat is much larger in size than the naked mole-rat and has hair covering its body. The breeding queen in a Damaraland colony is usually the largest female and has a significantly longer body than other female colony members (Young and Bennett, 2010). What is striking about the Damaraland mole-rats is that they live in much smaller groups and do not show the significant inbreeding that is seen in the naked mole-rats (Bennett and Faulkes, 2000). Because of these smaller numbers, the Damaraland mole-rat may not have been evolutionarily pushed to adapt to an extreme environment like the naked mole-rat.

I had previously shown that naked mole-rats do not avoid ammonia when allowed to roam freely while rats did (LaVinka et al, 2009). I now wanted to expand the project

to include other comparison species (mice, Damaraland mole-rats) as well as another noxious fume, acetic acid. I tested the animals' reaction to acetic acid in varying concentrations, hypothesizing that naked mole-rats would not avoid the noxious fumes while the other species would. I wanted to use acetic acid to observe the animals' reactions to acidic fumes. This is biologically relevant as naked mole-rats have been shown to live in environments with high levels of carbon dioxide (Bennett and Faulkes, 2000). Carbon dioxide has been shown to stimulate nociceptors within the nasal cavity, causing a painful, stinging sensation (Anton et al, 1991b; Sekizawa and Tsubone, 1994). It was shown that carbon dioxide stimulates TRPA1 receptors on trigeminal ganglion neurons through intracellular acidification (Wang et al, 2010). These same receptors are stimulated by weak acids like acetic acid (Wang et al, 2011). So living in high levels of CO2, like naked mole-rats, would lead to constant pain and irritation through trigeminal activation. To test if naked mole-rats have a tolerance to acidic fumes, like those from carbon dioxide, I used acetic acid-soaked sponges. If naked mole-rats showed a tolerance to the acidic fumes, this would indicate the natural tolerance they might have within their acidosis-inducing underground burrows.

B. Methods

1. Subjects

Experiments were conducted on naked mole-rats (*Heterocephalus glaber*), Damaraland mole-rats (*Fukomys damarensis*), Sprague-Dawley and Long-Evans rats, and C57BL/6 mice. The naked mole-rats were non-breeding animals between 20 and 40 grams in weight and at least one year in age. The naked mole-rats were housed under

semi-natural conditions in an artificial burrow system within a colony room. Because naked mole-rats are poikilotherms, the room in which they were housed was maintained at 82° F and 45–65% relative humidity (Artwohl et al., 2002). Rats, mice, and Damaraland mole-rats (which are also African mole-rats but not poikilothermic) were housed under conventional laboratory vivarium conditions. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

2. Behavioural Experiments: Avoidance test

Animals were placed in a four-arm arena with a sponge affixed to the end of each arm **(figure 4)**. In one test, one of the sponges was saturated with acetic acid, and the remaining three sponges were saturated with water. Animals were placed into the arena one at a time and allowed to explore freely for 20 minutes which they readily did. During this time, we recorded the cumulative amount of time spent within 10 cm of each sponge. The idea was that if an animal perceived acetic acid fumes as aversive, then it would spend less time near the sponge saturated with acid solution compared to the other sponges. After each individual animal was tested the arena was wiped clean with ethanol, rotated 90 degrees, and the sponges were re-positioned such that a given sponge (e.g. the acetic acid sponge) was not consistently in the same arm or the same spatial location relative to landmarks in the testing room.

We tested three concentrations of acetic acid solutions: 10, 20, and 50%. We also tested animals with 10% ammonia which is the concentration of household cleaning ammonia.

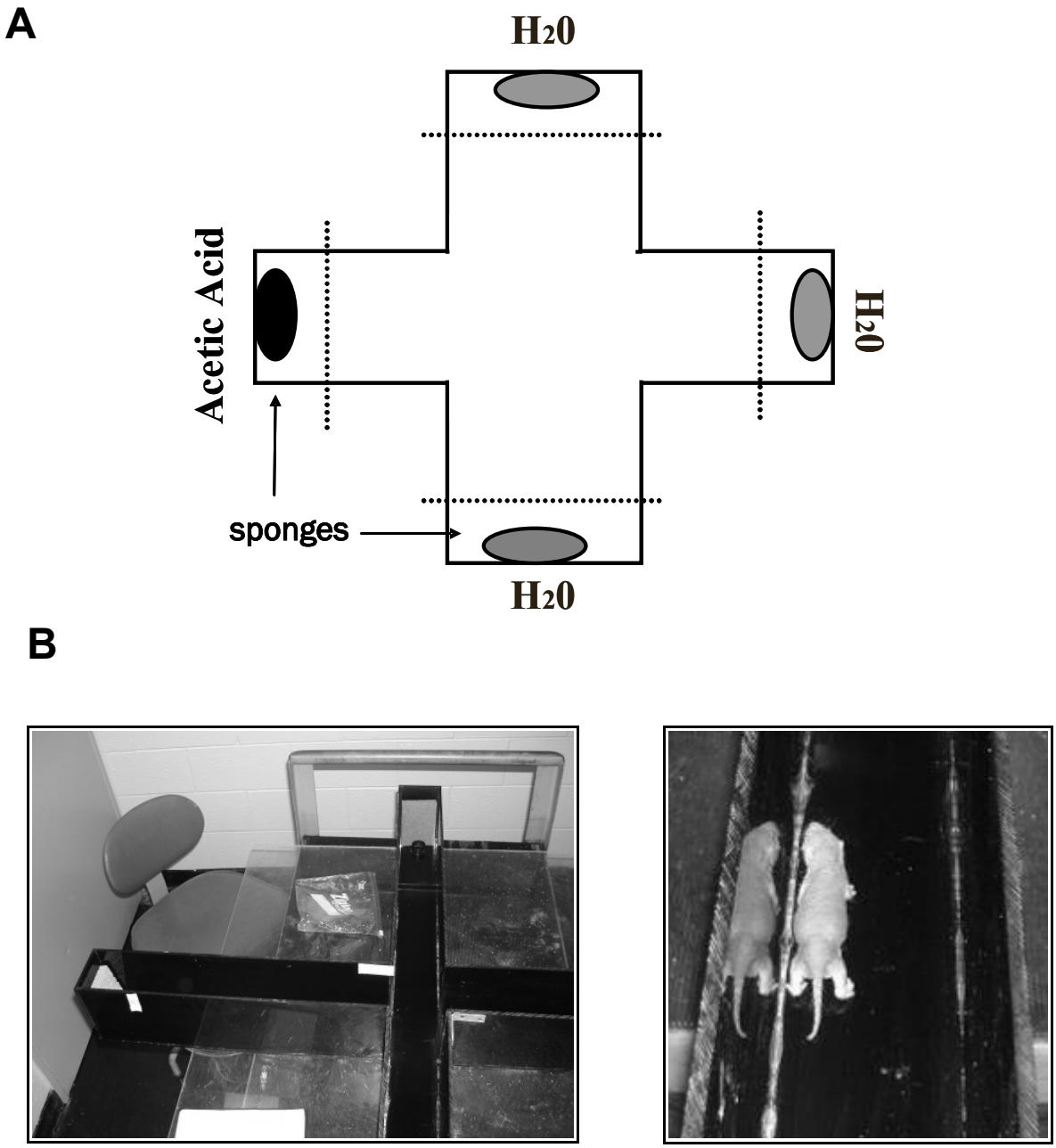


Figure 4. Avoidance test setup.
A. Diagram of the four-arm plus maze used in the avoidance test. At the end of each arm is a soaked sponge, three with water and one with a noxious stimulant. Time spent across the dotted line and close to soaked sponge was recorded. B. Pictures of the actual maze used and a naked mole-rat within the testing setup.

In a previous study, we reported results from testing naked mole-rats and laboratory rats with ammonia (LaVinka, et al., 2009). To complete the comparison across species and stimuli, we tested mice and Damaraland mole-rats with ammonia in the present study.

C. Results

In a previous study, we showed that naked mole-rats did not avoid fumes from 10% ammonia, whereas laboratory rats did avoid ammonia fumes (LaVinka, et al., 2009). The first objective of the present study was to test responses to another relevant noxious airborne chemical irritant, acidic fumes. Acetic acid fumes, like ammonia fumes, stimulate nasal trigeminal C fibers as well as trigeminal C fibers innervating the cornea and conjunctiva (Bryant, 2005; Wang, et al., 2011; Carstens, et al., 1998). The second objective was to expand our comparison species to include laboratory mice and another species of African mole-rat, the Damaraland mole-rat.

We tested animals in a 4-arm "plus maze" where they were allowed to move about freely. Animals were tested with fumes from three concentrations of acetic acid, 10, 20, and 50%. **Figure 5** shows the results for the four species tested with each acidic concentration, as well as ammonia for comparison. Each bar graph displays the average amount of time spent within 10 cm of the water-soaked sponges (grey bars) versus the irritant-soaked sponge (black bars). In each case, we pooled the data from the three water sponges. Significance was determined with an unpaired t-test.

We found that the naked mole-rats only showed a significant aversion to the highest acid concentration, 50% (**Figure 5, top row**). For 50% acetic acid, the naked mole-rats spent an average of 117 seconds near each of the water-saturated sponges, but

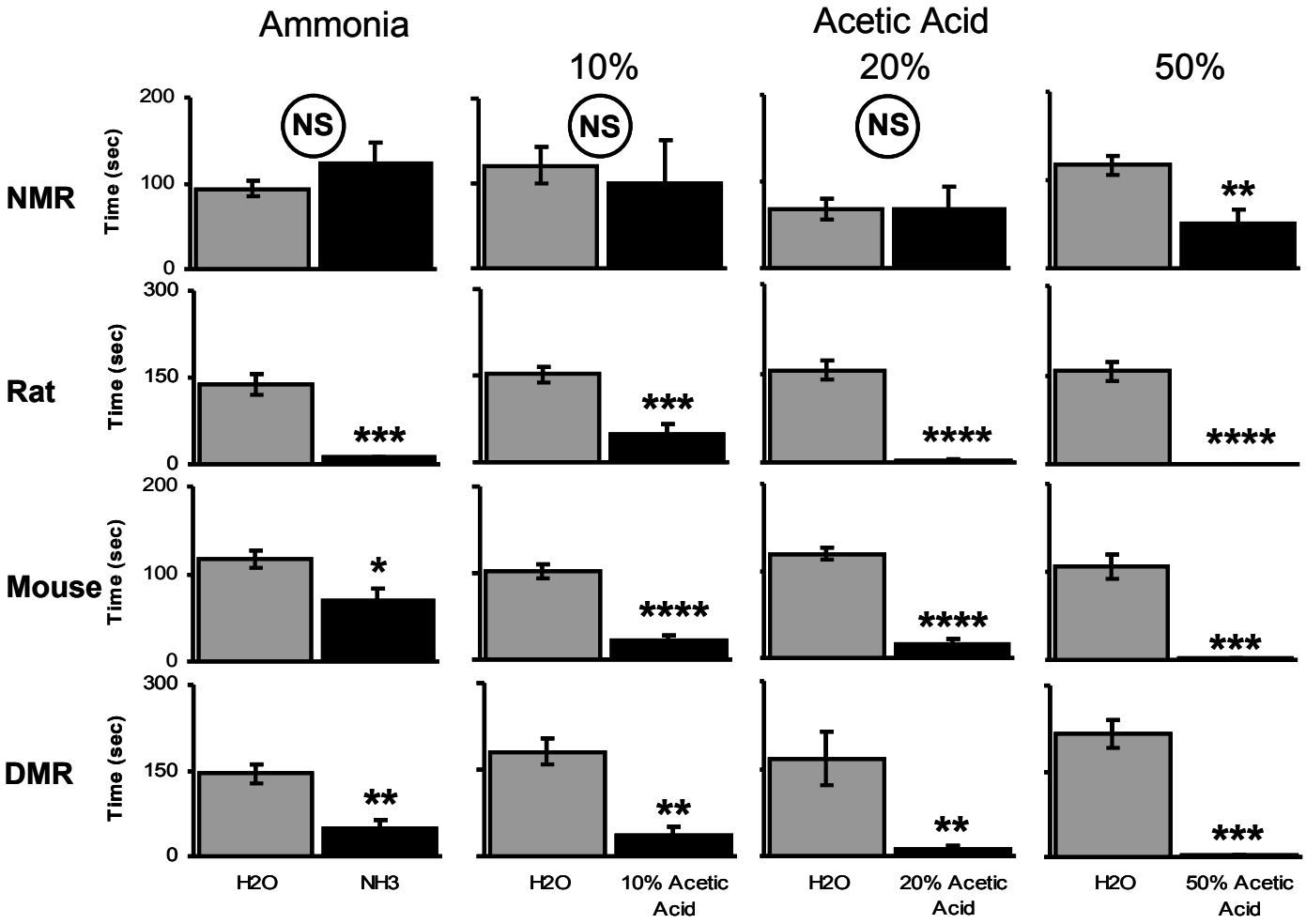


Figure 5. Behavioral avoidance testing with ammonia and acetic acid fumes. Animals explored a 4-arm arena that included one sponge saturated with an irritant and three sponges saturated with water. Gray bars represent the average time spent within ten centimeters of water-soaked sponges. Water data was pooled from three arms. Black bars represent the average time spent within ten centimeters of the irritant soaked sponge. Top row, naked mole-rats did not avoid fumes from ammonia, 10% or 20% acetic acid compared to water. They did significantly avoid 50% acetic acid. Bottom three rows, rats, mice and Damaraland mole-rats showed significant avoidance to all the irritants that we tested. Error bars are standard errors of the mean.

only 51 seconds near the 50% acetic acid-saturated sponge (**Figure 5, top row, far right**; t= 2.98 (df=38), p<.01). The naked mole-rats did not avoid 10% or 20% acetic acid, or 10% ammonia. They spent the same amount of time near the sponge saturated with each of these irritants as they did with sponges saturated with water. In contrast, the rats, mice, and Damaraland mole-rats showed a significant aversion to each acid concentration and the ammonia (**Figure 5, bottom three rows**). Table 1 shows the statistical data for each species and stimulus.

D. Discussion

This experiment showed that naked mole-rats do show aversion to acidic fumes but only at much higher concentrations when compared to other species. This data is consistent with previous findings that showed that acidic saline was incapable of driving action potentials in C fibers (Park et al, 2008). If the nociceptors are not activated by acid, then no corresponding behavioural avoidance will be shown. This also fits with the recent publication showing that naked mole-rats have Nav 1.7 channels that are inhibited by acid (Smith et al, 2011). However, if acid inhibits naked mole-rats nerves, then why do they eventually show avoidance at 50% acetic acid? It makes sense that naked mole-rats would still want to be able to sense such high levels of acid as these levels would start to cause tissue damage upon long enough exposure. If low levels of acid inhibit the nerves through the Nav 1.7 channels, higher levels of acid would seem to continue inhibiting the nerves.

Table I: Behavioural response of naked mole-rats, rat, mice, and Damaraland mole-rats to noxious fumes

Species	**N**	**Avg time near H2O**	**Avg time near NH3**	**T value**	**Df**	**P value**
NMR	10	93.47	122.7	-1.39	38	NS
Rat	5	138.6	11	4.17	18	P<.001
Mouse	5	117.06	69.46	2.49	18	P<.05
DMR	8	144.97	47.62	3.15	30	P<.01

Species	**N**	**Avg time near H2O**	**Avg time near 10% Acetic Acid**	**T value**	**DF**	**P value**
NMR	6	120.53	100.08	0.43	22	NS
Rat	5	152	47.54	4.01	18	P<.001
Mouse	5	102.45	21.22	5.45	18	P<.0001
DMR	7	180.58	36.8	3.56	26	P<.01

Species	**N**	**Avg time near H2O**	**Avg time near 20% Acetic Acid**	**T value**	**DF**	**P value**
NMR	10	68.36	67.7	.02	38	NS
Rat	10	155.8	3.27	5.48	38	P<.0001
Mouse	5	119.74	16.21	7.27	18	P<.0001
DMR	5	167.45	11.93	3.26	18	P<.01

Species	**N**	**Avg time near H2O**	**Avg time near 50% Acetic Acid**	**T value**	**DF**	**P value**
NMR	10	116.88	51.2	2.98	38	P<.01
Rat	4	156.16	0	5.53	14	P<.0001
Mouse	5	105.31	1.6	4.19	18	P<.001
DMR	5	214.63	2.95	4.91	18	P<.001

How are the naked mole-rats sensing to avoid the 50% acetic acid then? Perhaps naked mole-rats are not avoiding the 50% acetic acid due to pain signals from the trigeminal but signals coming from another nerve. This will be explored in the next chapter.

In contrast to naked mole-rats, all other species tested showed avoidance at all concentrations of acetic acid, including Damaraland mole-rats who are a close relative of naked mole-rats and also live underground.

Naked mole-rats also differed from comparison species in their behaviour towards ammonia. Naked mole-rats do not avoid ammonia while rats, mice and Damaraland mole-rats do. This lack of aversion cannot be explained by the naked mole-rat Nav 1.7 channels that are inhibited by acid (Smith et al, 2011), but not other chemical irritants (Park et al, 2008). Instead, the lack of avoidance to ammonia is likely related to the naked mole-rats' lack of the neuropeptides Substance P and CGRP from their C fibers. This ablation of neuropeptides seems to dampen the noxious signal from ammonia enough to not cause avoidance. Naked mole-rats also did not respond to topical application of capsaicin to their nose (LaVinka et al, 2009). This agrees with the naked mole-rats' tolerance to ammonia fumes. Ammonia, like capsaicin, stimulates trigeminal C-fibers (Lindberg et al., 1987). The other species tested all avoided ammonia and all have Substance P and CGRP in their C fibers. Also, when topical capsaicin was applied to mice' noses, they exhibited irritated behaviour like rubbing and licking (LaVinka et al, 2009). Interestingly, while the mice still avoided ammonia, they did not show quite as high aversion to ammonia as they did to acetic acid. The mice spent an average of 69.46

seconds near the ammonia sponge but only 21.22 seconds near the lowest concentration of acetic acid. From personal experience, I have noticed that mice are surrounded by high levels of ammonia in our mouse vivariums. Indeed, studies have shown that, in the laboratory, mouse cages accumulate much higher concentrations of ammonia than rat cages (Burn et al, 2006; Silverman et al, 2009). It would be likely that these high levels of ammonia are found in nests of wild mice also and as such, mice may have developed an increased tolerance to ammonia but not to acidic conditions.

III. Physiological Response to Noxious Trigeminal Stimulation

A. Introduction

My second aim was to examine the physiological response of the naked mole-rats' trigeminal nerve upon stimulation with acidic fumes. I previously showed that while naked mole-rats showed no aversive behaviour upon exposure to ammonia, there was a significant increase in post-synaptic activity within the Sp5c (LaVinka et al, 2009). I wanted to expand upon the ammonia exposure with another comparison species, C57BL6 mice as well as expose all species (mice, rats, naked mole-rats) to acidic fumes and examine the postsynaptic activity of the respective spinal trigeminal nuclei. My hypothesis was that all species other than naked mole-rats would show an increase in activity within the spinal trigeminal nucleus upon exposure to noxious fumes. Naked mole-rats, I hypothesized, would show no increase in post-synaptic trigeminal activity since previously studies had shown a lack of action potentials in sensory skin fibers upon exposure to acidic saline (Park et al, 2008).

B. Methods

1. C fos Experiments

a. Stimulation with noxious chemical fumes

Animals were deeply anesthetized with 50mg/kg sodium pentobarbital, IP. They were placed on their backs, with their nose easily accessible. A sterile cotton swab was submerged in the chemical and then placed in close proximity to the subjects' external nares. They were exposed to the chemical fumes until the subject flinched or twenty

seconds passed, whichever occurred first. The swab was then removed so the subject could not habituate to the stimulant. Stimulation occurred every minute for one hour. To obtain background Fos levels, control subjects were either anesthetized for one hour without stimulation or they were administered a lethal dose of sodium pentobarbital and immediately perfused. All efforts were made to maintain a similar degree of anesthia across animals.

b. Perfusion and Immunohistochemistry

After stimulation, the animal was given a lethal dose of sodium pentobarbital, (100mg/kg, IP) and was transcardially perfused with saline followed by 4% formaldehyde in phosphate-buffered saline (PBS). The brain and upper spinal cord was removed, post-fixed for an hour at 4°C, then rinsed and stored in 30% sucrose solution in PBS at 4°C. The brains were sliced into transverse serial sections, 40 μm-thick. The tissue was cut on a table-top microtome, cooled with dry ice. Once cut, the tissue was stored overnight in PBS at 4°C before being processed. Serially sliced sections were incubated in Anti-c-Fos (Ab-5) (4-17) Rabbit pAB for 48 hours (Oncogene Sciences/Calbiochem, Cambridge, MA, AB5, 25,000×). After incubation, tissue was rinsed three times in PBS for 15 minutes. The tissue was then incubated in biotinylated anti-rabbit IgG for an hour and a half and rinsed again. The tissue was processed using the avidin-biotin complex (ABC) method for an hour and a half using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). All tissue was rinsed in PBS and the visualized with diaminobenzidine with hydrogen peroxide to obtain a brown-black stain.

After processing, tissue was mounted on subbed slides and cover-slipped. The labeled Fos cells were manually counted within the spinal trigeminal nucleus using a

light microscope. Pictures were taken using an Amscope trinocular compound microscope with a 3.1 megapixel camera. Brain areas were defined by the Paxinos and Watson rat brain atlas and the stereotaxic naked mole-rat atlas by Xiao et al (2006).

C. Results

We compared C fos labeling in naked mole-rats, laboratory rats, and mice. We chose not to pursue these terminal experiments with Damaraland mole-rats because the Damaralands responded much more like rats and mice than naked mole-rats in the behavioral avoidance assay.

Figure 6 shows cross sections through the brain stem of an example control and stimulated mouse and naked mole-rat. The stimulus was 20% acetic acid fumes. The number of positively labeled cells was substantially greater for the stimulated mouse (**Figure 6b, low magnification; 6d, high magnification**) compared to the control mouse (**Figure 6a,c**). The results for the mouse are consistent with previous studies that have used ammonia (McCulloch and Panneton, 1997; LaVinka, et al., 2009) or mustard oil (Anton et al, 1991; Takeda, et al, 1999) as noxious stimulants. There were also numerous labeled cells in the nucleus tractus solitarius (NTS) which likely reflect activation of pulmonary vagal nerve fibers (Lipski et al, 1991). Sections from the control naked mole-rat (**Figure 6e,g**) had more positively labeled cells in the spinal trigeminal nucleus compared to the mouse overall (**Figure 6a,c**). However, the number of positively labeled cells was not greater for the stimulated naked mole-rat compared to the control naked mole-rat (**Figure 6g,h)**.

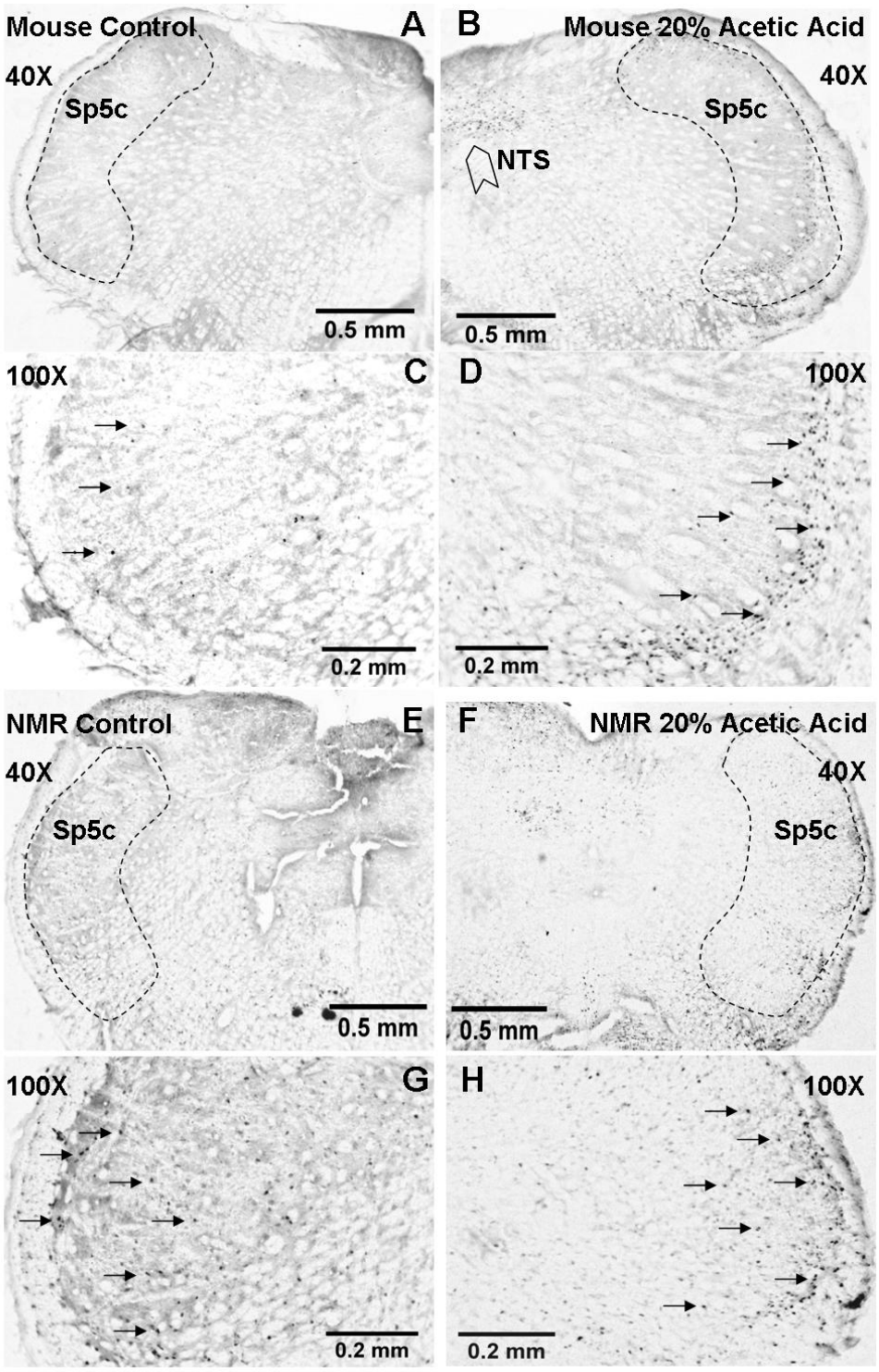


Figure 6. C fos labeled sections at the level of the trigeminal pain nucleus (spinal trigeminal nucleus, caudal part, Sp5c) from mouse and naked mole-rat. A, C. Example from a control mouse at low (40X) and high (100X) magnification. Arrows in C indicate examples of C fos positive labeled neurons. The dashed line indicates anatomical boundary of Sp5C. B,D. Example from a mouse stimulated with 20% acetic acid. Note the prominent increase of Fos labeled cells in D compared to C. In B, NTS indicates the nucleus tractus solitarius of the vagus nerve. E,G. Example from a control naked mole-rat at low and high magnification. F, H. Example from a naked mole-rat stimulated with 20% acetic acid. Note the similarity in the number of Fos labeled cells in H compared to G.

Summary data on the number of positively labeled neurons for each species and both stimuli is shown in **Figure 7**. Each panel in **Figure 7** shows average cell counts for each

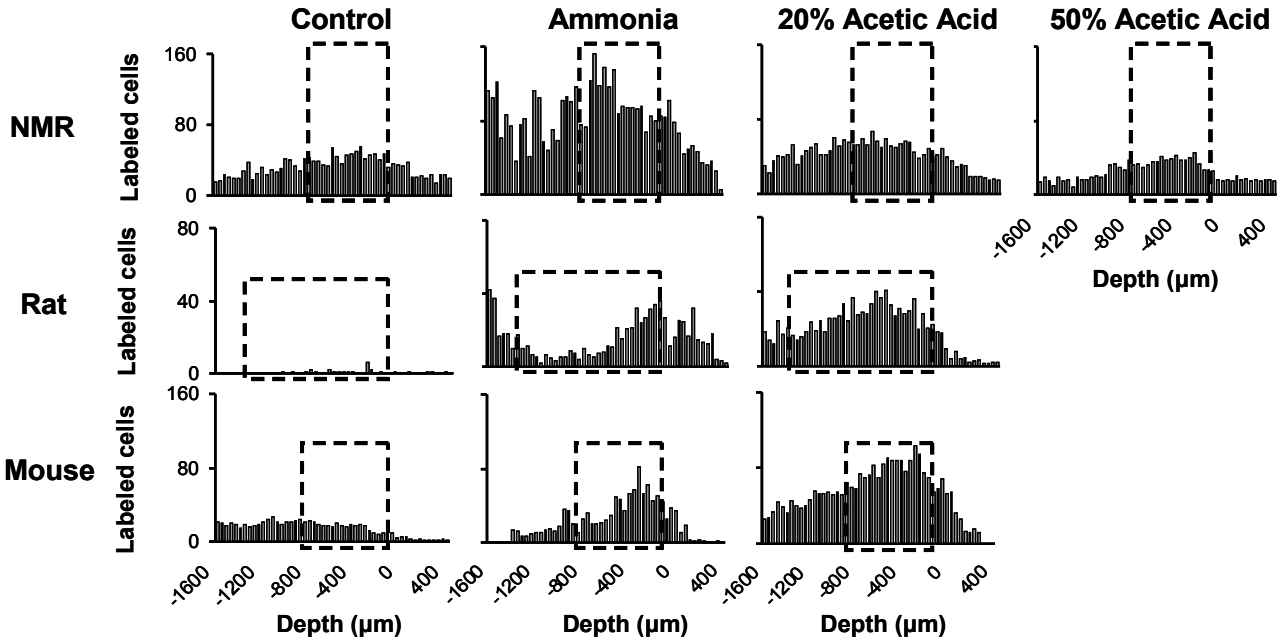


Figure 7. Average number of labeled cells per section through the brain stem of control and stimulated naked mole-rats, rats, and mice. Dashed boxes correspond to the range of Sp5c, the area used for statistical comparisons and summarized in **Figure 8**. The area to the left of the dashed boxes corresponds to cervical spinal cord. The area to the right of the dashed boxes corresponds to the interpolar region of the spinal trigeminal nucleus.

40 µm section through the brain stem from cervical spinal cord (left) to the interpolar region of the trigeminal nucleus (right). The dashed boxes indicate the trigeminal pain nucleus (spinal trigeminal nucleus, caudal part, Sp5c). For each species, a greater number of labeled cells can be observed in the Sp5c for the ammonia group compared to the control group. For stimulation with 20% acetic acid, only the rats and mice showed a

substantial increase compared to their respective control groups while the naked mole-rats remained near control values. An additional group of naked mole-rats was tested with 50% acetic acid, and that group showed a reduction in the number of labeled neurons.

The average numbers of labeled neurons across all trigeminal Sp5c slices are shown in **Figure 8**. For naked mole-rats (**Figure 8a)**, there are significantly more positively labels cells in the ammonia group compared to the control group (on average, 96.6 vs 45.8, $t=-6.28$, $df=8$, $p<.001$). Importantly, there is no significant difference between the average number of labeled neurons for the naked mole-rats stimulated with 20% acetic acid compared to those in the control group (48.9 vs 45.8, $t=-0.44$, $df=8$, NS).

The fos data from stimulating naked mole-rats with acidic fumes appears to be inconsistent with the fos data from ammonia and the behavioral avoidance results: Behaviorally, naked mole-rats respond in the same way to both the ammonia and the 20% acetic acid in that they do not avoid either. Yet, the group stimulated with ammonia had significantly more labeled neurons than control, while the group stimulated with 20% acetic acid did not.

The results from naked mole-rats that were stimulated with 50% acetic acid not only failed to show an increase in fos labeling, they showed a significant decrease compared to the control group (**Figure 8a**). The average number of labeled neurons for the 50% acetic acid group was 31.8 compared to 45.8 for control ($t=-6.28$, $df=10$, $p<.05$). Hence, the only irritant stimulus that we tested that evoked avoidance in naked mole-rats actually decreased labeling in the trigeminal pain nucleus.

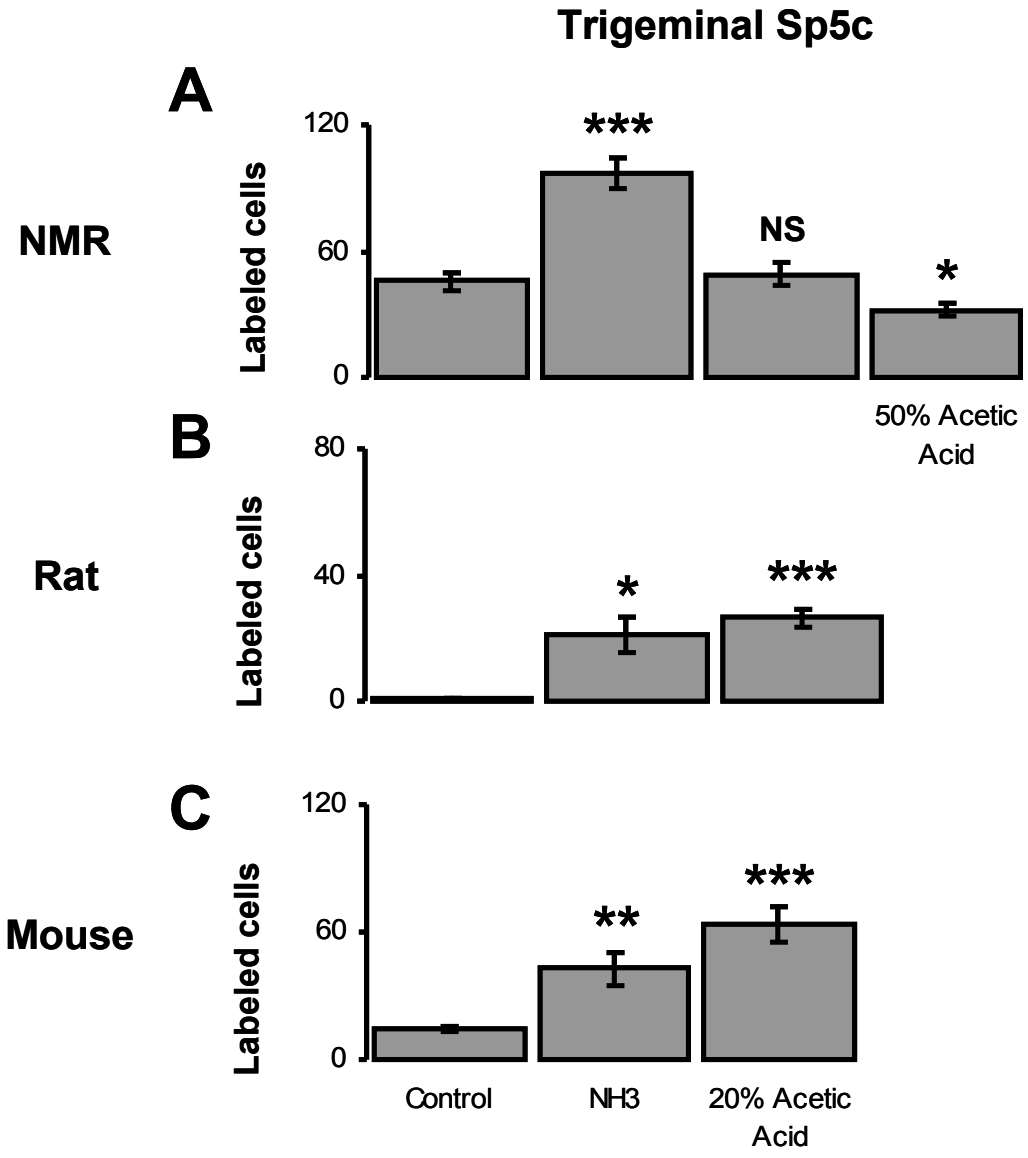


Figure 8. Average number of Fos labeled cells in trigeminal Sp5c in control and stimulated naked mole-rats, rats and mice. Averages correspond to the area in the dashed boxes in **Figure 7**. A. Naked mole-rats show a significant increase in the number of C fos labeled neurons after stimulation with ammonia. Naked mole-rats show no increase from stimulation with 20% acetic acid and remarkably, they show a significant decrease in number of labeled cells in response to stimulation from 50% acetic acid. B and C. Rats and mice show a significant increase in the number of labeled cells in response to stimulation from ammonia and 20% acetic acid.

The summary data for laboratory rats and mice is more straightforward (**Figure 8b,c**). Both rats and mice showed significantly more labeled neurons in their respective ammonia and acetic acid groups, compared to their control groups.

The average numbers of labeled neurons in the vagal NTS for naked mole-rats, rats, and mice are shown in **Figure 9**. For the naked mole-rats (**Figure 9a**), 50% acetic acid was the only stimulant tested that resulted in a significant increase in labeled neurons compared to control (27.6 vs 16.8, $t=-2.96$, $df=9$, $p<.05$). The number of labeled neurons in naked mole-rats tested with ammonia and 20% acetic acid were not significantly different from the number of labeled neurons in control naked mole-rat. Hence, fumes from 50% acetic acid were able to evoke action potentials in vagal nerves and neurons in the NTS, which was surprising to us given the mutation in naked mole-rat Nav1.7.

In contrast to the naked mole-rats, laboratory rats showed significant increases in the number of labeled neurons for both ammonia and 20% acetic acid (**Figure 9b,c**). Interestingly, laboratory mice showed a significant increase in NTS activity in response to 20% acetic acid, but not in response to ammonia stimulation. Coincidentally, mice showed a higher behavioural tolerance to ammonia fumes than acetic acid fumes, spending relatively more time near the ammonia sponge (**Figure 5**). Previous studies have shown that, in the laboratory, mouse cages accumulate much higher concentrations of ammonia than rat cages (Burn et al., 2006; Silverman et al., 2009). Hence, it seems possible that mice in the wild live under chronically high levels of ammonia in their nests which might result in a higher tolerance to ammonia behaviourally and physiologically in their vagal sensory system.

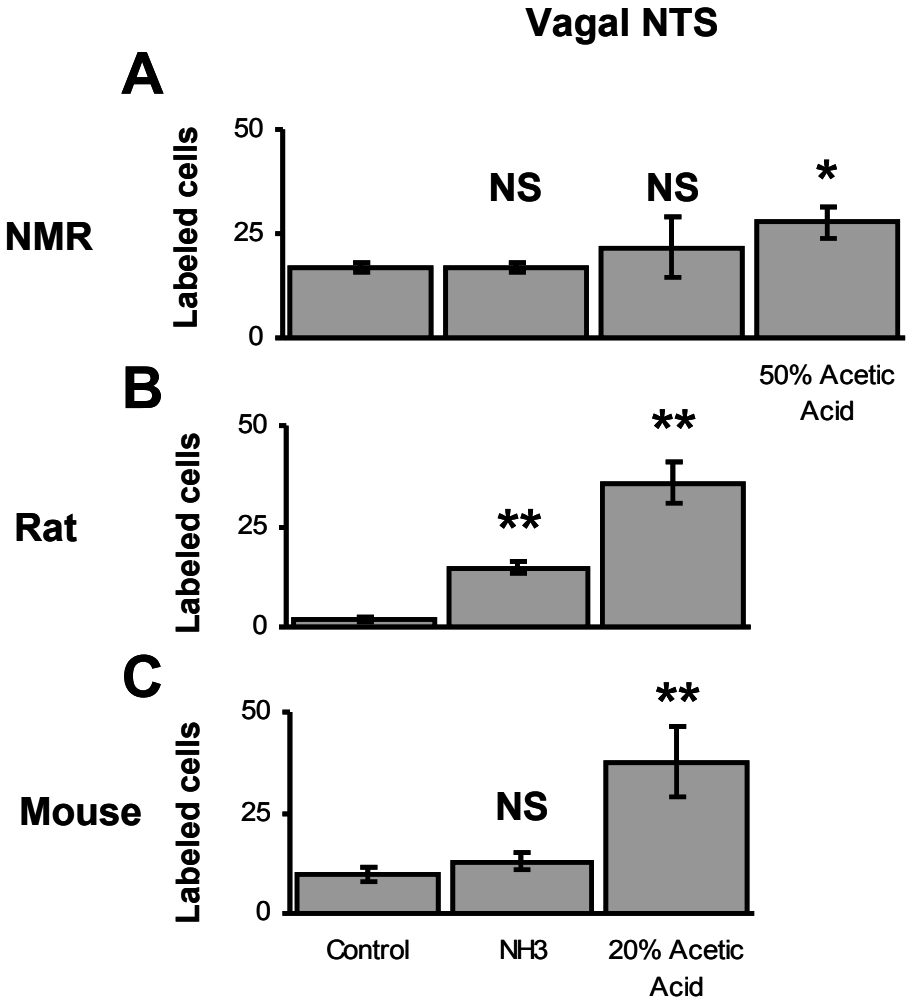


Figure 9. Average number of Fos labeled cells in vagal NTS of naked mole-rats, rats, and mice. A. Naked mole-rats show no significant increase in Fos labeled cells for ammonia or 20% acetic acid. However, they do show a significant increase in vagal activity upon stimulation with 50% acetic acid. This Fos data corresponds well with our behavioural data for naked mole-rats in **Figure 5**, which shows that they only avoided 50% acetic acid. B. Rats show significant increases in Fos activity in the NTS upon stimulation with ammonia and 20% acetic acid. C Mice show a significant increase in Fos labeled cells in their NTS upon stimulation with 20% acetic acid. They show no increase in activity in the NTS in response to ammonia

D. Discussion

Naked mole-rats display a number of putative adaptations for living under chronic, low O_2/high CO_2 conditions in their crowded burrows. For low O_2, these include a low resting metabolic rate (O'Connor et al. 2002), hemoglobin with a high affinity for O_2 (Johansen et al. 1976), and hypoxia tolerant brain tissue (Larson and Park, 2009; Peterson, et al., 2012). With regards to living under high CO_2/acidosis, putative adaptations include voltage-gated sodium channels that inhibit spiking in pain fibers under acidic conditions (Smith et al,2011) and a lack of neuropeptides in pain fibers (Park et al, 2003, Park et al, 2008) which would be released during acidosis in other species, generating a painful burning sensation (Anton et al. 1992; Chen et al. 1995).

The present study focused on behavioral, trigeminal, and vagal responses to acidosis from exposure to acetic acid fumes in naked mole-rats. The main findings were: 1) naked mole-rats showed behavioral aversion to acidic fumes, but only at a much higher concentration compared to rats and mice; 2) aversion in the naked mole-rats appears to have been driven not via activation of the trigeminal brainstem pain pathway as in other mammals. However, the data suggest that the vagus nerve may play a role in high threshold aversion; and 3) the patterns of behavioral aversion and trigeminal Fos labeling were reversed for naked mole-rats tested with 50% acetic acid compared to naked mole-rats tested with ammonia. Exposure to 50% acetic acid fumes generated aversion and a decrease in Fos labeling in the trigeminal pain nucleus, whereas ammonia generated no aversion but an increase in Fos labeling in the trigeminal nucleus. This is intriguing considering that in the mammalian nasal epithelium, c fiber nociceptors that

signal pain/irritation usually respond to both acid and ammonia (and capsaicin) (Leffler, et al, 2006; Sekizawa and Tsubone 1994; Lundblad et al. 1983; Taylor-Clark et al. 2005).

The naked mole-rat trigeminal pain pathway has several anomalies that can account for this species' apparently inconsistent behavioral and c fos responses to acetic acid and ammonia. The most recently identified anomaly is a gene variant in the naked mole-rat voltage-gated sodium channel, Nav1.7 which causes inhibition of spike initiation under acidic conditions (Smith et al., 2011). This finding is consistent with a previous report that low pH saline failed to drive spikes in cutaneous c fibers, whereas capsaicin triggered normal spiking in these fibers (Park, et al., 2008). This can account for the Fos results from acidic stimulation of the trigeminal nerve in the present study in that fumes from 20% acetic acid apparently prevented acid-induced spiking in trigeminal c fibers, and hence there was no increase in post-synaptic, activity-driven Fos labeling in the trigeminal nucleus of naked mole-rats. Remarkably, the significant decrease in labeled trigeminal cells from 50% acetic acid suggests that acidification can even suppress baseline activity.

The gene variant in naked mole-rat Nav1.7 can account for high behavioral thresholds and low c Fos labeling in the trigeminal nucleus for acidic stimuli, but it does not have a role in responses to other irritants such as ammonia and capsaicin. However, another striking anomaly has been identified in the naked mole-rat pain pathway that can have a role in responses to these irritants. C fibers in the trigeminal (and dorsal root) ganglia of naked mole-rats naturally lack certain neuropeptides associated with pain/irritant signaling (Park, et al., 2003). These neuropeptides include Substance P and Calcitonin Gene-Related Peptide, and their selective lack appears to involve specific

deletions in gene promoters (Kim, et al., 2011). However, c fibers in naked mole-rats retain glutamate (Brand, et al., 2010), which is able to trigger activity in post-synaptic cells in the dorsal horn (Park, et al., 2008) and trigeminal nucleus (LaVinka, et al., 2009). Hence, irritants such as ammonia and capsaicin can trigger spikes in c fiber afferents and activity in post-synaptic targets which can be detected via Fos labeling (present study) or patch clamp recording (Park, et al., 2008).

Even though irritants other than acid can activate c fibers and post-synaptic targets in naked mole-rats, without the neuropeptides, pain and aversive behaviors are extremely reduced or lacking. Thus, injecting the foot skin with capsaicin (Park, et al., 2008), or stimulating the nasal epithelium with ammonia fumes results in virtually no behavioral responses. We previously showed the important role for one of the lacking neuropeptides, Substance P, in c fiber-mediated pain behavior. Introducing Substance P into the spinal cord of naked mole-rats by gene therapy or by intrathecal injection resulted in rescue of pain behaviors in response to capsaicin application (Brand, et al., 2010; Park, et al., 2008), as well as rescue of itch behaviors to application of histamine (Smith et al., 2010). These procedures had no effect on acid insensitivity, which is consistent with a different mechanism (Nav1.7) for acid insensitivity.

An interesting evolutionary question is, why do naked mole-rats have what appear to be redundant mechanisms for stifling c fiber-mediated pain? Our working hypothesis is that the anomalies in the naked mole-rat pain pathway are adaptations for living in a chronically high CO2/acidic environment. Under this hypothesis, insensitivity to ammonia, and for that matter capsaicin, may be a byproduct since many of the same fibers respond to CO_2, ammonia, and capsaicin. It would seem that either the lack of

neuropeptides, or the altered Nav1.7 would achieve acid insensitivity, so why both? It is possible that altered Nav 1.7 channels led to the loss of neuropeptides from C fibers due to relaxed selective pressure on the neuropeptide genes. The vomeronasal organ, an organ responsible for detecting pheromones, is greatly deteriorated in humans. This deterioration is thought to be due to relaxed selective pressure 25-40 million years ago when trichromacy, visual signaling, appeared (Liman, 2006). Visual confirmation of sexual interest such as swelled sexual organs led to less need to recognize pheromones. Similarly, mutated Nav 1.7 channels resulting in insensitivity to acid could have relaxed evolutionary pressure on the neuropeptide genes. This relaxation may have led to loss of neuropeptides within the pain C fibers of the naked mole-rat as they were no longer needed to transmit acid pain.

The result that naked mole-rats do avoid fumes from 50% acetic acid is interesting. The results from Fos labeling in the vagus solitary nucleus suggest that this pathway may be involved in mediating this species' aversion behavior based on the correlation between labeling and behavior. In naked mole-rats, 50% acetic acid drives both behavioral aversion and a significant increase in the number of Fos labeled neurons in the solitary nucleus. For a lower concentration of acetic acid (20%), there was no aversion and no increase in labeling. Alternatively, an increase in Fos labeling in the solitary nucleus may be related to pH effects on respiration centers (Miura, et al., 1994; Takada, et al., 2011).

Acid-driven activity in the solitary nucleus of the naked mole-rat is very interesting given the gene variant in naked mole-rat Nav1.7. One might have expected that acidity would be unable to drive action potentials in vagal nerves, similar to the

inability to drive action potentials in trigeminal nerves. The difference in how sensory fibers in these two cranial nerves respond to acidification may result from differences in distributions and/or densities of Nav1.7 and other sodium channels (Nav1.8, Nav1.9). Future studies using lesion techniques to inactivate the NTS could further elucidate the role of NTS in the naked mole-rats' detection of acid. If inactivation of NTS led to loss of avoidance of acid, then the NTS would be shown to be essential in acid perception in naked mole-rats.

In conclusion, naked mole-rats have a high threshold for behavioral avoidance to acidic fumes and an apparent lack of acid-driven activity in the trigeminal pain nucleus. However, activity in the solitary nucleus is consistent with behavioral avoidance. The high threshold for airborne acidic fumes is consistent with an adaptation for living under chronically acidic conditions.

IV. Substance P Knockout Mice

A. Introduction

My third aim was to more closely examine the role of Substance P in trigeminal pain. I previously showed that naked mole-rats have an increased tolerance to ammonia stimulation of their trigeminal nerve (LaVinka et al, 2009). This is thought to be due partly to the absence of neuropeptides from their trigeminal C fibers (LaVinka et al, 2009). To examine the effect of removing one neuropeptide from trigeminal nociceptors, I tested Substance P knockout mice in the behavioral and c fos procedures described in Chapters 2 and 3. This transgenic mouse has its preprotachykinin-A (PPTA) gene, the gene which codes for Substance P, disrupted. My hypothesis was that PPTA-/- mice would show blunted responses to noxious stimulation of their trigeminal nerve, both behaviourally and physiologically.

B. Methods

1. Subjects

Subjects used were PPTA-/- knockout mice. Substance P knockout mice were obtained from Jackson Laboratories from a strain created by Allan Basbaum. These mice have a targeted deletion in the PPT-A genomic locus. The SP coding region was replaced with pGK-neo and the neurokinin A (NKA) region was deleted. The deletion only affected production of Substane P and NKA with NK1 receptor expression remaining unchanged (Cao et al, 1998). They were housed under conventional laboratory vivarium conditions. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

2. Behavioural Tests

a. Avoidance Test

Substance P knockout mice were tested using the avoidance test described in chapter 2. See chapter 2 for complete description of the four-arm plus maze and stimulants used in behavioural testing.

b. Capsaicin Irritant Test

Capsaicin solution (1.5%) or 0.9% saline was topically applied to the openings of the nostrils with a saturated cotton tip applicator. Capsaicin was prepared by dissolving 25 mg of capsaicin in 2mL of 100% ethanol. The capsaicin/ethanol solution was mixed into 9.3 mL of saline and 0.7 mL of Tween 80. Then the solution was heated to evaporate off the ethanol. Mice were momentarily restrained in their home cage for application of the capsaicin solution and then observed for 10 minutes immediately after. Cumulative time spent wiping the nose was recorded with a stopwatch to the nearest tenth second. Each animal was tested with both capsaicin and saline in a randomized order.

3. C fos Experiments

a. Stimulation with noxious chemical fumes

Animals were deeply anesthetized with 50mg/kg sodium pentobarbital, IP. They were placed on their backs, with their nose easily accessible. A sterile cotton swab was submerged in the chemical and then placed in close proximity to the subjects' external nares. They were exposed to the chemical fumes until the subject flinched or twenty seconds passed, whichever occurred first. The swab was then removed so the subject

could not habituate to the stimulant. Stimulation occurred every minute for one hour. To obtain background Fos levels, control subjects were either anesthetized for one hour without stimulation or they were administered a lethal dose of sodium pentobarbital and immediately perfused.

b. Perfusion and Immunohistochemistry

After stimulation, the animal was given a lethal dose of sodium pentobarbital, (100mg/kg, IP) and was transcardially perfused with saline followed by 4% formaldehyde in phosphate-buffered saline (PBS). The brain and upper spinal cord was removed, post-fixed for an hour at 4°C, then rinsed and stored in 30% sucrose solution in PBS at 4°C. The brains were sliced into transverse serial sections, 40 μm-thick. The tissue was cut on a table-top microtome, cooled with dry ice. Once cut, the tissue was stored overnight in PBS at 4°C before being processed. Serially sliced sections were incubated in Anti-c-Fos (Ab-5) (4-17) Rabbit pAB for 48 hours (Oncogene Sciences/Calbiochem, Cambridge, MA, AB5, 25,000×). After incubation, tissue was rinsed three times in PBS for 15 minutes. The tissue was then incubated in biotinylated anti-rabbit IgG for an hour and a half and rinsed again. The tissue was processed using the avidin-biotin complex (ABC) method for an hour and a half using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). All tissue was rinsed in PBS and the visualized with diaminobenzidine with hydrogen peroxide to obtain a brown-black stain.

After processing, tissue was mounted on subbed slides and cover-slipped. The labeled Fos cells were manually counted within the spinal trigeminal nucleus using a light microscope. Pictures were taken using an Amscope trinocular compound

microscope with a 3.1 megapixel camera. Brain areas were defined by the Paxinos and Watson rat brain atlas and the stereotaxic naked mole-rat atlas by Xiao et al (2006).

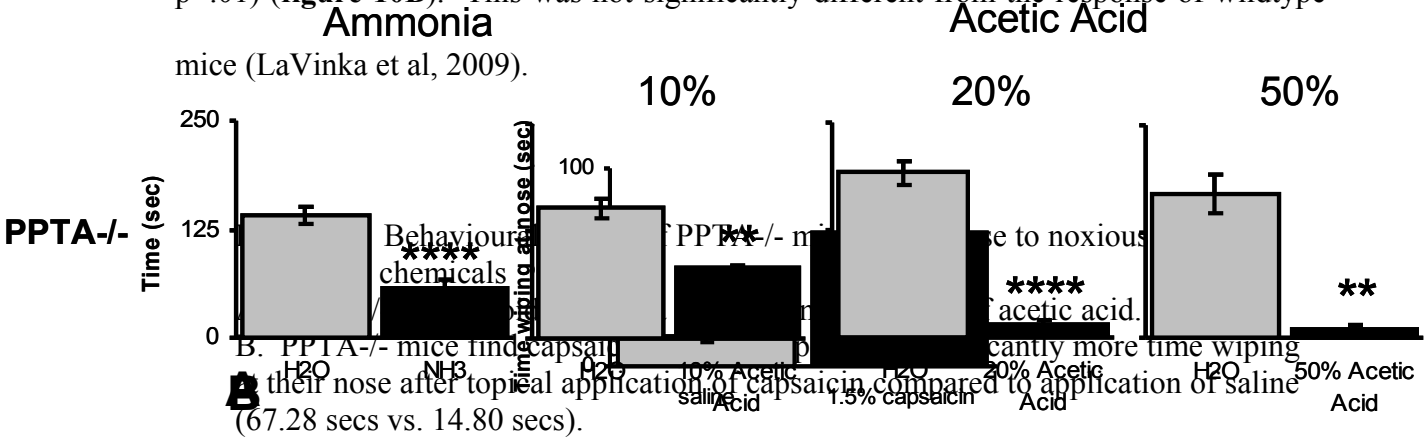
C. Results

Substance P knockout mice avoided all noxious irritants, similar to wildtype mice. PPTA-/- mice spent significantly less time close to sponges saturated with ammonia, 10%, 20%, and 50% acetic acid when compared to the time spent near the sponges saturated with water (**table 2, figure 10A).**

TABLE II: Behavioural avoidance of PPTA-/- mice to noxious fumes

Irritant	N	Avg time near H2O	Avg time near Irritant	T value	Df	P value
NH3	6	141.29	56.74	4.99	22	P<.0001
10%	5	151.55	81.34	3.61	18	P<.01
20%	5	191.50	15.24	7.03	18	P<.0001
50%	5	169.46	10.02	3.92	18	P<.01

These avoidance results indicate that Substance P knockout mice found all stimulants to be noxious. Upon topical application of capsaicin to their nose, Substance P knockout mice spent significantly more time rubbing and scratching their nose compared control mice which received saline application (67.28 secs vs. 14.80 secs, t=-4.12, df=8, p<.01) (**figure 10B**). This was not significantly different from the response of wildtype mice (LaVinka et al, 2009).



Behavioural [illegible] of PPTA-/- mice to noxious chemicals [illegible] acetic acid.

B. PPTA-/- mice find capsaicin [illegible] cantly more time wiping their nose after topical application of capsaicin compared to application of saline (67.28 secs vs. 14.80 secs).

Post-synaptic activity showed no difference in the spinal trigeminal nucleus between control, ammonia, or 20% acetic acid (48.45, 38.12, 56.89) (**figure 11**). No difference was seen in the nucleus tractus solitarius either (21.44, 24.69, 23.48) (**figure 11**).

D. Discussion

Substance P knockout mice showed significant aversion to ammonia and acetic acid, as did wildtype mice. Their behaviour indicates that the lack of Substance P from their trigeminal nerve did not cause a decrease in aversive response to noxious stimulants. However when Fos activity was examined in their spinal trigeminal nucleus, no difference in activity was seen upon stimulation of ammonia or 20% acetic acid when compared to control Substance P knockout mice.

I examined the activity in the nucleus tractus solitarius to see if chemical activation of the respiratory system could account for the PPTA-/- mice avoidance of ammonia and acetic acid. However, the NTS showed no difference in activity upon stimulation with the irritants compared to activity in the controls. These findings were not expected. It has been shown in the literature, that the role of Substance P is not clear cut. In the original paper by Cao et al (1998), the group responsible for creating this knockout, they showed mixed results in pain tests. When the knockout mice were tested thermally, they only showed reduced responses at 55.5°C. They showed comparable aversive responses to wildtype mice at 52.5°C and 58.5°C (Cao et al, 1998). PPTA-/-

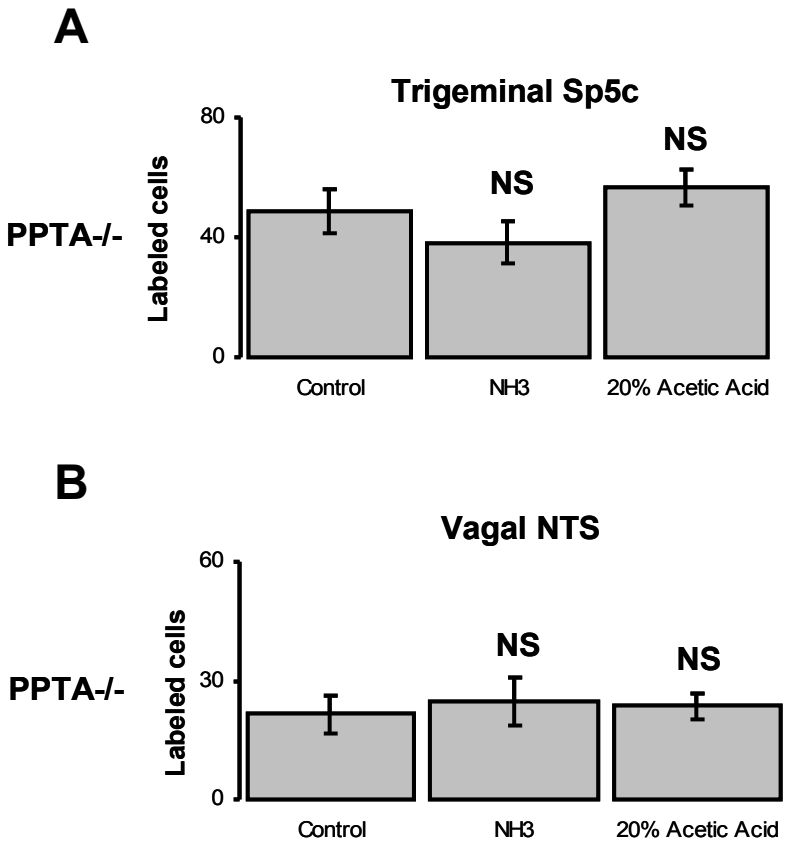


Figure 11: Average number of Fos labeled cells in the Sp5c and NTS in control and stimulated PPTA-/- mice
A. PPTA-/- mice showed no difference in Fos activity in their Sp5c upon stimulation with ammonia or 20% acetic acid compared to control.
B. No differences in Fos activity were found in the NTS either.

mice showed no altered responses to mechanical stimulation by von Frey hairs but did have decreased sensitivity to mechanical stimulation by tail clip (Cao et al, 1998). Chemical stimulation also showed mixed results. Injection of 0.6% formalin resulted in similar responses for both wildtype and PPTA-/- mice. Increasing the concentration to 1.2% formalin showed decreased responses in PPTA-/- mice. However increasing the concentration to 2% formalin showed similar responses in both wildtype and knockout (Cao et al, 1998). This data shows that removing Substance P does not lead to clear distinctions in pain processing. Our behavioural tests showed no difference in avoidance of noxious chemicals in PPTA-/- mice compared to wildtype. This avoidance would be expected to correspond with an increase in activity within Sp5c. However, this was not the case. No change was seen in the Fos activity of Sp5c of PPTA-/- in response to ammonia or 20% acetic acid. We did notice that the PPTA-/- mice had much higher numbers of labeled Fos cells within the spinal trigeminal nucleus in the control compared to BL6 wildtype mice (48.45 vs 14.15, $t=3.32$, $df=7$, $p<.05$). This is a 242% increase in activity. The NTS showed a 121% increase from wildtype control to Substance P knockout control (9.71 vs 21.44, $t=2.55$, $df=7$, $p<.05$). This increased level of background activity could be masking a change in Sp5c activity upon noxious stimulation.

V. Selective Lack of Substance P in the Trigeminal Nerve of the African Naked Mole-Rat

A. Introduction

My final aim was to examine the naked mole-rat's trigeminal nerve. It was shown that naked mole-rats lack Substance P in their dorsal root and trigeminal ganglion (Park et al, 2003). I wanted to examine the localization of Substance P within the naked mole-rat brain. Naked mole-rats have been shown to have Substance P positive fibers on the blood vessels of their viscera, so they are not a complete knockout for the neuropeptide (Park et al, 2003). The vasculature of the viscera is innervated by the vagus nerve, suggesting that there might be Substance P in different brain areas. However, nothing is currently known about Substance P expression within the naked mole-rat brain. I examined five areas known to express substance P in rats and mice: 1. spinal trigeminal nucleus; 2. nucleus tractus solitarius; 3. substantia nigra; 4. habenular nucleus; and 5. globus pallidus (Ribiero-da-Silva and Hokfelt, 2000; Cuello et al, 1978; Mounir and Parent, 2002). We found that the naked mole-rat had Substance P in all brain areas examined except for the spinal trigeminal nucleus. Substance P has been implicated in pain, neurodegeneration, neuroendocrine control of reproduction, sleep, and positive reinforcement (Seybold et al, 1982; Fernandez et al, 1996; Lasaga and Debeljuk, 2011; Silva and Palmer, 2011; Haun et al, 1992; Kertes et al, 2010).

Substance P is released by C fibers in the trigeminal nerve. One possible explanation for lack of Substance P in the naked mole-rats' spinal trigeminal nucleus would be lack of C fibers within their trigeminal nerves. To test this hypothesis, I used

electron microscopy to examine the fibers comprising the naked mole-rat trigeminal nerves. My results show that naked mole-rats have a comparable number of C fibers to wildtype mice.

B. Methods

1. Perfusion and Immunohistochemistry

Subjects were given a lethal dose of sodium pentobarbital, (100mg/kg, IP) and transcardially perfused with saline followed by 4% formaldehyde in phosphate-buffered saline (PBS), two minutes at low pH 6.5 and ten minutes at high pH, 9.5. The brain and upper spinal cord was removed, post-fixed for an hour, pH 7.4 at 4°C, then rinsed and stored in 30% sucrose solution in PBS at 4°C. The brains were sliced into transverse serial sections, 40 μm-thick. The tissue was cut on a table-top microtome, cooled with dry ice. Once cut, the tissue was stored overnight in PBS at 4°C before being processed. Serially sliced sections were pre-blocked for a half hour in 10% normal goat serum in PBS, pH 7.4 with 0.3% Triton X-100. Tissue was rinsed three times in PBS, ten minutes a rinse and then incubated in anti-Substance P Rabbit pAB for 18-24 hours (ImmunoStar, Hudson, WI, 10,000X). After incubation, tissue was rinsed three times in PBS. The tissue was then incubated in biotinylated anti-rabbit IgG for an hour and a half and rinsed again. The tissue was processed using the avidin-biotin complex (ABC) method for an hour and a half using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). All tissue was rinsed in PBS and then visualized with diaminobenzidine with hydrogen peroxide to obtain a brown-black stain.

After processing, tissue was mounted on subbed slides and cover-slipped. Brain areas were defined by the Paxinos and Watson rat brain atlas and the stereotaxic naked mole-rat atlas by Xiao et al (2006).

2. Transmission Electron Microscopy

Animal subjects used were adult African naked mole-rats (*Heterocephalus glaber*) and wildtype C57BL6 mice. Animals were injected with a sacrificing dose of sodium pentobarbital (50mg/kg) diluted with physiological saline in a 9:1 ratio. Animals were intracardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.2-7.4. After fixation, samples of trigeminal nerve were taken. Samples were post-fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for two hours at room temperature. Samples were rinsed three times for ten minutes each in 0.1 M sodium cacodylate buffer, pH 7.4 at room temperature. Samples were then placed in 1% osmium in 0.1 M sodium cacodylate buffer, pH 7.4. Tissue was rinsed three times and stored overnight in sodium cacodylate buffer at 4°C. Samples were dehydrated in 25% ethanol and 50% ethanol for fifteen minutes each. Then samples were placed in 5% uranyl acetate in 50% ethanol for 15 minutes on a rotator. The tissue was further dehydrated in 75% and 95% ethanol, fifteen minutes each. Samples were placed in anhydrous 100% ethanol over molecular sieve for five changes, four minutes each, for a total of twenty minutes.

All samples were covered and infiltrated in 1 part 100% ethanol to 1 part Spurr's resin overnight on a rotator. Samples were covered and infiltrated with pure Spurr's resin, with two changes over twenty-four hours. Samples were embedded in fresh

Spurr's resin in Beem capsules and placed in oven to polymerize at 70°C for forty-eight hours. Resin blocks were removed from capsules and trimmed with a razor blade until tissue was exposed. Blocks were then faced off with a Microstar Histo Diamond knife on a Reichert-Jung Ultra Cut E ultramicrotome. Semithin and ultrathin sections (1.9 micrometers, 90 nanometers) were collected using a DIATOME Diamond knife.

Semithin sections were collected and mounted on subbed glass slides and stained using Toluidine blue. Ultrathin (90nm) sections were collected on 200 hex mesh nickel grids coated with parlodion/carbon. Sections were stained with 4% aqueous uranyl acetate for forty-five minutes and then rinsed three times with distilled water. Sections were then stained with Reynold's lead citrate for five minutes then rinsed with distilled water three times. Grids were allowed to air dry and sections were observed using a JEOL 1200EX Transmission Electron Microscope. Pictures were taken on Kodak 4489 electron microscopy film and developed. Photos were scanned into a computer using an Umax Power Look III scanner and images were analyzed using Image J. Myelinated and unmyelinated fibers were counted within eight random high magnification (X12000) photographs per nerve, with a total of four nerves examined. Semithin sections from each nerve had their cross-sectional areas calculated with Image J and total A and C fiber counts were extrapolated.

C. Results

I found that the spinal trigeminal nucleus in naked mole-rats was virtually devoid of Substance P positive fibers. This was completely different from what I found for wildtype mice which showed abundant labeling in the spinal trigeminal nucleus. Figure

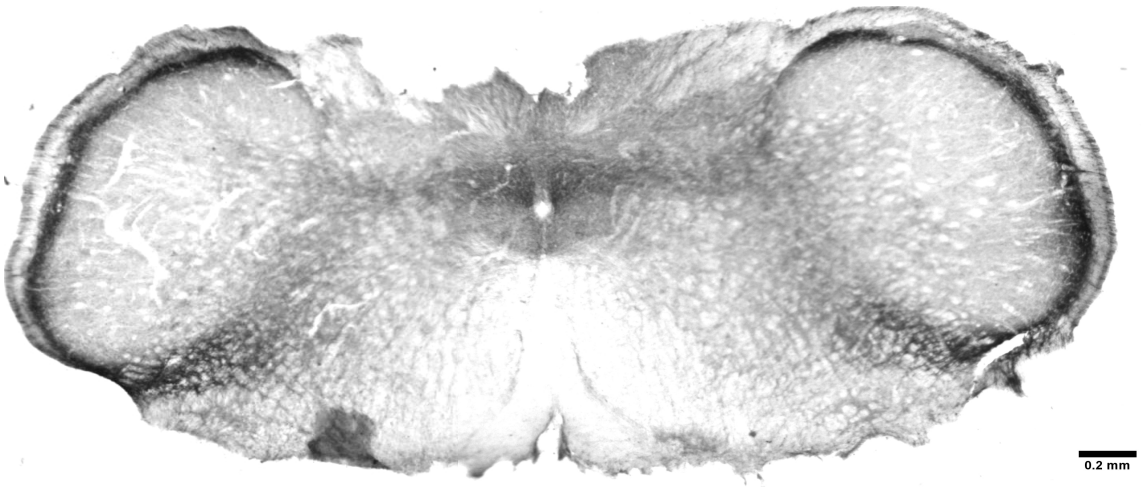
12 shows examples images of sections through the brainstem of a naked mole-rat and mouse, both are labeled for Substance P. Note the prominent dark band of Substance P positive fibers in the spinal trigeminal nucleus of the mouse, located at the outer edges of the slice. No such dark band is evident for the naked mole-rat.

Among the brain regions that I examined in naked mole-rats, the spinal trigeminal nucleus was the only region which showed a lack of Substance P, indicating that the lack of Substance P in this species is selective to that region. I found positive label for Substance P in the nucleus tractus solitarius of both the naked mole-rat and mouse. The NTS is located medially, near the central canal. Darkened tissue around the central canal indicates the presence of Substance P (**figure 12).** The dark lateral areas in figure 13 are the left and right substantia nigra. Both the naked mole-rat and mouse showed dark Substance P staining in this brain region (**figure 13)**. The habenula is the pointed medial structure within figure 14. The habenula is divided into medial and lateral nuclei. The darkened lines show the positive Substance P staining in the medial nuclei of the naked mole-rat and mouse (**figure 14)**. The globus pallidus is dark staining seen medially in the upper lateral, baguette shaped structure within figure 15. Also note, the dark staining the medial amygdala located below the globus pallidus in both naked mole-rat and mouse (**figure 15)**.

I also assessed slices from Damaraland mole-rats, a close relative to the naked mole-rat. Like the mice, the Damaraland mole-rats showed positive label for Substance P in all brain areas examined (**figure 16)**.

To test the Substance P antibody for non-specific staining, I also stained a Substance P knockout mouse which was processed with tissues from other subjects in this study. The knockout mouse showed no positive labeling in any region, indicating good specificity of the antibody (**figure 17 and figure 18)**.

A



B A

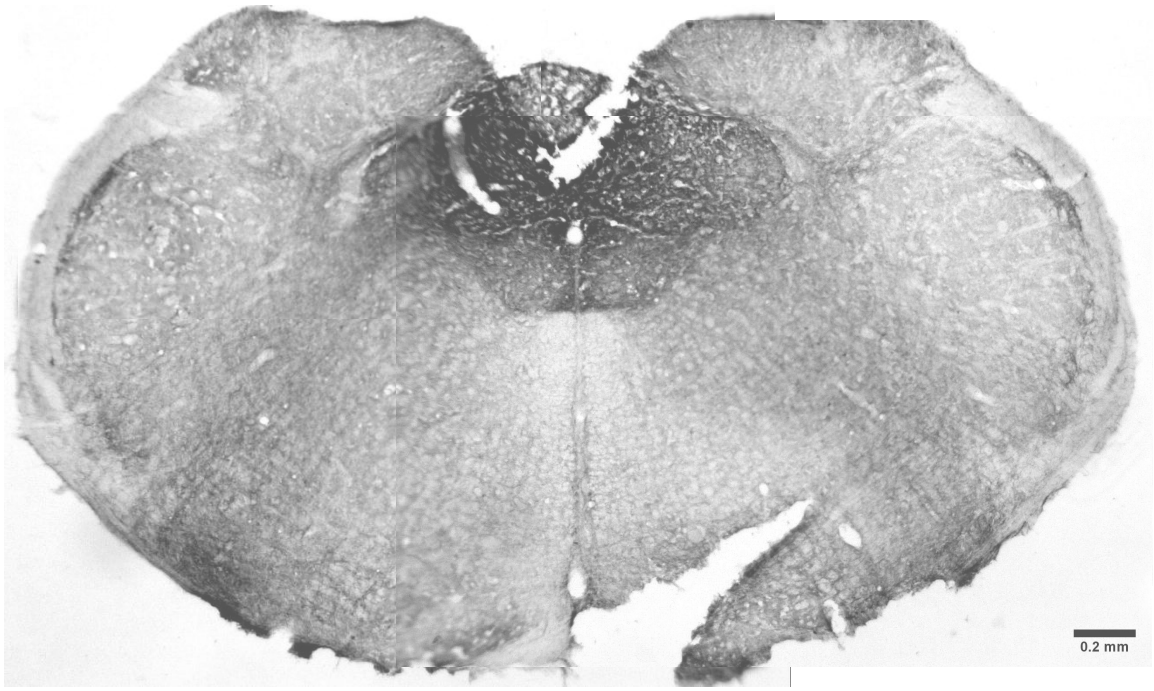


Figure 12: Substance P staining within the spinal trigeminal nucleus
A. Wildtype mouse
B. Naked mole-rat

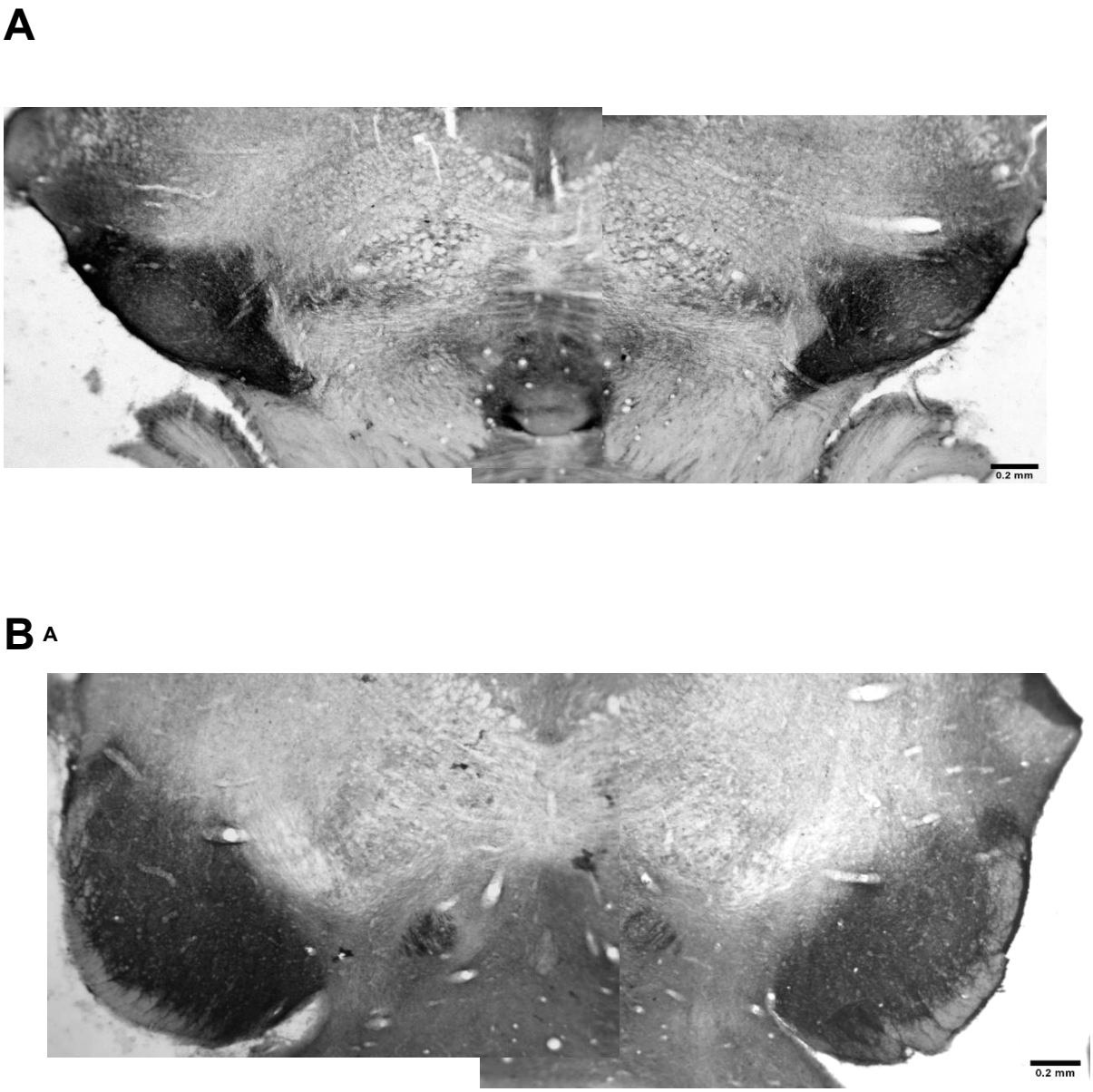
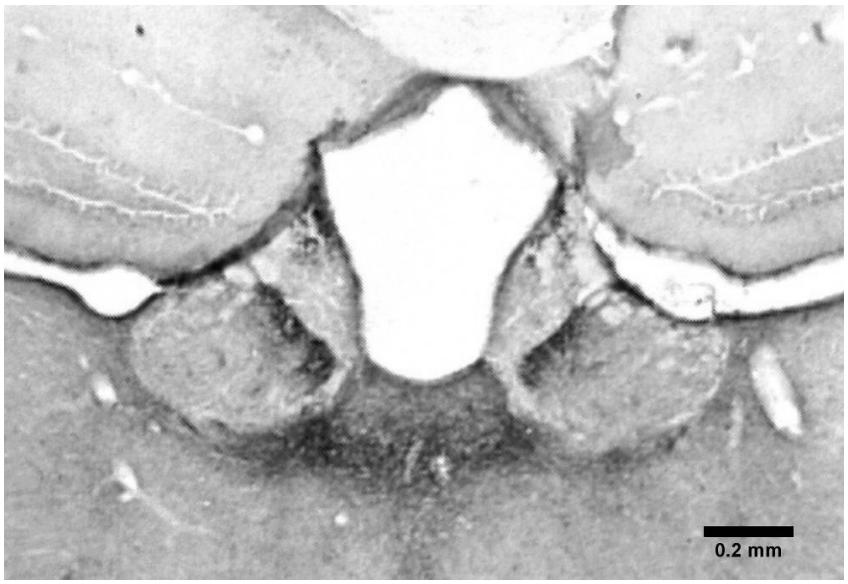


Figure 13: Substance P staining within the substantia nigra
A. Wildtype mouse
B. Naked mole-rat

A



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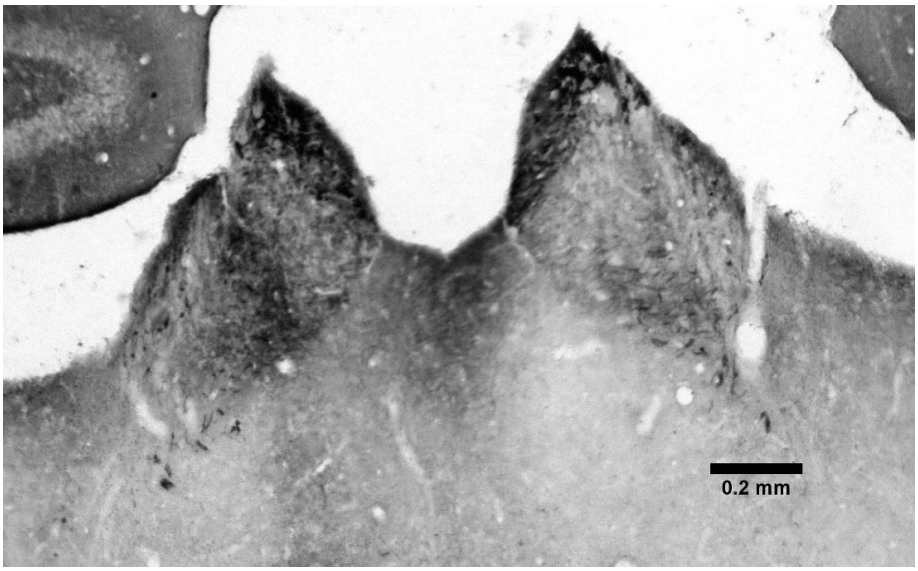
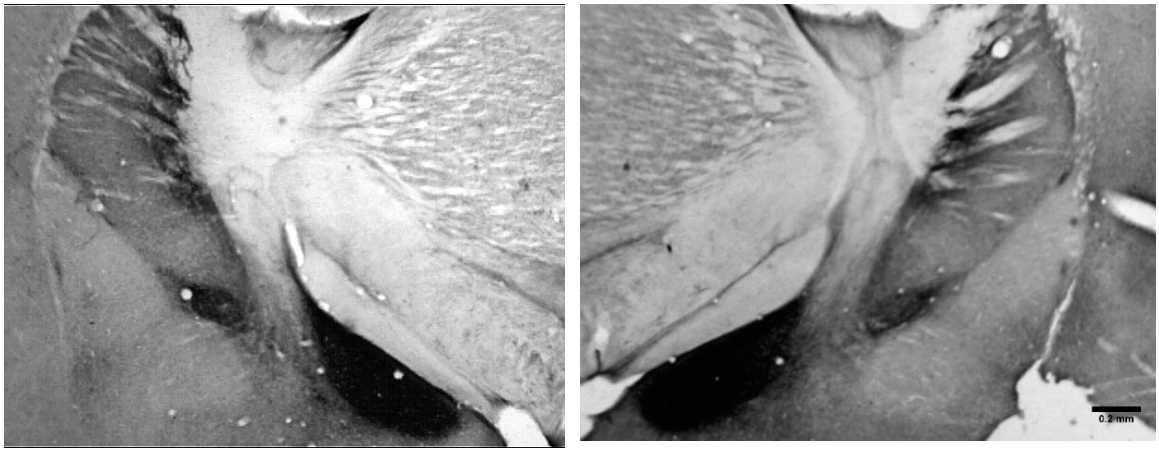


Figure 14: Substance P staining within the habenula
A. Wildtype mouse
B. Naked mole-rat

A



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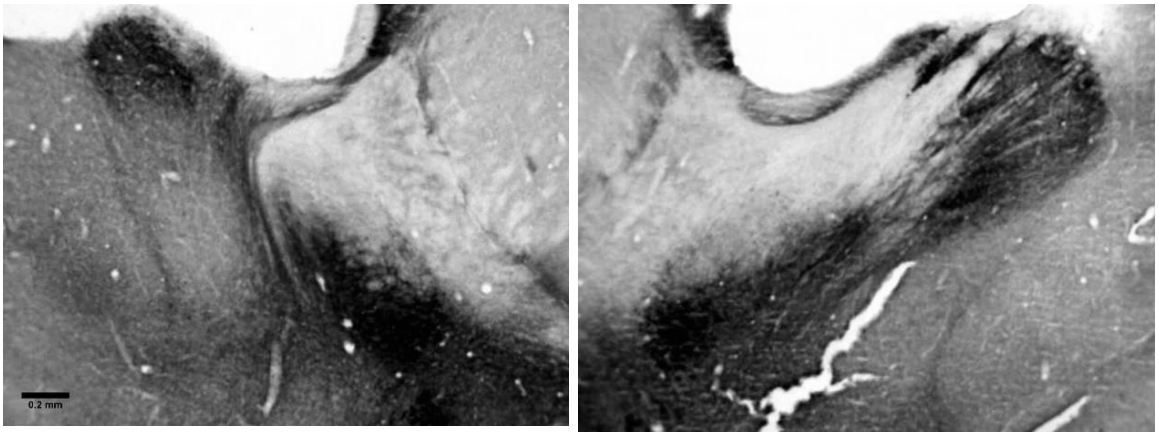


Figure 15: Substance P staining within the globus pallidus
A. Wildtype mouse
B. Naked mole-rat

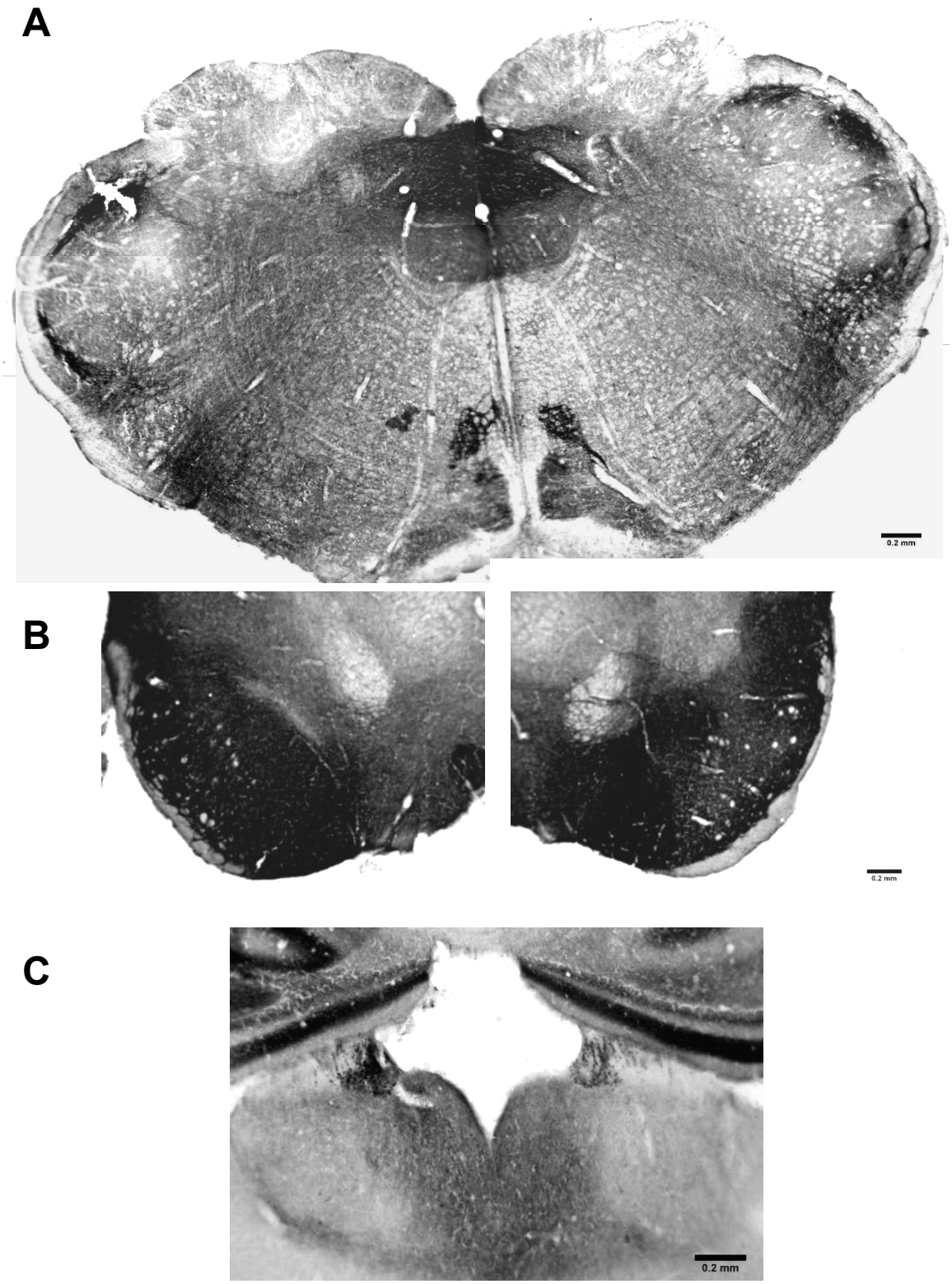
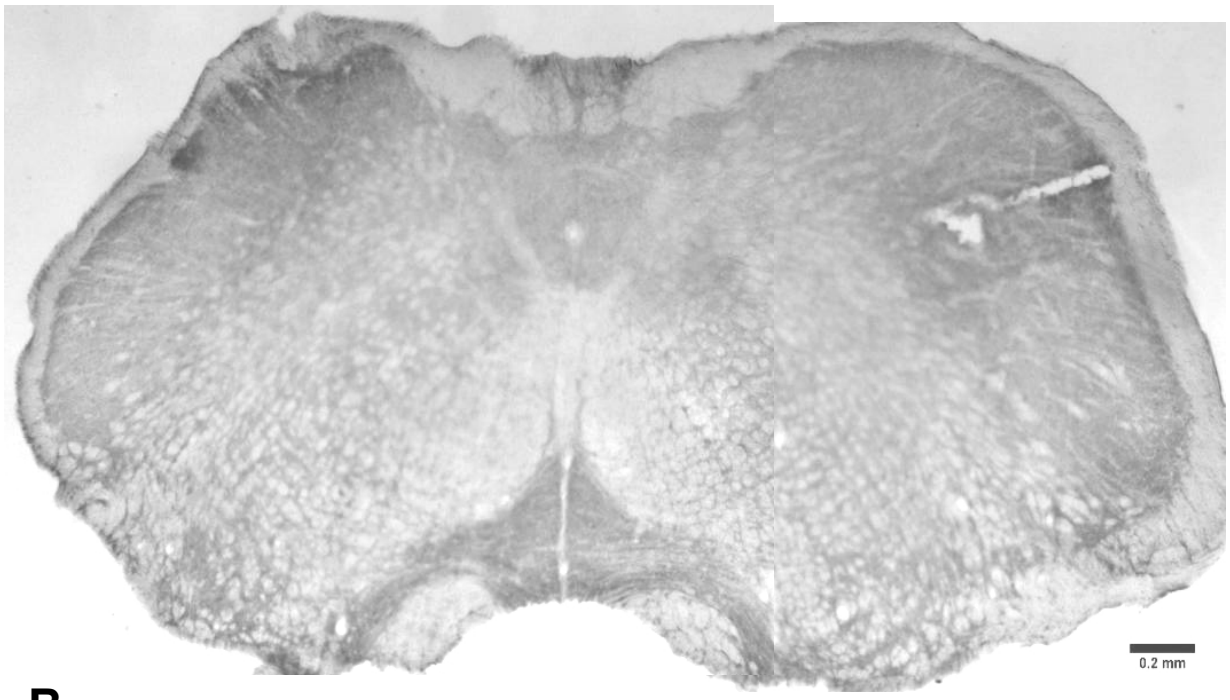


Figure 16: Substance P staining of Damaraland mole-rat
A. Sp5c and NTS
B. Left and right substantia nigra
C. Habenula

A



B

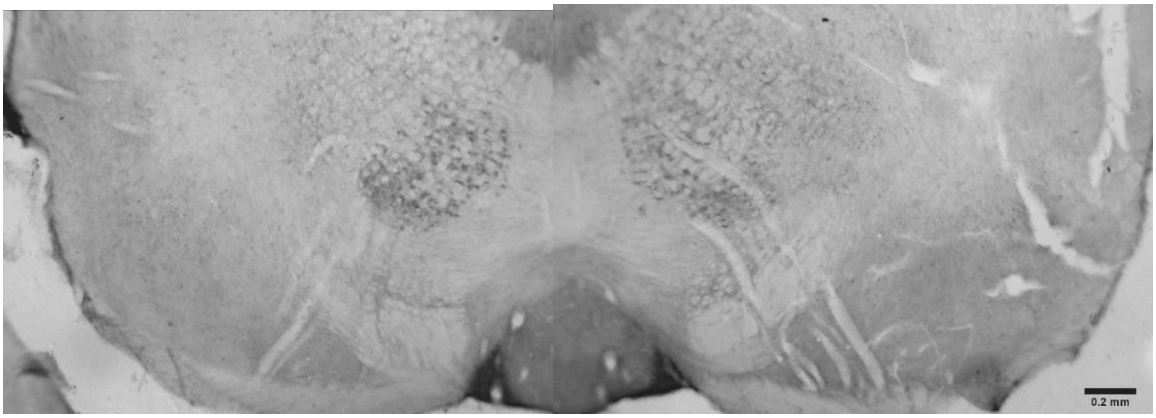


Figure 17: Substance P staining of PPTA-/- mouse
A. Sp5c and NTS
B. Left and right substantia nigra

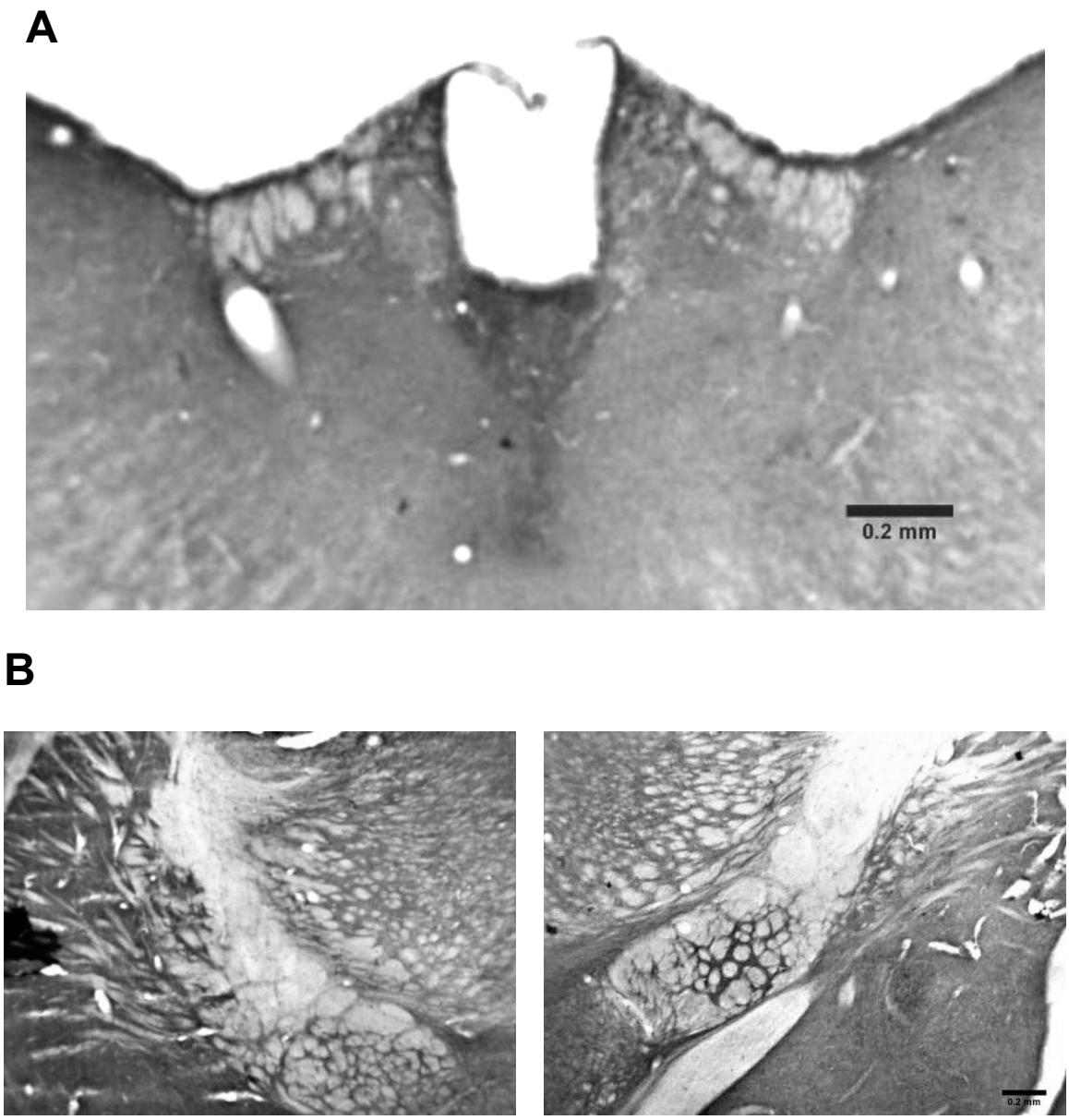


Figure 18: Substance P staining of PPTA-/- mouse
A. Habenula
B. Left and right globus pallidus

I next addressed the possibility that lack of Substance P positive fibers in the naked mole-rat spinal trigeminal nucleus could be due to a lack of C fibers (the fiber type where most Substance P is found) in the trigeminal nerve. To do so, I examined sections through the trigeminal nerve of naked mole-rats and mice with transmission electron microscopy and I counted both C and A fibers in both species. I found that naked mole-rats have comparable numbers of A and C fibers to wildtype mice. I counted the number of fibers within each 54 μm² image (**figure 19**), and I found that naked mole-rats had an average of 2.25 A fibers and 1.56 C fibers per image. Mice had an average of 2.89 A fibers and 1.63 C fibers per image (**figure 20A**). Comparison between mouse and naked mole-rat A fiber counts and C fiber counts showed no significant differences. ($t=1.53$, $df=57$, $p=0.13$ for A fibers; $t=0.11$, $df=57$, $p=0.91$ for c fibers).

The average total cross-sectional area of the trigeminal nerves from the naked mole-rats was 664,042.4 μm. For the mice it was 170,558.5 μm. By extrapolating, I was able to estimate the total number of fibers for both species. Naked mole-rats have approximately 27,680 myelinated (A) fibers per trigeminal nerve, and mice have approximately 20,070 A fibers. Regarding unmyelinated C fibers, naked mole-rats have approximately 19,222 and mice have approximately 11,322 (**figure 20B**).

These strait forward fiber counts answer the question of whether or not naked mole-rats have c fibers in their trigeminal nerves: they do. However, fiber counts by themselves may miss more subtle differences since species differences in the size of a nerve (and hence total fiber counts) can mask a significant difference. To avoid the

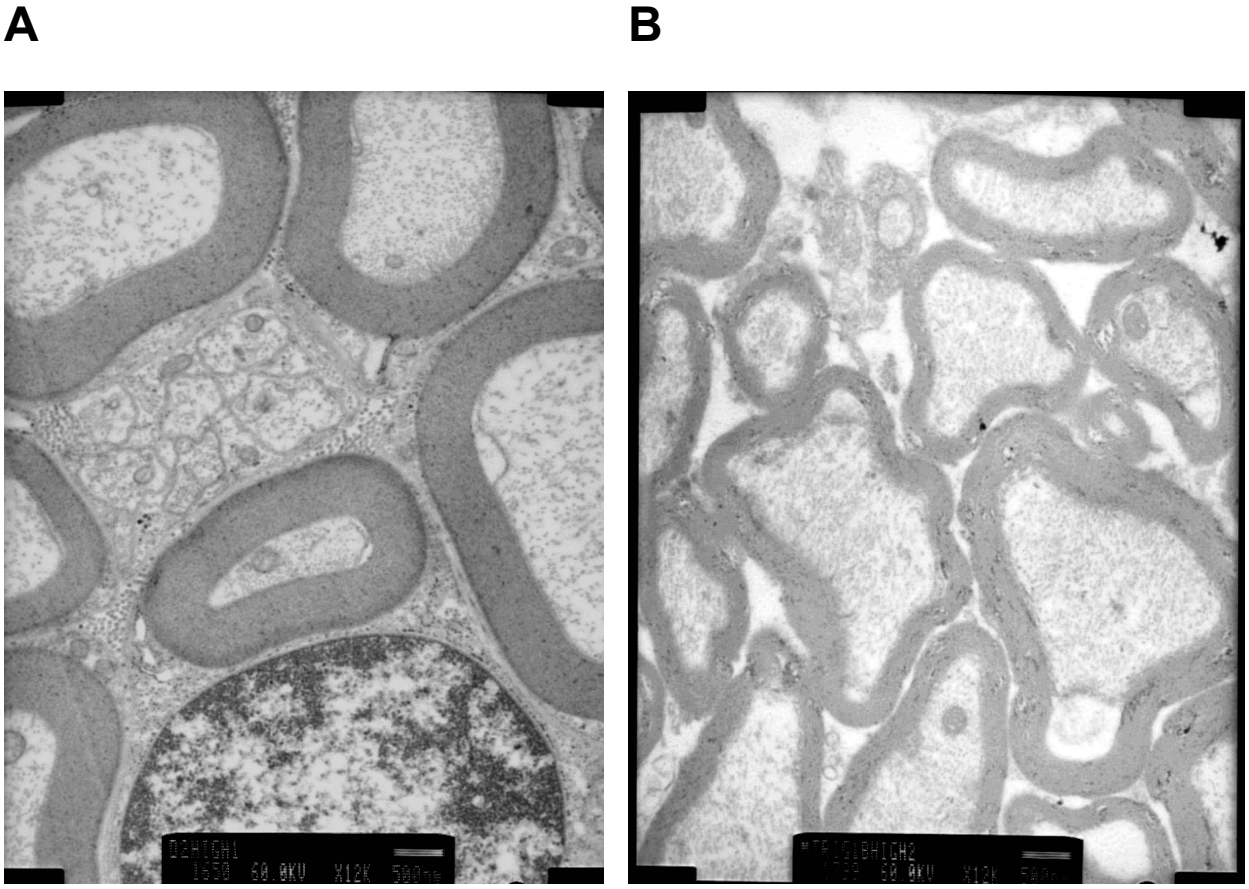


Figure 19. TEM of naked mole-rat and mouse trigeminal nerve
A. Naked mole-rat trigeminal nerve
B. Mouse trigeminal nerve

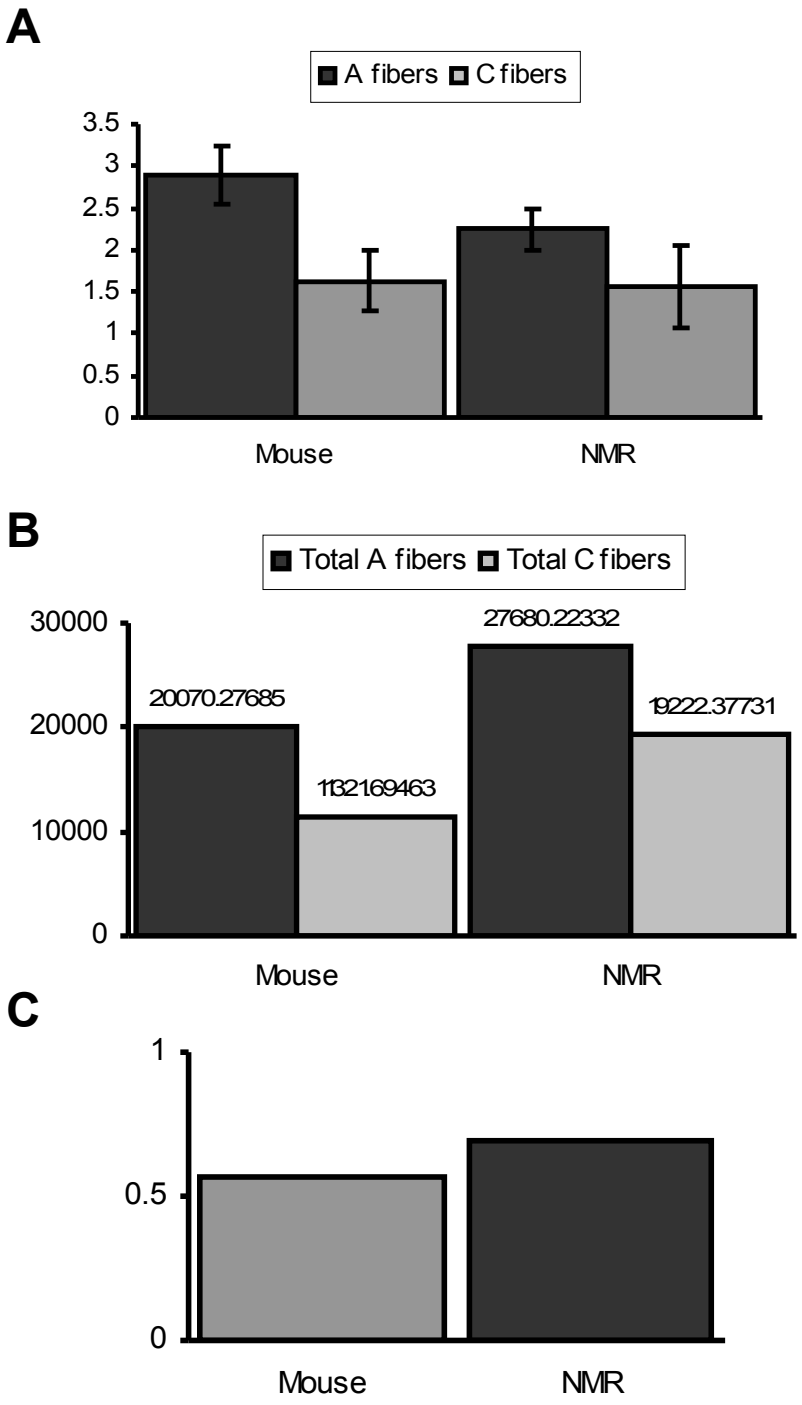


Figure 20. Innervation of trigeminal nerve in African naked mole-rat and mouse
Average A and C fibers counted in 54 μm² area.
Total A and C fiber counts in trigeminal nerve
Ratio of C to A fibers in the trigeminal nerve of mice and NMRs.

possible masking effects of overall differences in counts, previous studies have compared the ratios of C to A fibers when comparing nerve composition across species. Hence I also calculated C to A fiber ratios for the naked mole-rat and mouse trigeminal nerves in this study. I found similar ratios for both species. For mouse trigeminal nerves, the ratio of C to A fibers was 0.56 compared to naked mole-rat trigeminal nerves which had a ratio of C to A fibers of 0.69 (**figure 20C**).

D. Discussion

1. Substance P staining

The naked mole-rat showed an absence of Substance P labeled fibers in the spinal trigeminal nucleus but mouse-like labeling in the nucleus tractus solitarius, substantia nigra, habenula, and globus pallidus.

a. Spinal Trigeminal Nucleus

Upon painful stimulation, the trigeminal nerve releases Substance P into the spinal trigeminal nucleus (Mitsikostas et al. 1999). For more detail, refer to Chapter 1.

b. Nucleus Tractus Solitarius

The nucleus tractus solitarius is the primary integration site for visceral sensory information, including information from arterial chemoreceptors and vagal afferents (Jordan and Spyer, 1986). High levels of Substance P are found in chemoafferents terminating in the NTS (Douglas et al, 1982). Levels of Substance P are found to increase during and after hypoxia and injections of Substance P into the NTS increases

ventilation (Lindefors et al, 1986;Srinivasan et al, 1991; Mazzone et al, 1998; Mazzone and Geraghty, 1999).

c. **Substantia Nigra**

Substantia nigra has been shown to heavily label for Substance P (Ribiero-da-Silva and Hokfelt, 2000). Substance P is also found in striatal afferents terminating in the substantia nigra (Bolam and Smith, 1990). Substance P within the substantia nigra has been shown to be related to Parkinson's Disease. Patients suffering from Parkinson's Disease show decreased Substance P levels in their substantia nigras (Mauborgne et al, 1983; Fernandez et al, 1996; Llorens-Cortes et al, 1984). When Substance P is injected into the substantia nigra, increased dopamine release is shown in the striatum (Reid et al, 1990).

d. Habenula

The habenular complex is a diencephalic structure located on the dorsomedial surface of the caudal thalamus. (Lecourtier and Kelly, 2007). It is divided into the medial and lateral habenular nuclei. The medial habenular nucleus has very different afferents and efferents from the lateral habenular nucleus. The medial habenular nucleus has substance P positive neurons dorsally (Cuello et al, 1978). These neurons project to the interpeduncular nucleus (Lecourtier and Kelly, 2007). Substance P from the habenula has been implicated in sleep behaviours such as muscle tone and duration of sleep (Haun et al, 1992). Injection of Substance P decreases cAMP levels within the habenula (Palkovits et al, 1990).

e. Globus Pallidus

Another place Substance P has been found is the globus pallidus (Ribiero-da-Silva and Hokfelt, 2000; Shults et al, 1984). Striatofugal fibers project Substance P-containing fibers to the globus pallidus (Mounir and Parent, 2002). Substance P levels within the globus pallidus have been implicated in playing a role in the severity of symptoms within Parkinson's disease (de Ceballos et al, 1993; de Ceballos et al, 1999). The globus pallidus reacts stronger to reward-related events than to aversive events (Joshua et al, 2009). It has been shown that Substance P could play a role in positive reinforcement. When Substance P was injected into the globus pallidus, rats showed place preference (Kertes et al, 2010). Also, cAMP levels were decreased with Substance P injection (Palkovits et al, 1990).

2. TEM examination of the Trigeminal Nerve

The results of the behavioral experiments previously published and presented in Chapter 2 show that naked mole-rats are extremely tolerant to noxious stimulation of their trigeminal nerve (LaVinka et al, 2009; present study). Tolerance to ammonia and acetic acid fumes is thought to be due to lack of neuropeptides within the trigeminal nerve C fibers as well as the inability of acid to drive action potentials in the trigeminal nerve. I wanted to know if the lack of neuropeptides and insensitivity might be due to a reduction or absence of C type pain fibers (the main fibers associated with neuropeptides) in trigeminal nerve of naked mole-rats compared to the trigeminal nerve of mice. A previous study showed that in the purely sensory saphenous nerve that innervates the skin of the hind paw, naked mole-rats had C fibers but significantly lower numbers of C fibers

compared to mice (Park et al, 2008). Naked mole-rats had a C to A fiber ratio of 1.1 compared to the mouse C to A fiber ratio of 3.8. I examined the naked mole-rat trigeminal nerve to see if 1) naked mole-rat trigeminal nerves has C fibers at all, and 2) if they did, was there a substantial reduction in the number/proportion of C fibers compared to mice. I found that there was no significant difference in numbers of C and A fibers in naked mole-rat compared to mouse.

The difference between naked mole-rat trigeminal and saphenous nerves – reduction in C fibers in saphenous but not trigeminal nerves compared to mice – may reflect differences in motor versus sensory composition of the nerves. The saphenous nerve is purely sensory, while the trigeminal nerve is a mixed nerve, with motor and sensory fibers (Purves et al, 2001; Park et al, 2008). Since motor neurons are all A type, myelinated fibers, a deficit in the number of C fibers could be masked. In any case, the overall result that naked mole-rats have many C fibers in their trigeminal nerve dissuades the notion that lack of Substance P is due to a lack of C fibers.

The experiments described in chapter five show 1) naked mole-rats' lack of Substance P is specific to their spinal trigeminal nucleus and 2) the lack of Substance P is not due to the absence of C fibers within the naked mole-rat trigeminal nerve. These results support the notion that naked mole-rats have specific adaptations to survive within their extreme environments.

VI. Conclusion

Naked mole-rats live in an extreme environment of low oxygen and high carbon dioxide. This toxic environment has lead to a number of putative adaptations. Carbon dioxide is a known painful stimulus. My interest is in the naked mole-rats adaptation to blunt this type of pain.

My behavioural experiments (**Chapter 2**) show that naked mole-rats have much higher tolerances to noxious airborne irritants when compared to rats, mice, and Damaraland mole-rats.

My c Fos experiments (**Chapter 3**) show that acidic fumes do not activate the trigeminal pain pathway compared to other rodents. While the trigeminal pain pathway is not activated by acidic fumes, the vagal chemosensory pathway is activated. The vagal pathway may mediate the high threshold avoidance of acidic fumes.

My experiments with Substance P knockout mice (**Chapter 4**) show that the lack of Substance P alone is insufficient to blunt behavioural avoidance to the extent we see in naked mole-rats. The unusual Fos results of these animals show that the role of Substance P in trigeminal nociception is a complicated one.

My Substance P labeling experiments (**Chapter 5**) show that naked mole-rats have a specific peptide omission in their trigeminal pain nucleus. Electron microscopic examination of the naked mole-rat trigeminal nerve shows that the absence of Substance P is not due to the lack of C fibers.

My research supports the hypothesis that naked mole-rats have adapted their pain pathways in order to survive their noxious environments. By learning about how natural model systems blunt pain, we can discover new targets for therapeutic intervention.

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Bios 240 - Homeostasis: The Physiology of Plants and Animals
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Bios 335 – History of Life
Teaching Assistant

Bios 443 – Animal Physiological Systems
Guest lecturer
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SCIENTIFIC TECHNIQUES

Behavioural Studies
Histology
Immunohistochemistry
Electron Microscopy
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PROFESSIONAL AFFILIATIONS

Society for Neuroscience 2009-2010

PUBLICATIONS

Peer Reviewed Scientific Articles

LaVinka PC and Park TJ (2011) The insensitivity of the trigeminal nerve to acid in African naked mole-rats: a behavioural and physiological study (in submission)

LaVinka PC, Brand A, Landau V, Wirtshafter D, and Park TJ (2009) Extreme tolerance to ammonia fumes in African naked mole-rats: animals that naturally lack neuropeptides from trigeminal chemosensory nerve fibers J Comp Physiol A 195:419–427

Published Meeting Abstracts

LaVinka PC, Landau V, Ragozzino M, Brand A, Park TJ (2004) Naked Mole-Rats Can Detect Presence Of Ammonia, But They Do Not Perceive It As Painful. Midwest Neurobiology Meeting, Chicago.

LaVinka PC, Brand A, Landau V, Comer C, Ragozzino M, Smith TD,Schofield BR, Bhatnagar KP and Park TJ (2004). Distribution and Function of the nasal epithelium in naked mole rats - Animals that naturally lack substance P in epithelial nociceptors. Society for Neuroscience abstracts.

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Lavinka PC, Grauslyte K, Amoroso VG, Park TJ (2009). Tolerance to high CO2 and acetic acid fumes in Naked Mole-Rats. Society for Neuroscience Abstracts Online.

Honors and Awards

Acknowledged in Nature paper – Kim, EB et al.(2011) Genome sequencing reveals insights into physiology and longevity of the naked mole rat Nature 479:223-227

Featured in a pain documentary for NTV Channel Russia 2010

Graduate Student Council Travel Award 2009

LIN Minigrant Award 2007

Graduate Student Council Travel Award 2004

Undergraduates mentored in Bios 399 Independent Study

Victoria Landau 2003-2005
Jasmine Dowell 2005
Abby Peters 2006-2007
Roman Tulis 2006-2007
Kelly Coussee 2006-2007
Karolina Grauslyte 2008-2010
Diana Maniev 2009-2010
Catherine Barone 2009-2010