ACCELERATING DEVELOPMENT OF TREATMENTS FOR TRIGEMINAL NEURALGIA USING INTRANASAL DELIVERY AND A NOVEL BEHAVIORAL SCREENING DEVICE FOR RATS.

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Chapter 2

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Chapter 3

DEDICATION

This dissertation is dedicated to my mentor Dr. Leah Hanson and my family Marie,

Clara and baby #2.

ABSTRACT

Orofacial pain disorders are challenging to treat because of poor targeting to the trigeminal, orofacial and brain structures involved. Intranasal administration targets these structures while minimizing systemic exposure and side effects. Lidocaine was intranasally administered to rats, and the trigeminal nerve and trigeminally-innervated structures (teeth, temporomandibular joint (TMJ), and masseter muscle) accumulated up to 20-fold higher tissue concentrations of lidocaine than the brain and blood as measured by ELISA. Intranasally administered infrared dye reached the trigeminal nerve and brain within 10 minutes. Intranasal administration distributed the dye to the trigeminal nerve through three regions with high drug concentrations in the nasal cavity: the middle concha, the maxillary sinus and the choana. These findings indicate that intranasal delivery has the potential to target and treat trigeminal pain disorders with fewer side effects. Such disorders include: tooth pain, TMJ disorder, trigeminal neuralgia, headache, and even certain brain diseases. Although intranasal delivery may aid in targeting approved medications, better novel treatments need to be developed for orofacial pain disorders, specifically trigeminal neuralgia.

A new testing device (TrigeminAir) was developed to assist in the screening of trigeminal neuralgia models and treatments. The TrigeminAir device assesses orofacial sensitivity in rats over time by measuring the sip rate of sweetened condensed milk in the presence of a normally non-painful air puff stimulus on the whisker pad. This device detected, 5 to 12 hours after infraorbital nerve injection, that carrageenan (2% or 4%)

reduced sip rate by inflaming the whisker pad. During the same time period, intranasal 10% lidocaine (8mg) reversed this reduced sip rate by anesthetizing the inflamed whisker pad. In the first four hours after 4% carrageenan injection, intranasal lidocaine (4% and 10%) decreased neuronal activity in nerves innervating the maxillary teeth, palate, whisker pad and snout. Sensory and motor activity was unaffected after intranasal lidocaine administration, except for anesthesia of the whiskers affecting balance. In conclusion, these results suggest the TrigeminAir device is an efficient and reliable screening method of preclinical models and treatments for orofacial pain and demonstrate that intranasal lidocaine effectively reduces tactile allodynia in rodents. Intranasal delivery and the TrigeminAir device could accelerate the development of new orofacial pain treatments by improving targeting and screening of treatments.

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1   Introduction

Orofacial pain is relayed through the trigeminal nerve and includes dental pain,

headache/migraine, temporomandibular pain, and rare neuropathic pain disorders. The

trigeminal nerve consists of three main branches that innervate the upper, middle and

lower portion of the mouth and face in both rats and humans (see Figure 1 and 2). The

peripheral trigeminal nerve synapses with trigeminal nuclei, and brain centers can

become sensitized in chronic orofacial pain conditions and be challenging to treat.

Current treatments for orofacial pain use either oral administration or local needle

injection delivery. This thesis investigates the use of intranasal delivery as an

alternative delivery method that better targets orofacial and brain structures while

minimizing side effects.

1.1   Oral Delivery poorly targets the brain and orofacial structures

In general, oral delivery of therapeutics to the trigeminal nerve and brain is restricted by

the blood-brain barrier, resulting in poor targeting to these tissues. In addition, to reach

these targets, oral drugs need to circumvent the acidity (pH 2) of the stomach, the

basicity (pH 9) of the intestine, and numerous gut bacteria and enzymes. All of these

barriers limit the delivery of potential therapeutics for orofacial pain such as proteins,

oligonucleotides, viral vectors, and stem cells. For the limited scope of drugs that can

cross the digestive lining into the blood, the drug immediately undergoes 1st pass

metabolism by the liver, typically eliminating half the drug. Additionally, the drug

enters the circulatory system and is diluted throughout the body with a small fraction

reaching orofacial structures and crossing the blood-brain barrier to target brain pain

centers such as the trigeminal nucleus. The blood-brain barrier blocks delivery of drug

into the brain by tight junctions not usually found on blood vessels outside of the central

nervous system and by proteins that pump drug out of the brain.[1-3] Although oral

delivery is a simple method of administration, it poorly targets the trigeminal nerve and

brain pain centers.

1.2   Local injection delivery is painful and manifests anxiety

Targeted local anesthesia used to treat dental pain is typically delivered by injection, but

is painful and sometimes insufficient as an anesthesia option. Injection is painful due to

the initial penetration of the needle and then due to the change in pressure and pH in the

tissues.[4-7] Injection into the maxillary palate to anesthetize the lingual dental nerve

fibers is especially painful because the soft tissues are firmly attached to the periosteum

of the bone with very little free space for the drug to flow.[4] It is ironic that the method

used to reduce pain for dental procedures still remains one of the most feared and

painful parts of visiting the dentist.[8, 9] In some cases, local anesthesia may not

sufficiently anesthetize the area due to infection or allow the patient to treat post-

operative pain in the targeted area at home.[4, 10] Additional issues limiting the utility of

local anesthesia delivery with injection include: challenges for patients who cannot

remain still such as anxious pediatric patients and patients with Parkinson's disease,[11, 12]

the inherent risk of a dentist or staff receiving a needle stick that can carry hepatitis or

AIDS from a patient,[13-15] and exacerbation of dental pain due to needle and dental

anxiety formed by past experiences and perceptions.[16, 17]   Local anesthesia provides targeted delivery but is painful, requires skill, and compliant patients.

1.3   Intranasal delivery targets orofacial and brain structures while not being painful.

Intranasal delivery provides an alternative to local delivery and oral delivery by painlessly targeting orofacial and brain structures while minimizing side effects. Intranasal delivery bypasses the blood-brain barrier, and has been shown to treat brain disorders both in preclinical models and in humans. [2, 18-22] Intranasal administration has been shown to deliver a wide variety of therapeutics to the brain, such as hydrophilic drugs, oligonucleotides, proteins, viral vectors, and stem cells.[23-27] Intranasal delivery into the brain occurs rapidly (in minutes) via several routes including the olfactory and trigeminal nerve pathways.[28, 29] During intranasal administration, drug deposited onto the nerve endings of the trigeminal nerve, which innervates the nasal mucosa, can target the brainstem, TMJ, maxillary teeth and palate.[30] In addition to targeted delivery, one of the most important advantages of intranasal administration of therapeutics is its non-invasiveness. Intranasal delivery is painless, doesn't elicit needle-associated anxiety, doesn't pose a risk of needle stick, and can be self-administered by patients to treat post-operative pain.[10, 31] Intranasal delivery of anesthetic has been used to numb anterior maxillary teeth, and more importantly, the anterior maxillary palate.[32, 33] Intranasal anesthetic has also been used to treat trigeminal neuropathic pain and migraine.[34, 35] Intranasal delivery of fentanyl can provide comparable analgesia to IV morphine for pre- and post-operative pain.[36-38] Intranasal delivery provides a promising alternative to

oral and local delivery that is painless, doesn't elicit anxiety, and rapidly targets orofacial and brain structures while minimizing side effects.

1.4 Current novel nasal delivery devices target the olfactory epithelium to target the brain.

A variety of nasal delivery devices are in development to target the brain by depositing drug on the upper nasal cavity in order to utilize the olfactory nerve pathway to the brain. Factors that improve nasal delivery and aid in reaching the upper nasal passage are angle, inspiration, particle size and head position.[39, 40] Pulsatile flow has also been shown to improve delivery.[41-44] The ViaNase device, which vortexes the fluid or powder, has been shown to improve deposition onto the nasal passages.[45, 46] The OptiNose device uses a blowing reflex to close the soft palate to confine drug to the nasal cavity, and has been shown to dramatically improve deposition onto the nasal passages.[47-50] Since these devices target the forebrain via the olfactory nerve pathway, it is unclear if these devices improve delivery to the trigeminal nerve, TMJ and hindbrain by depositing more widespread drug across the nasal passages.

1.5 Administration to the maxillary sinus may improve targeting to orofacial structures.

Based upon the results discussed in chapter 2, a possible future direction of intranasal delivery for targeting of the trigeminal nerve is administration to the maxillary sinus. The maxillary sinuses are two air filled golf ball sized cavities located behind the cheeks. The maxillary sinus is lined by a thin absorbent pseudoepithelium surrounded

by trigeminal nerve branches, specifically the anterior, middle and posterior alveolar nerves that innervate the teeth (Figure 1).[51] The roots of the posterior maxillary teeth and palatine nerves form the base of the sinus. The maxillary sinus is a poorly vascularized structure that has the potential to restrict diffusion of drug and focus delivery into the surrounding trigeminal nerve branches and the structures they innervate: the palate, upper lip, and all the maxillary teeth. Furthermore, maxillary sinus delivery of an anesthetic could restrict anesthesia just to one side by administering to just one sinus. This would prove helpful and provide better targeting for neuropathic pain disorders, which usually occur only on one side of the face.[52-54] Moreover, the infraorbital nerve, which innervates the lip and is most typically involved in trigeminal neuralgia (discussed later), runs along the superior wall of the maxillary sinus.[34] The opening of the maxillary sinus is located on the superior wall located closer to the midline, and fluid in the sinus is naturally swept out of the opening into the nasal cavity. This cilia propelled movement could provide a natural mechanism to slowly deposit drug (9 mm/minute) into the upper-to-middle nasal cavity if the sinus is filled.[51] Drug deposited into the upper nasal cavity has been shown to transport to the brain via the olfactory nerve pathway following intranasal delivery.[23, 24] Utilizing this mechanism, it is possible that filling the maxillary sinus could deliver drug along both the olfactory nerve pathway and the trigeminal nerve pathway. Partially filling the sinus would theoretically deliver only along the trigeminal nerve pathway, providing the flexibility of targeting trigeminally innervated structures and brain regions. In rats, the opening between the maxillary sinus and nasal cavity is proportionally larger compared to

humans allowing intranasal administration to deposit drug into the maxillary sinus delivering drug into the trigeminal nerve and maxillary teeth, as discussed in chapter 2. Due to the unique anatomical properties of the maxillary sinus in humans, depositing drug into the maxillary sinus may improve targeting of the maxillary teeth and trigeminal nerve, and slowly deposit drug into the nasal cavity to target the brain for prolonged periods of time.

1.6   Therapeutic development for trigeminal neuralgia is needed.

Although intranasal devices and methods of delivery may improve targeting of current orofacial pain treatments, new therapeutics need to be developed to effectively treat these disorders, specifically trigeminal neuralgia. Trigeminal neuralgia is a rare disorder (1.5 per 10,000 prevalence) characterized by intense pain triggered by light touch or vibration as subtle as a puff of air followed by a facial spasm or tic.[54, 55] Trigeminal neuralgia most commonly occurs unilaterally along the maxillary trigeminal nerve branch, which innervates the maxillary sinus and nasal cavity.[54]

The mechanism of trigeminal neuralgia is challenging to determine clinically because of spontaneous remission and low prevalence. The development of an animal model could facilitate research to understand the pain mechanisms involved.[52, 56-59] There is debate whether the pain originates from compression or irritation of the peripheral trigeminal ganglion or from seizures within the CNS, mainly the trigeminal nucleus which spans the cervical spinal cord, brainstem, pons and midbrain (Figure 1).[52, 56-59] The newer peripheral mechanism theory describes that the trigeminal ganglion is the pain center

with irritation caused by microvascular pressure of the superior cerebellar artery on the trigeminal nerve causing trigeminal neuralgia.[56, 57] The older central mechanism theory was established with the discovery that an anti-epileptic had some efficacy treating the CNS, most likely the trigeminal nucleus as a pain center.[60]

Trigeminal neuralgia was so excruciating that it was called "the suicide disease" before current treatments like carbamazepine.[52] Oral carbamazepine is an anti-epileptic that manages pain for ~75% of trigeminal neuralgia patients by modulating sodium channels, and is the most studied and inexpensive trigeminal neuralgia medication.[61] Despite dramatic pain reduction oral carbamazepine has limiting serious systemic side effects including hepatic toxicity and induction, hematopoietic suppression, electrolyte imbalances, multiple drug interactions and cognitive impairment.[52] Other anticonvulsants and muscle relaxants are used to treat trigeminal neuralgia, but are typically not as effective, also have side effects, and cost 4-20 fold more.[54] As our aging population lives longer and trigeminal neuralgia typically onsets in ones 40s and 50s, managing this debilitating pain disorder is becoming challenging.[11, 54, 62, 63] Clearly new treatments, and treatments that better target the affected areas need to be developed.

1.7 Intranasal formulations of current medications may improve treatment of trigeminal neuralgia.

It is possible that intranasally administered carbamazepine could reduce the systemic side effects of oral carbamazepine, while targeting the trigeminal nerve and brainstem. For example in the treatment of migraine and dental analgesia, intranasal delivery has

been shown to have a similar therapeutic effect with administration of 1/5 the dose compared to oral formulations, thus limiting systemic drug exposure. [64-76] Chapter 2 describes how drug is transported following intranasal delivery to the trigeminal nerve and brainstem, which are the main pain targets for trigeminal neuralgia. Administering carbamazepine directly into the maxillary sinus on the affected side could further isolate and target the delivery to the trigeminal nerve on just that side of the face. Improved delivery methods of current medications would be a rapid way of improving treatment for trigeminal neuralgia, but clinical trials of new therapeutics can be slow and costly.

1.8 More efficient preclinical screening methods could accelerate drug development.

One factor that could accelerate the development of new therapeutics is better screening methods in preclinical models of orofacial pain. Current methods include the von Frey test, formalin test, and thermal operant behavior device, which have various advantages and disadvantages discussed in Chapter 3 (Table 1). [77-81] We developed the TrigeminAir device, which can efficiently and objectively screen numerous pre-clinical orofacial pain models and treatments over time by using a stimulus specific to trigeminal neuralgia, an air puff. The TrigeminAir device is an apparatus that uses a computer to measure the sip rate of sweetened condensed milk in the presence of an air puff on the rat's whisker pad over time. The TrigeminAir device was used to detect the efficacy of intranasal lidocaine, which is effective in humans at blocking trigeminal neuralgia mediated pain,[34] in the presence of inflammatory orofacial pain in the whisker pad over a 12-hour period.

1.9 Conclusions

Orofacial pain disorders are challenging to treat because current administration methods poorly target the trigeminal nerve and connected structures. Development of new treatments is challenging because current preclinical screening techniques for new treatments are inefficient. The major conclusions in the following chapters are:

1. **Intranasal delivery targets** - Following intranasal administration of lidocaine to rats, the trigeminal nerve and trigeminally-innervated structures (teeth, temporomandibular joint (TMJ), and masseter muscle) were found to have up to 20-fold higher tissue concentrations of lidocaine than the brain and blood as measured by ELISA. These results suggest that intranasal delivery could be used to target and treat disorders such as tooth pain, TMJ disorders, trigeminal neuralgia, and headache, with fewer side effects.

2. **Intranasal delivery is rapid** - Infrared dye reached the brain within 10 minutes after intranasal administration.

3. **A new target for intranasal administration was identified** - Intranasal administration distributed dye to the trigeminal nerve through three regions with high drug concentrations in the nasal cavity: the middle concha, the maxillary sinus and the choana.

4. **TrigeminAir detects facial sensitivity in a rat model** - The TrigeminAir device was developed to assess orofacial sensitivity in rats over time by measuring the sip rate of sweetened condensed milk in the presence of a normally non-painful air puff stimulus on the whisker pad. Infraorbitally

injected carrageenan (2% or 4%) reduced sip rate by inflaming the whisker pad 5 to 12 hours after infraorbital nerve injection.

5. **Intranasal lidocaine reduced facial sensitivity** - During the same time period (5 to 12 hours after infraorbital nerve injection), intranasal 10% lidocaine (8 mg) reversed this reduced sip rate by anesthetizing the inflamed whisker pad.

6. **Intranasal lidocaine reduced neuronal excitation** - In the first four hours after 4% carrageenan injection, intranasal lidocaine (4% and 10%) decreased c-fos activity in neurons innervating the maxillary teeth, palate, whisker pad and snout.

7. **Intranasal lidocaine had few side effects** - Sensory and motor activity was unaffected after intranasal lidocaine administration, except for anesthesia of the whiskers affecting balance.

8. **The TrigeminAir device is an efficient and reliable method of screening** preclinical models and treatments for orofacial pain that has the potential to accelerate therapeutic development.

1.10 Figures:

A)

Infraorbital
nerve (V2)

Maxillary
sinus

Anterior, middle, posterior
alveolar nerves (V2)

B)

Internal nasal
branches (V1)

Trigeminal
ganglion

Olfactory nerves (I)
and
cribriform plate

Pterygopalatine
ganglion (V2)

Illustrations modified from Netter FH. Atlas of Human Anatomy. 2006
and Hendelman WL. Atlas of Functional Neuroanatomy. 2006

C)

1° somatic
sensory
cortex

VPM

Trigeminal ganglion

Spinal trigeminal tract

Trigeminothalamic
tract

Lower Midbrain

Mid Pons

Upper Medulla

Mid Medulla

Lower Medulla

Figure 1: Human Trigeminal nerve and CNS pathway

The maxillary branch (V2) of the trigeminal nerve innervates the nasal epithelium and surrounds the maxillary sinus that relay to the trigeminal ganglion, trigeminal nuclei in the brainstem, ventral posterior medial nucleus, and the primary somatic sensory cortex. A) The infraorbital nerve, alveolar nerves (anterior superior, middle superior and posterior superior), and pterygopalatine ganglion surround the maxillary sinus and innervate the upper lip, cheek, maxillary teeth, and palate. B) The branches of the pterygopalatine ganglion innervate the nasal mucosa. In addition, the internal nasal branches (V1) and olfactory nerves innervate the olfactory epithelium and pass through the cribriform plate directly into the brain. C) The afferent branches of the trigeminal ganglion enter the brainstem at the level of the pons and synapse the trigeminal nucleus via the spinal trigeminal tract. Secondary neurons relay information to the ventral posterior medial nucleus (VPM) via the trigeminothalamic tract and tertiary neurons relay to the primary somatic sensory cortex.
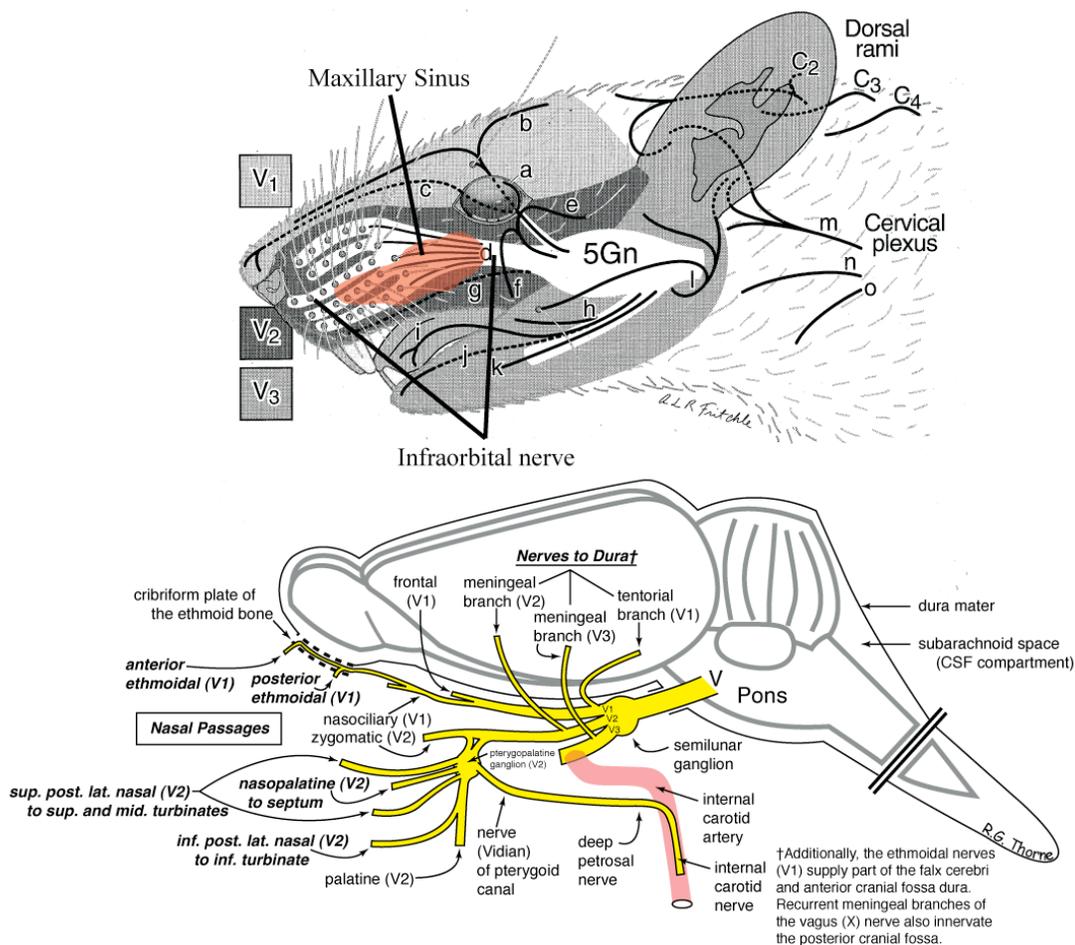
Figure 2: Rat trigeminal nerve anatomy
Similar to humans the maxillary branch of the trigeminal nerve in the rat surrounds the maxillary sinus and innervates the nasal mucosa. In rats the maxillary sinus opening is proportionally larger compared to humans. A) The proportionally larger infraorbital nerve passes through the lateral wall of the maxillary sinus. The alveolar nerves (not shown) also surround the maxillary sinus. B) The branches from the pterygopalatine ganglion, ethmoidal nerves (analogous to the internal nasal nerves in humans), and olfactory nerves similarly innervate the nasal mucosa. The ethmoidal nerves and olfactory nerves pass through the cribriform plate bypassing the blood-brain barrier. Illustrations modified from Thorne RG. The nasal pathway for drug delivery to the central nervous system : studies with protein tracers and therapeutics. 2002

2 Trigeminal pathways deliver a low molecular weight drug from the nose to the brain and orofacial structures.

Intranasal delivery has been shown to non-invasively deliver drugs from the nose to the brain in minutes along the olfactory and trigeminal nerve pathways, bypassing the blood-brain barrier. However, no one has investigated whether nasally applied drugs target orofacial structures, despite high concentrations observed in the trigeminal nerve innervating these tissues. Following intranasal administration of lidocaine to rats, trigeminally-innervated structures (teeth, temporomandibular joint (TMJ), and masseter muscle) were found to have up to 20-fold higher tissue concentrations of lidocaine than the brain and blood as measured by ELISA. This concentration difference could allow intranasally administered therapeutics to treat disorders of orofacial structures (i.e. teeth, TMJ, and masseter muscle) without causing unwanted side effects in the brain and the rest of the body. In this study, an intranasally administered infrared dye reached the brain within 10 minutes. Distribution of dye is consistent with dye entering the trigeminal nerve after intranasal administration through three regions with high drug concentrations in the nasal cavity: the middle concha, the maxillary sinus, and the choana. In humans the trigeminal nerve passes through the maxillary sinus to innervate the maxillary teeth. Delivering lidocaine intranasally may provide an effective anesthetic technique for a non-invasive maxillary nerve block. Intranasal delivery could

be used to target vaccinations and treat disorders with fewer side effects such as tooth pain, TMJ disorder, trigeminal neuralgia, headache, and brain diseases.

Abbreviations: PBS, phosphate buffered saline; CNS, central nervous system; IN, intranasal; TMD, temporomandibular disorder; DTI, drug-targeting index; CSF, cerebrospinal fluid

2.1   Introduction

Intranasal delivery provides a non-invasive method of bypassing the blood-brain barrier to rapidly deliver therapeutic agents to the brain, spinal cord, lymphatics and to the vessel walls of the cerebrovasculature for treating CNS disorders such as Alzheimer's disease, brain tumors, and stroke.[2, 18-22] This novel delivery method allows drugs, therapeutic proteins, polynucleotides, and viral vectors that do not normally cross the blood-brain barrier to be delivered to the central nervous system.[23, 25] Additionally, intranasal targeting of drugs to the CNS avoids first pass elimination by the liver allowing a lower therapeutic drug dose and fewer systemic side effects. Delivery from the nose to the central nervous system occurs within minutes along both the olfactory and trigeminal nerves.[23, 24, 82] Delivery occurs by an extracellular route and does not require that the drugs bind to any receptor or undergo axonal transport. Intranasal delivery has been reported to effectively bypass the blood-brain barrier and treat neurologic disorders in mice, rats, primates, and humans.[19, 24, 82-84] In humans, intranasal

delivery has been documented to transport neuropeptides to the CSF.[85] Furthermore, intranasal insulin has been shown to improve memory in healthy adults, obese men, and Alzheimer's patients.[83, 86-92] Although considerable intranasal studies have focused on nose to brain delivery, no studies have investigated if drug delivery occurs to orofacial structures innervated by the trigeminal nerve despite evidence that high drug concentrations are observed in the trigeminal nerve.

Evidence suggests that after intranasal administration, the olfactory and trigeminal nerves receive high concentrations of drug from the nasal cavity and transport it to the brain and other connected structures.[23] Intranasally administered drug can reach the trigeminal nerves and perineural space from the absorbent respiratory and olfactory pseudoepithelium because they are innervated by the trigeminal nerve. Chemuturi & Donovan 2007 demonstrated that intranasal delivery rapidly fluxes dopamine (a small molecule) across the nasal pseudoepithelium where large trigeminal nerve branches pass.[30] Additionally, the trigeminal nerve covers and travels through the maxillary sinus, which is connected to the nasal cavity and is also lined by a thin pseudoepithelium. It is not known where drug enters the trigeminal nerve from the nasal cavity, but Thorne et al 2004 reported an increase in IGF-I concentrations in the three major branches of the trigeminal nerve and in the brain stem where the trigeminal nerve enters following intranasal IGF-I administration. This suggests that intranasal delivery uses the trigeminal nerve pathway as a conduit to transport drug to the brainstem beginning at the entry through the pons and then through the rest of the

hindbrain.[93] A portion of the trigeminal nerve that passes through the cribriform plate may also contribute to delivery of drug from the nasal mucosa to the forebrain.[23] If the trigeminal nerve can transport drug to the forebrain and hindbrain, connected structures, then trigeminally innervated structures such as the teeth, masseter muscle, and TMJ may also receive drug from the trigeminal nerve. Even though drug is transported to the hindbrain, treatment of the trigeminal nerve without affecting the brain may be possible because a 20-fold higher drug concentration has been reported in the trigeminal nerve as compared to the brain.[23, 24] Understanding where drug enters the trigeminal nerve from the nasal cavity after intranasal administration would help target the trigeminal nerve for clinical use.

Intranasal drugs have been used to treat orofacial structures clinically, yet these studies did not describe the trigeminal nerve as a mechanism of drug transport.[34, 35, 64-76, 94, 95] Although local injections can effectively target orofacial structures, there are adverse effects including pain, dental anxiety, and the patient cannot self-administer medication. Although topical and gas anesthesia are helpful in decreasing pain before injection, intranasal delivery may provide a better approach by being rapid, painless, targeted, and patient administered. Intranasal migraine medications (sumatriptan, zolmitriptan) require 1/5 the dose of oral formulations with a more rapid onset and few side effects.[64-76] Furthermore, lidocaine, a drug not usually indicated for migraine or trigeminal neuralgia treatment, was found to be therapeutic when delivered intranasally.[34, 35, 94, 95] Finding specific regions in the nasal cavity where drug enters the trigeminal nerve could

help develop better devices in clinic to target the trigeminal nerve and connected structures.

In this study, intranasal administration of lidocaine resulted in rapid delivery to the brain, trigeminal nerve and orofacial structures while minimal drug was delivered to the blood as compared to intravenous delivery. The biodistribution of the dye showed high concentrations at three locations near the trigeminal nerve (the middle nasal concha, the maxillary sinus, and choana). Targeting these nasal sites could improve delivery to the brain, trigeminal nerve, or orofacial structures.

2.2 Materials and Methods

2.2.1 Experiment 1: Concentrations of lidocaine in rat tissues following intranasal and intravenous administration

2.2.2 Experimental design

Rats were administered the same lidocaine dose intranasally or intravenously. After approximately 30 minutes, rats were perfused, and a variety of brain, orofacial and body structures were dissected to determine lidocaine concentrations. Tissues were pulverized and supernatant fractions were analyzed using an ELISA, as described in detail below.

2.2.3   Animals

Adult male Sprague-Dawley rats (ordered 200-250g from Charles River Laboratories, Wilmington, MA, USA) were group housed under a 12-h light/dark cycle. Food and water was provided ad libitum. Animals were cared for and experiments were approved in accordance with institutional guidelines (Regions Hospital, HealthPartners Research Foundation Animal Care and Use Committee approved protocol 05-052) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). Lidocaine (8 mg) was either intranasally (n=6) or intravenously (n=6) administered to two groups, while another group (n=3) was given intranasal vehicle (phosphate buffered saline, PBS) to collect tissue for the ELISA standard curve and tissue to be spiked with known concentrations of lidocaine to detect the specificity and sensitivity of the assay.

2.2.4   Intranasal and intravenous delivery

Lidocaine HCl was purchased from Sigma Aldrich (cat# L5647-15G). Animals were anesthetized with pentobarbital sodium (Nembutal, 40 mg/kg I.P.; Abbott Laboratories, North Chicago, IL USA). Core body temperature was maintained at 37 ˚C using a rectal temperature probe and heating pad. A 20-gauge cannula was inserted into the descending aorta for blood access. For intranasal delivery, rats were laid on their back with a rolled 2"x2" gauze under the neck to maintain a horizontal head position. During intranasal delivery, a cotton swab wrapped in paraffin was used to occlude one nostril

while an 8 μL drop of 10% lidocaine dissolved in phosphate buffered saline (PBS) was

placed onto the opposite nostril and naturally sniffed in by the rat. Five drops were

administered in each nostril, alternating every two minutes over 18 minutes. A total of

8 mg of lidocaine was given in 80 μL. For intravenous delivery, 80 μL of 10%

lidocaine was combined with 0.5 mL of saline, and 1/10 doses were injected through the

descending aorta cannula every two minutes for 18 minutes. Every 5 minutes, a 0.25

mL sample of blood was removed from the descending aorta. At 10 & 20 minutes 0.5

mL of saline was administered through the descending aorta cannula to replace lost

blood volume.

2.2.5   Tissue collection

After 25 minutes from delivery onset, 120 mL of cold saline was perfused through the

descending aorta cannula of the anesthetized rat at 15 mL/min using a syringe pump.

After perfusion and death, the head was removed using a guillotine, and tissues were

dissected on ice. Blood samples were centrifuged at 16,000 G for 30 minutes and

supernatant removed. The deep cervical lymph nodes were removed as described in

Thorne et al. 2004. The skin dorsal to the brain cavity was incised at the midline. The

whole brain was removed starting caudally and progressing rostrally using a hemostat to

remove the skull. The whole brain was dissected in the following order in a petri dish:

cerebellum, brainstem, midbrain, diencephalon, and cortex. The olfactory bulb was

removed last to minimize contamination. After removing the whole brain, the

trigeminal ganglion was removed from the base of the skull without opening the nasal

cavity. The lacrimal gland, eye, and masseter muscle were dissected by first removing the overlying skin. The temporomandibular disc was dissected from the mandibular condyle and glenoid fossa. All teeth were extracted with nerves inside pulp. The tongue and tissue from the maxillary sinus were dissected. The nasal epithelium was removed from the cribriform plate after splitting the skull along the midline. The spinal cord was removed whole and dissected into cervical, thoracic, and lumbar segments without removing the dura. Each liver lobe and the kidney cortex and medulla were dissected. Urine was collected from the bladder using a 1 mL needle syringe. All tissues were placed in tubes, weighed, flash frozen in liquid nitrogen, and stored at -70 °C until tissue preparation for ELISA.

2.2.6   *Tissue preparation for ELISA*

Tissue was diluted 1:5 (tissue weight (g) : homogenization buffer (ml)). Homogenization buffer consisted of Tris HCl (100 mM), sodium chloride (400 mM), 2% bovine serum albumin, and 0.05% sodium azide. Two dozen 1.0 mm glass and zirconium microbeads were added to each tube of tissue and shaken vigorously for three 5-minute increments at ~10 Hz using a motorized microbead shaker. After pulverizing, samples were sonicated until the solution was homogenous. After sonication, the samples were centrifuged at 16,000 G for 30 minutes. Supernatant was removed and flash frozen in liquid nitrogen and stored at -70 °C.

2.2.7   Lidocaine ELISA

Supernatant was allowed to thaw to room temperature and diluted 1:500 in EIA buffer

supplied in the lidocaine ELISA kit (Neogen Corporation, Lexington KY). An 8 µL

sample was used in the lidocaine ELISA kit well, otherwise the kit instructions were

followed. Briefly, enzyme conjugate was diluted 1:180 in EIA buffer, and 180 µL was

incubated with the 8 µL sample in each ELISA well (duplicates of each tissue) for 45

minutes. Using a multi-channel pipettor, the wells were washed with buffer, and 150

µL of substrate was incubated for 1.5 hours (all supplied in the kit). At 1.5 hours

samples were read using a plate reader (Molecular Devices) at 450 nm after adding

50µL of 1.0 N HCl, which doubles the color intensity read by the plate reader. Samples

were compared against a standard curve of lidocaine spiked brain supernatant from

vehicle treated animals processed the same way as experimental samples.

Supernatant from vehicle treated animals were spiked with known concentrations of

lidocaine to validate the specificity and sensitivity of the assay. A variety of tissues

showed the same concentration measured by the assay as the amount injected into the

tissues. The assay was very specific to lidocaine as tissues without lidocaine were

below the standard curve's sensitivity and had the same optical density as wells filled

with distilled water. The lowest detectable concentration was ~10 nM. The standard

curve consistently had an R squared value of ≥ 0.99.

2.2.8   Statistical analysis

Data was analyzed and graphed using Prism statistical software (version 5.0; GraphPad, La Jolla, CA). The lidocaine concentrations were quantified using standard curves. An unpaired t-test assuming equal variance was used to compare each tissue between the intranasal and IV groups. P values were determined and represented as <0.05 (*), <0.01 (**), and <0.001(***). Targeting of lidocaine for intranasal or intravenous delivery was calculated by dividing the tissue concentration by the blood's lidocaine concentration at 25 minutes within the same delivery method. A drug-targeting index (DTI) was used to measure improved CNS targeting through intranasal delivery as compared to intravenous delivery and was calculated by determining the ratio of intranasal to intravenous targeting of lidocaine.

2.2.9   Experiment 2: In vivo imaging of intranasally administered IRdye 800 and biodistribution at 30 minutes.

2.2.10 Experimental design

IRdye 800 was intranasally administered to rats and imaged with an infrared imaging system for 30 minutes prior to euthanasia and tissue collection. IRdye 800 was used because it can be imaged in vivo and is easily visible by the naked eye, enabling tracking of the dye (as opposed to lidocaine which is clear in solution). In addition, IRdye 800 fluoresces at 800 nm, a unique wavelength where tissue is relatively transparent because of minimal absorption by blood and water. IRdye 800 had the closest molecular weight to lidocaine of any of the available infrared dyes.

2.2.11 Animals

Adult male Sprague-Dawley rats (n=3, 200-250g; Charles River Laboratories,

Wilmington, MA, USA) were housed under a 12-h light/dark cycle and were cared for

as described in Experiment 1.

2.2.12 IRdye 800 and Odyssey infrared imaging system

IRdye 800 (Li-Cor Bioscience, Lincoln, NE) was used for in vivo imaging after

intranasal administration. IRdye 800 conjugation sites were neutralized by dilution in

PBS (pH 8.5) for greater than two hours. IRdye 800 was imaged using a Li-Cor

Odyssey infrared imaging system (Lincoln, NE) equipped for in vivo imaging. Rats

were shaved completely with an electric razor to limit the hair absorbing infrared light,

which can interfere with imaging. The infrared background was scanned prior to

delivery.

2.2.13 Intranasal administration and in vivo imaging

Rats were anesthetized as described in Experiment 1. Drops (8 µL) of 1 mM IRdye 800

in saline were given every 2 minutes for 18 minutes alternating nostrils using the same

intranasal technique as experiment 1 for a total of 80 µL administered. The head and

neck were scanned between each 2-minute delivery interval. During in vivo imaging,

the Odyssey scanner resolution was 337 μm^2/pixel with a scanning depth of 3 ± 0.5 mm

for both the 700 nm and 800 nm filters. After 25 minutes the rat was removed from the

imaging device and transcardially perfused with 60 mL cold saline followed by 360 mL

4% paraformaldehyde at a rate of 15 mL/min using a syringe pump.

2.2.14 Dissection and subsequent imaging

Following perfusion, the brain was removed, sectioned in 1 mm coronal brain slices

using a brain matrix from the olfactory bulb to the upper cervical spinal cord, and

imaged. The spinal cord was dissected and imaged whole. The head with brain

removed was cut mid-sagittally so the cross-section could be imaged. The trigeminal

nerve and respiratory epithelium were removed, and the head was then re-scanned. The

trigeminal maxillary nerve branch was dissected from the whisker pad to the brainstem

nerve root. The following tissues were also removed and imaged: kidneys, heart, lungs,

digestive tract, spleen, liver, urine (1 mL), epididymis, and testes. For all tissues, the

scanning resolution after dissection was 21 μm^2/pixel for both 700 nm and 800 nm

filters. The scanning depth for thin tissues such as coronal brain slices, spinal cord, skin

and aponeurosis was 1 ± 0.5 mm. The rest of the tissues were imaged at 4 ± 0.5 mm

scanning depth. Alongside experimental tissues, a standard curve was imaged by

scanning known concentrations of IRdye 800 in glass tubes. The known concentrations

detected by the Odyssey scanner ranged from 10 nM to 100 μM, otherwise IRdye seen

by the naked eye was defined as >100 μM.

2.3 Results

2.3.1 *Experiment 1: Tissue concentrations of lidocaine following intranasal and intravenous administration.*

2.3.2 *Blood bioavailability of lidocaine was lower following intranasal delivery as compared to intravenous delivery.*

Although the nose is well vascularized, blood levels of lidocaine were significantly lower following intranasal administration as compared to intravenous administration across the entire 25-minute period (Figure 1). The area under the curve of the lidocaine concentration in the blood was also significantly lower (p=0.0002) following intranasal application (173 µM*minutes) than following intravenous injection (3,916 µM*minutes). Blood concentration of lidocaine peaked at 5 minutes after intravenous administration vs. 10 minutes after intranasal administration; then both steadily decreased. At 25 minutes, the final sample point, lidocaine blood concentrations were significantly lower (p=0.0168) following intranasal (7.9 µM) vs. intravenous administration (21 µM). The kidney was the only systemic organ where intranasal and intravenous administration was not significantly different (p=0.6525). Intravenous administration resulted in significantly higher lidocaine concentrations in the urine (p=0.0012) and liver (p=0.0015). Intranasal administration resulted in significantly higher concentrations at the drug administration sites such as the nasal epithelium (p=<0.0001). All of the lidocaine concentration data for each group can be found in Table 1.

2.3.3 Brain structures had higher lidocaine concentrations following intranasal delivery as compared to intravenous delivery.

In spite of the much higher blood lidocaine levels observed with intravenous administration, intranasal delivery of lidocaine to the brain was significantly higher for all structures except the lumbar spinal cord (Figure 2). Following intranasal administration, the olfactory bulb, connected to the olfactory epithelium via olfactory sensory neurons, received the highest concentration of lidocaine (266 µM), and concentrations decreased in a rostral-to-caudal direction from the cortex (33 µM) to the diencephalon (16 µM) then increased from the midbrain (23 µM) to the brainstem (45 µM), where the trigeminal nerve root and ganglion enters running along the base of the skull (147 µM). The lidocaine concentration decreased in a dorsal direction from the brainstem to the cerebellum (35 µM). Progressing caudally the concentration decreased from the brainstem to the lower cervical spinal cord (24 µM) to the thoracic spinal cord (21 µM) to the lumbar spinal cord (19 µM). In contrast, intravenous administration resulted in lower and fairly similar concentrations throughout the central nervous system structures (7-10 µM), except in the diencephalon (2.0 µM; p= 0.0004).

2.3.4 Orofacial structures had higher lidocaine tissue concentrations than brain structures following intranasal delivery.

Intranasal administration delivered significantly more lidocaine to all orofacial structures compared to brain structures, except the facial structures innervated by the ophthalmic branch of the trigeminal nerve (lacrimal gland, eye, and skin on the head).

Following intranasal administration, the maxillary sinus (3,508 µM) received the

highest lidocaine concentration besides the nasal epithelium (4,549 µM) where the drug

is directly deposited. The trigeminal nerve, which passes through the maxillary sinus

and the nasal epithelium, had a concentration of 147 µM before it enters the brainstem

(at the base of the skull). The trigeminal nerve segment was cut at the base of the skull

because dissecting through the nasal epithelium in an unfixed animal would have

contaminated the trigeminal nerve. In the IRdye 800 study discussed later, the

trigeminal nerve was dissected more rostrally to the teeth and nasal cavity where the

concentration was dramatically higher (~100 µM) compared to near the brainstem (~10

µM). The trigeminal nerve concentration near the maxillary teeth is ~1470 µM. This

higher concentration in maxillary teeth nerves compared to the rostral part of the

trigeminal nerve is due to the close proximity to the maxillary sinus (3,508 µM) and

nasal epithelium (4,549 µM). Of the maxillary teeth, incisors (803 µM) received the

highest concentration of lidocaine followed by the maxillary molars (476 µM). The

maxillary incisors are in closer proximity than the maxillary molars to the middle

concha, where the drug is directly deposited. The mandibular teeth are innervated by

the trigeminal nerve (147 µM) and close to the mandibular molar branch that innervates

the tongue (651 µM). Of the mandibular teeth, the molars (199 µM) received the

highest lidocaine concentration of the mandibular trigeminal nerve branch followed by

the mandibular incisors (148 µM), which is located further from the tongue (651 µM)

and the maxillary nerve branch (147 µM). The mandibular teeth lidocaine

concentrations were not significantly different from that of the trigeminal ganglion,

while the maxillary teeth had significantly higher concentrations ($p<0.05$) than the trigeminal ganglion suggesting maxillary teeth are receiving lidocaine from some other source. The intranasal IRdye experiment suggests the maxillary sinus is the main source of drug to the maxillary teeth. The teeth had the highest drug targeting indices (145 to 701) of all the tissues collected. Intranasal administration delivered less lidocaine to facial structures as compared to oral structures. The temporomandibular joint (72 µM) has a higher density of trigeminal nerve fibers than the masseter muscle (15 µM). The lacrimal gland was not significantly higher in lidocaine concentration following intranasal delivery (58 µM) as compared to intravenous administration (43 µM).

2.3.5   *Oral structures had the highest drug-targeting index, followed by brain structures and facial structures.*

The drug-targeting index (DTI) was highest among the maxillary sinus (856), teeth (maxillary incisors 701; maxillary molars 413; mandibular incisors 145; mandibular molars 270) and tongue (213). Facial structures had a lower DTI; these include temporomandibular joint (27), masseter muscle (8.2), lacrimal gland (3.6), eye (3.4), and skin on the head (2.7). All the central nervous system structures had a drug targeting index (DTI) greater than 1. The highest DTI central nervous system structures were the olfactory bulb (84), diencephalon (22), and brainstem (14). The diencephalon had a significantly lower concentration ($p=0.0004$) compared to other brain structures during intravenous delivery. The DTI of the remaining central nervous system

structures are cerebellum (11), lower cervical spinal cord (9.0), cortex (8.9), midbrain (8.4), thoracic spinal cord (6.8), and lumbar spinal cord (3.8).

2.3.6   Experiment 2: In vivo imaging of intranasally administered IRdye 800 and biodistribution at 30 minutes.

2.3.7   IRdye 800 was visualized in the brain at 10 minutes following intranasal administration.

Prior to intranasal treatment with IRdye 800, rat tissue is almost transparent at 800 nm (Figure 3a). The neck and body were less transparent compared to the head. The body was visualized using the 700 nm filter and appears red in images (Figure 3b-e). IRdye 800 can be seen within the nasal cavity as green or yellow. Yellow results from the overlap of the red 700 nm filter with the green 800 nm filter. At 0 minutes immediately after the first intranasal drop, IRdye 800 was visualized well in contrast to the transparent rat tissue (Figure 3b). At 5 minutes the IRdye 800 extends into the more caudal nasal cavity (Figure 3b). Within 10 minutes dye appears at the beginning of the olfactory bulb (Figure 3c). At 15 minutes, IRdye 800 is imaged in the rostral portion of the frontal lobe (Figure 3d). Not all of the dye in the brain imaged in figure 2 is seen in figure 3 because the Odyssey infrared imaging system cannot detect the deep ventral structures of the brain.

2.3.8   Trigeminal nerve and nasal cavity had high concentrations of IRdye 800 at the

entry of the choana, the middle nasal concha and the maxillary sinus.

Dye was visible in three locations of a mid-sagittal section of the head: the middle nasal

concha, the maxillary sinus, and choana (Figure 4). High concentrations of IRdye 800

(>100 µM) were also seen at the same locations where the trigeminal nerve passes next

to the middle nasal concha, maxillary sinus, and choana (Figure 4). The nerve under the

middle nasal concha (>100 µM) innervates the maxillary incisors (~100 µM) and also

passes through the maxillary sinus (>100 µM). Most of the nerves passing through the

maxillary sinus (>100µM) innervate the whisker pad (~100 µM). The maxillary sinus

(>100 µM) then connects with the nerves that pass near the choana (>100 µM) which

connect to the maxillary molar nerves (~100 µM). The trigeminal nerve then passes

through the cerebrospinal fluid eventually to synapse at the brainstem (~10 µM). All

these values are on the same order of magnitude as the lidocaine data collected using

ELISA.

2.3.9   Intranasal administration delivered high concentrations of IRdye 800 to the

brain, particularly cerebrospinal-fluid-contacting ventral brain structures.

Coronal brain sections had an IRdye 800 concentration range from 100 nM to >10 µM

(Figure 2 inset), as determined by comparison to a scanned standard curve of known

dye concentrations (data not shown). Generally, ventral brain structures, near

cerebrospinal fluid (CSF) were higher in IRdye 800 concentration compared to more

dorsal non-CSF contacting structures. IRdye 800 distributed to the entire olfactory bulb,

ventral and midline portions of the anterior olfactory nucleus, hypothalamus, medial and ventral portions of the cortex, ventral portion of the pons, and entry of trigeminal nerve roots. High concentrations of IRdye 800 concentrated in the trigeminal nuclei located on the lateral sides of the caudal brainstem and rostral cervical spinal cord.

2.3.10 Structures in the rest of the body received low concentrations of IRdye 800 compared to the brain and trigeminal nerve following intranasal administration. Blood contained an IRdye 800 concentration of 10 nM-100 nM. Remaining body structures, including the gastrointestinal tract, pancreas, liver, gallbladder, spleen, heart, spinal cord, kidneys, urine, epididymis and testis, had dye concentrations less than 10 nM (data not shown).

2.4 Discussion

These experiments demonstrate that intranasal delivery can target therapeutics like lidocaine to orofacial structures even more than to brain structures. This likely occurs because the trigeminal nerve acts as a conduit to transport drug from the nasal cavity to the orofacial structures. Similar to other intranasally administered molecules, low molecular weight drugs like intranasal lidocaine (234 Da) and IRdye 800 (962 Da) target the olfactory bulb, ventrally located brain structures, and the brainstem surrounding the trigeminal nerve root relative to the blood and other organs. Furthermore, this study identified three novel results. 1) Following intranasal

administration, the therapeutic agent enters the trigeminal nerve and trigeminal neural pathway at three points from the nasal cavity: choana, middle nasal concha, and maxillary sinus. 2) The trigeminal neural pathway acts as a conduit to transport drug not only to the brain but also in the opposite direction to other connected structures such as the teeth and temporomandibular joint. 3) Previous experiments demonstrated intranasal administration delivers drug to the brain within 10 minutes, but this is the first experiment to *image* this phenomenon, demonstrating this transport is a rapid process. These results may aid clinicians in targeting therapeutics to the trigeminal neural pathway by administering intranasally.

Intranasal administration to an anesthetized rat concentrates drug at three major locations in the nasal cavity, which subsequently results in rapid transport across the nasal epithelium into the trigeminal neural pathway. Our study found that a small molecule concentrates (>100 μM of IRdye at 30 minutes) at certain permeable anatomical locations within the nasal cavity following intranasal delivery, particularly the entry of the choana, the middle nasal concha and the maxillary sinus.[96] Furthermore, the high concentrations within the underlying trigeminal nerve were at these exact three locations, suggesting the three high concentration regions in the nasal cavity almost exclusively penetrate drug to the trigeminal nerve. This seems plausible since a small molecule (similar in size and properties to lidocaine i.e. dopamine) has been shown to rapidly flux across the respiratory and olfactory pseudoepithelium at 2-5 $\mu g/cm^2/min$.[30,]

[97] Once drug enters the trigeminal neural pathway, it appears to travel to the trigeminal nerve's connected structures.

These results suggest that the trigeminal nerve is a bidirectional conduit utilized by intranasal delivery to transport therapeutic agents to connected structures: the brain and orofacial structures. Previous studies have demonstrated that drug is transported via the trigeminal nerve in a rostral-to-caudal direction to the brain,[23, 24, 82] but this is the first study to demonstrate trigeminal drug distribution in the caudal-to-rostral direction to the maxillary teeth. Based on IRdye 800 distribution data, the maxillary teeth, infraorbital nerve, temporomandibular joint, and masseter muscle receive therapeutic via the trigeminal nerve from the choana, middle nasal concha, and maxillary sinus (see Figure 4). The maxillary incisor, a more rostral structure, showed dwindling IRdye 800 concentration emanating from the middle nasal concha and maxillary sinus, suggesting the therapeutic in the more rostral maxillary incisor originated from the more caudal middle nasal concha and maxillary sinus. The source of IRdye 800 to the maxillary molars was the choana, although there was contribution from the trigeminal nerve originating from the maxillary sinus and middle nasal concha. The infraorbital nerve had IRdye 800 concentration emanating mostly from the maxillary sinus due to the infraorbital nerve's large surface area exposed to the maxillary sinus. The IRdye 800 also appeared to transport in a rostral-to-caudal direction to the brainstem followed by a caudal-to-rostral direction to reach the temporomandibular joint and masseter muscle. All these conclusions were also supported based on the dramatically higher lidocaine

tissue concentrations following intranasal delivery as compared to intravenous delivery. Thus, the trigeminal nerve, a large bundle of nerves, can be used to rapidly and non-invasively transport therapeutics to all trigeminally connected structures. One future direction of this research could include further exploration of bidirectional transport along the trigeminal nerve by injecting lidocaine into the gums or the pulp of teeth and measuring delivery to connected structures such as other teeth, the temporomandibular joint or the brainstem.

In conclusion, following intranasal administration, drug not only travels from the nasal cavity to the brain via the trigeminal neural pathway but also in the opposite direction to orofacial structures. Targeting the trigeminal neural pathway is an effective method of targeting its connected orofacial and brain structures. Intranasal delivery to the trigeminal nerve and connected orofacial structures may provide a more effective and targeted method for treating postoperative dental pain/anxiety, trigeminal neuralgia,

2.5   Tables and Figures:
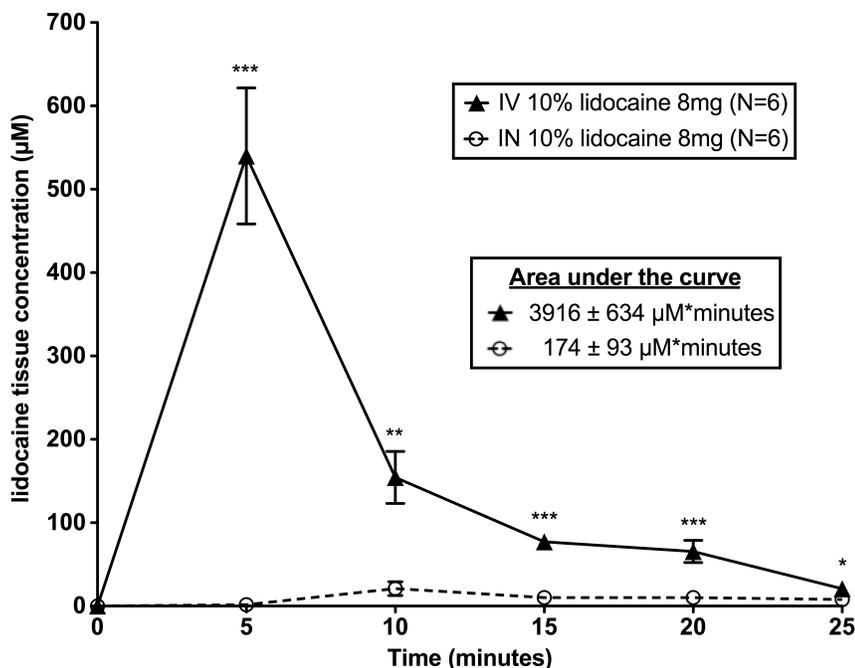
Figure 1. Intranasal administration of lidocaine leads to less drug delivery to the blood as compared to intravenous administration. Following intranasal or intravenous delivery of 8 mg of 10% lidocaine, lidocaine concentrations in the blood were measured every 5 minutes. For intranasal delivery the area under the curve of lidocaine concentration in the blood over the 25 minute period was 174±93 µM*seconds, and intravenous delivery had an area under the curve of 3916±634 µM*seconds. Intranasal delivery had significantly less lidocaine from 0-25 minutes compared to IV delivery ($p<0.001$). The points and error bars represent the mean ± SEM (N=6). P values are represented as follows: <0.05 (*), <0.01 (**), and <0.001(***).
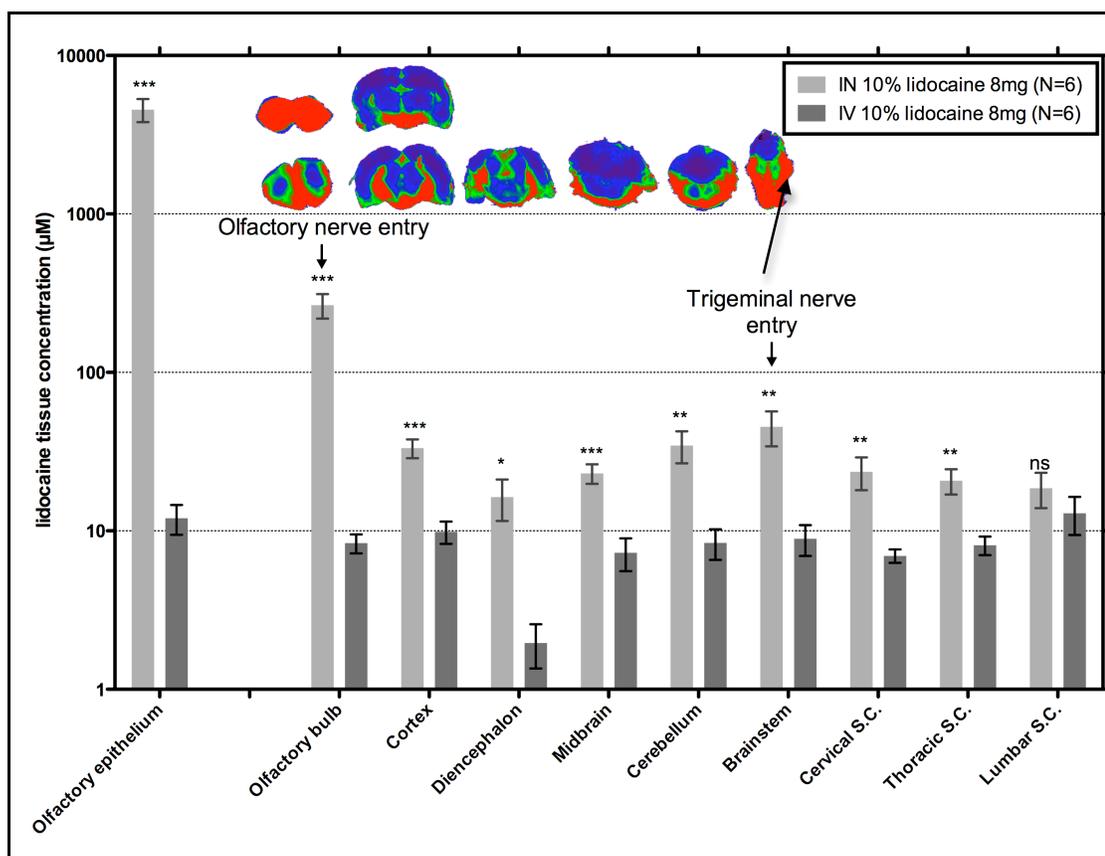
Figure 2. Brain structures had higher lidocaine tissue concentrations following intranasal delivery as compared to intravenous delivery, and accumulated around the olfactory nerves, trigeminal nerves, and cerebrospinal fluid. The bars are grouped by tissue comparing delivery method (intranasal or intravenous) of 8 mg of a 10% lidocaine solution with the error bars as SEM (N=6). P values are represented as follows: <0.05 (*), <0.01 (**), and <0.001 (***). The coronal brain sections in the inset illustrate the distribution within these brain structures where high concentrations are visualized in regions of the brain in contact with CSF, olfactory nerve, and trigeminal nerve.
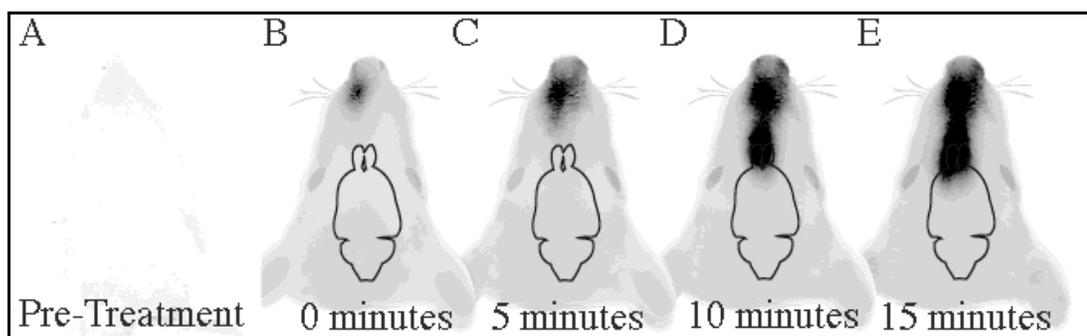
Figure 3. Distribution time course of intranasal delivery to the brain. At 800 nm an anesthetized rat is relatively transparent using an Odyssey infrared imaging system (A). At 0 minutes after the first 1mM drop was delivered it was clearly seen at an 800 nm wavelength (B). At 5 minutes the IRdye can be clearly seen in the nasal cavity (C). At 10 minutes IRdye 800 enters the olfactory bulb (D). At 15 minutes IRdye 800 enters the cortex (E). There were no significant changes in IRdye 800 distribution between 15, 20 and 25 minutes. Every 2 minutes an 8μL drop was administered.
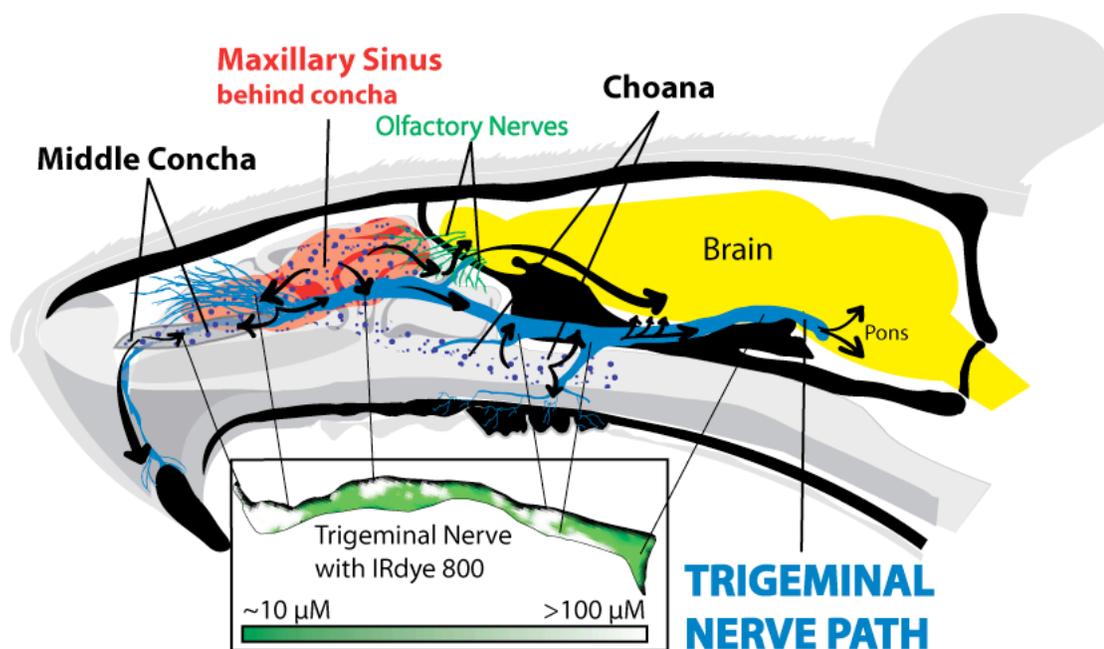
Figure 4. Intranasal administration delivers IRdye 800 to the trigeminal nerve and maxillary teeth via the middle concha, maxillary sinus, and choana. Intranasal delivery deposits IRdye 800 in three locations: middle concha, maxillary sinus and choana. After intranasal administration, IRdye 800 (purple dots) passes under the middle nasal concha and into the maxillary sinus (in red). Upon contact with the middle nasal concha and maxillary sinus, the underlying incisal nerve and multi-branched infraorbital nerve absorb >100 µM IRdye. After entering these structures the dye is transported in both directions along the trigeminal nerve. The remaining IRdye 800 is deposited in the choana where it is distributed to the maxillary molar and septal nerve branches. As IRdye is transported to the brainstem, IRdye 800 concentration plummets as it is deposited into the base of the skull and CSF. The cribriform plate also had a high concentration of IRdye 800. The trigeminal nerve (see inset) has high IRdye 800

concentrations in four locations: 1) the maxillary incisal nerve as it passes through the middle concha, 2) the infraorbital nerve as it passes through the maxillary sinus, 3) the septal branch and 4) maxillary molar branch as they passes through the choana. The maxillary teeth and trigeminal nerve receive IRdye based on its proximity from these trigeminally connected structures.

tissue	[Lidocaine] mean ± SEM (μM) IN	IV	significance[b]	lidocaine targeting: ratio of tissue concn (μM)/blood concn at 25 min (μM), mean ± SEM IN	IV	drug targeting index: ratio IN/IV, mean
blood						
blood 5 min	1.6 ± 0.7	540 ± 82	***			
blood 10 min	21 ± 8.3	154 ± 31	**			
blood 15 min	10 ± 4.6	77 ± 8.0	***			
blood 20 min	10 ± 3.2	65 ± 13	***			
blood 25 min	7.9 ± 1.8	21 ± 3.3	*			
total AUC[c] (0−25 min) μM·min	173 ± 93	3916 ± 634	***			
peripheral tissues						
deep cervical lymph nodes	30 ± 5.9	13 ± 1.6	*	3.8 ± 3.2	0.6 ± 0.5	6.2
nasal epithelium	4549 ± 756	12 ± 2.6	***	577 ± 409	0.6 ± 0.8	1003
urine	48 ± 13	1142 ± 318	**	6.1 ± 6.9	55 ± 97	0.1
kidney	80 ± 7.6	88 ± 16	ns	10 ± 4.1	4.2 ± 5.0	2.4
liver	44 ± 5.0	87 ± 11	**	5.6 ± 2.7	4.1 ± 3.2	1.3
brain and spinal cord tissue						
olfactory bulb	266 ± 47	8.4 ± 1.1	***	34 ± 25	0.4 ± 0.3	84
cortex	33 ± 4.5	9.9 ± 1.6	***	4.2 ± 2.4	0.5 ± 0.5	8.9
diencephalon	16 ± 4.8	2.0 ± 0.6	**	2.1 ± 2.6	0.1 ± 0.2	22
midbrain	23 ± 3.2	7.3 ± 1.7	***	2.9 ± 1.8	0.3 ± 0.5	8.4
cerebellum	35 ± 7.9	8.4 ± 1.8	**	4.4 ± 4.3	0.4 ± 0.6	11
brainstem and upper SC	45 ± 11	8.9 ± 2.0	**	5.8 ± 6.1	0.4 ± 0.6	14
lower cervical SC	24 ± 5.5	7.0 ± 0.7	**	3.0 ± 3.0	0.3 ± 0.2	9.0
thoracic SC	21 ± 3.8	8.1 ± 1.1	**	2.6 ± 2.0	0.4 ± 0.3	6.8
lumbar SC	19 ± 4.7	13 ± 3.5	ns	2.4 ± 2.5	0.6 ± 1.1	3.8
trigeminal ganglion[d]	147 ± 44	13 ± 1.5	**	19 ± 24	0.6 ± 0.5	29
oral structures with trigeminal innervations						
maxillary sinus	3508 ± 685	11 ± 2.4	***	445 ± 371	0.5 ± 0.7	856
maxillary incisor: SA nerve (V2)	803 ± 253	3.0 ± 0.5	**	102 ± 137	0.1 ± 0.2	701
maxillary molar: SA nerve (V2)	476 ± 116	3.1 ± 0.4	***	60 ± 63	0.1 ± 0.1	413
mandibular incisor: IA nerve (V3)	148 ± 31	2.7 ± 0.6	***	19 ± 17	0.1 ± 0.2	145
mandibular molar: IA nerve (V3)	199 ± 43	1.9 ± 0.8	***	25 ± 24	0.1 ± 0.2	270
tongue (V3)	651 ± 35	8.1 ± 1.6	***	83 ± 19	0.4 ± 0.5	213
facial structures with trigeminal innervations						
temporomandibular joint (V3)	72 ± 27	7.1 ± 1.0	***	9.1 ± 15	0.3 ± 0.3	27
masseter muscle (V3)	15 ± 2.7	4.8 ± 0.8	*	1.9 ± 1.4	0.2 ± 0.2	8.2
lacrimal gland (VII)	58 ± 14	43 ± 4.4	ns	7.4 ± 7.6	2.0 ± 1.4	3.6
skin and aponeurosis on head (V1)	3.6 ± 1.1	3.6 ± 0.5	ns	0.5 ± 0.6	0.2 ± 0.2	2.7
eye (V1 and CN II)	8.3 ± 1.5	6.5 ± 0.7	ns	1.1 ± 0.8	0.3 ± 0.2	3.4

[a] The table shows the mean and SEM ($N = 6$) of tissue concentrations following intranasal and intravenous administration of 8 mg of 10% lidocaine. The p value for each tissue is an unpaired t-test comparison between intranasal and IV delivery (<0.05 (*), <0.01 (**), and <0.001 (***). [b] Significance = <0.05 (*), <0.01 (**), <0.001 (***). [c] Area under the curve. [d] The trigeminal nerve was dissected at the base of the skull. See Figure 4 for concentration distribution in the trigeminal nerve.

Table 1. Lidocaine tissue concentrations following intranasal or intravenous administration. The table shows the mean and SEM (N=6) of tissue concentrations following intranasal and intravenous administration. The p values for each tissue is an unpaired t-test comparison between intranasal and IV delivery (<0.05 (*), <0.01 (**), & <0.001 (***).

3 A novel behavioral device uses an air puff stimulus to screen the efficacy of intranasal lidocaine in a rat model of orofacial pain.

Improved preclinical behavioral screening methods are necessary to accelerate development of new strategies for the treatment of trigeminal neuralgia, a rare yet painful disorder. Current therapeutics possess self-limiting side effects, including hepatic toxicity. Intranasal delivery bypasses first pass elimination of the liver while targeting the trigeminal nerve and connected orofacial structures. The purpose of this study was to test a newly developed automated behavioral device to measure the effectiveness of an intranasal anesthetic, lidocaine, in treating inflammatory orofacial pain. The TrigeminAir device assessed orofacial sensitivity in rats over time by measuring the sip rate of sweetened condensed milk in the presence of a normally non-painful air puff stimulus on the whisker pad. Five to 12 hours after infraorbital nerve injection, 2% or 4% carrageenan reduced sip rate by inflaming the whisker pad.[98, 99] During the same time intranasal 10% lidocaine (8 mg) reversed this reduced sip rate by anesthetizing the inflamed whisker pad. During the first four hours after 4% carrageenan injection, 4% and 10% intranasal lidocaine decreased c-fos activity in neurons innervating the maxillary teeth, palate, whisker pad and snout. Sensory and motor activity was unaffected after intranasal lidocaine administration, except for anesthesia of the whiskers affecting balance. In conclusion, these results suggest the

TrigeminAir device is an efficient and reliable screen of preclinical models and treatments for orofacial pain and demonstrate that intranasal lidocaine effectively reduces tactile allodynia in rodents.

3.1 Introduction

Trigeminal neuralgia (TN) is a rare pain disorder characterized by intense, lancinating pain when a small area of the face is stimulated by light touch or vibration, such as wind [100]. TN occurs in ~4.5 out of 100,000 people, most often with age of onset from the middle to late in life [101-105]. Carbamazepine is an anti-epileptic that is effective in 75% of TN patients, yet over time has reduced effectiveness and worsening side effects [61]. Other therapeutics used to treat TN include different anti-epileptics, muscle relaxants, and calcium channel blockers, but these orally administered treatments have various systemic side effects including hepatic toxicity, hematopoietic suppression, electrolyte imbalance, multiple drug interactions, and cognitive impairment [52]. Clearly there is a need for the development of new therapeutics with fewer side effects.

Intranasal drug administration has been shown to minimize systemic side effects, while targeting the trigeminal nerve and orofacial structures. Intranasal administration avoids first pass elimination by the liver, decreasing the overall dose necessary [2, 23, 24]. A wide range of therapeutics including polynucleotides, proteins, viral vectors, and stem cells have been administered nasally with improved targeting of central nervous system (CNS) structures compared to oral administration [23-27]. Moreover, the trigeminal nerve

and connected orofacial structures are targeted to a greater degree compared to CNS structures, following intranasal administration [23, 29]. Common intranasal therapeutics used clinically, such as lidocaine, tetracaine, and ketorolac, have effectively anesthetized tissues including the lip, cheek, maxillary anterior palate and teeth, to treat TN, migraine, and dental pain [10, 32-34, 95]. Even further targeting has been reported with intranasal formulation including vasoconstrictors, which limit absorption into the blood [28, 32]. Intranasal administration of currently approved TN therapeutics may improve orofacial targeting resulting in lower doses, fewer side effects, and fewer drug metabolites. Optimizing an intranasal formulation for treatment of TN will require preclinical screening and there are many limitations to the current screening methods.

Current preclinical orofacial pain screening methods are often inefficient, subjective, cannot be measured repeatedly, or do not use a stimulus relevant to TN (see Table 1). For example, von Frey filaments, thin flexible rods that exert a constant amount of pressure on the whisker pad, are often used to measure orofacial pain in rodents, yet reliable test results requires extensive investigator training [77, 78]. Video recording and counting the scratch response after injecting formalin into the whisker pad is a newer less subjective behavior test, yet requires extensive staff time, cannot be repeatedly measured on the same rat, and limits the pain model to formalin injection [79]. Operant behavior devices that measure sip rate in the presence of an aversive stimuli such as extreme temperatures or bite force are efficient, objective, and can repeatedly measure rodents in a cross-over design [80, 81, 106, 107]. However, existing operant devices do not

deliver a stimulus relevant to TN and other orofacial pain disorders, such as a mechanical stimulus like an air puff.

The TrigeminAir device was developed to measure an operant behavior and uses an air puff as a stimulus to mechanically activate facial skin receptors. In this study, the TrigeminAir device was used to determine the efficacy of intranasal lidocaine in a rat model of inflammatory orofacial pain. Intranasal lidocaine was used in this study because it had shown efficacy in a recent clinical study [34]. Other behavior tests in this study were used to detect potential side effects of treatment. The TrigeminAir device detected an increase in facial sensitivity in the inflammatory orofacial pain model by measuring sip rate, which was reversed by intranasal lidocaine during the 5-12 hour period after injection with few side effects. A neuronal activity marker, c-fos protein, measured in the trigeminal brainstem demonstrated reversal of increased activation with intranasal lidocaine during the first four hours. The TrigeminAir device efficiently detected the effects of intranasal delivery of lidocaine consistent with clinical results.

3.2   Materials and Methods

3.2.1   Animals

Adult male Sprague-Dawley rats (300-400 g from Harlan Laboratories, Indianapolis, IN) were group housed under a 12-hour light/dark cycle. A total of 87 rats were used in these experiments. Thirty-six rats were used to measure sipping behavior following injection of the thickening agent carrageenan (CGN) into the whisker pad. Six of these

rats received no air puff in the first 24 hours. Thirty-six rats were nasally administered lidocaine after CGN injection; sipping behavior was measured in 18 of these animals and c-fos positive cells in the brainstem labeled in the remaining rats. General behavior including sensory and motor tests was assessed in 15 rats to examine possible side effects of intranasal lidocaine administration. The protocols for handling and experimentation in animals were approved and in agreement with institutional guidelines (Regions Hospital, HealthPartners Research Foundation Animal Care and Use Committee approved protocol 06-037).

3.2.2   Orofacial pain model created with infraorbital nerve injection of carrageenan
For CGN injection, a 22-gauge needle was inserted ~1cm at the most rostral vibrissae to deposit the CGN at the infraorbital nerve branching point. The infraorbital nerve is a direct extension of the maxillary division of the trigeminal nerve (cranial nerve V). Lambda-CGN (Fluka, Allentown, PA) was diluted in distilled water and shaken vigorously in a bead beater (Biospec, Bartlesville, OK) for 15 minutes and spun for 5 seconds to eliminate bubbles in the viscous carrageenan solution. For the sipping behavior experiment where only CGN was administered, rats received 0.1 ml of 0%, 2%, or 4% CGN (0 mg, 2 mg, 4 mg respectively). For all intranasal lidocaine experiments, 4% CGN (4mg) was administered to all groups. No CGN was administered for the general behavior experiments examining possible side effects of IN lidocaine.

For the c-fos experiment, four hours after CGN injection the rats were anesthetized with pentobarbital sodium (Nembutal, 40 mg/kg I.P.; Abbott Laboratories, North Chicago, IL) and perfused with 60 mL of cold saline and 360 mL of 4% formalin. For the remaining experiments, the rats were anesthetized and euthanized with an overdose of pentobarbital sodium (100 mg/kg dosage) at the end of the experiment.

3.2.3 *Intranasal lidocaine treatment*

Intranasal administration was performed in groups of six rats under gas anesthesia using an isoflurane vaporizer (Matrix Medical Inc., Orchard Park, NY). Initial anesthesia was attained using an induction chamber and a vaporizer setting of 3%. Anesthesia was maintained using nose cones and a vaporizer setting of 2.5%. For intranasal lidocaine treatment, rats were laid on their backs with rolled 2"x2" gauze under the neck to maintain a flat horizontal head position to decrease drainage of lidocaine into the throat. Lidocaine HCl was obtained from Sigma Aldrich (St. Louis, MO). During intranasal delivery, a cotton swab wrapped in paraffin was used to occlude one nostril while an 8 µL drop of 0%, 4% or 10% lidocaine dissolved in phosphate buffered saline (PBS) was placed onto the opposite nostril and naturally inhaled by the rat. Five 8 µL drops were administered in each nostril, alternating every two minutes over 18 minutes for a total volume of 80 µL and total lidocaine dose of 0 mg (0%), 3.2 mg (4%), and 8 mg (10%).

3.2.4   C-fos immunohistochemistry

The whole brain was dissected and removed along with a portion of spinal cord

extending 2-3 vertebrae below the foramen magnum. The tissues were stored in 4%

formaldehyde overnight and sectioned the following day. The brainstem was cut into

two 2-mm blocks caudal to the obex. Tissue blocks were cut in cold PBS at 40μm

sections using a Vibratome 3000 sectioning system (St. Louis, MO) and collected in

PBS.

All procedures subsequent to incubation in primary antiserum were performed at room

temperature. Sections were incubated for 60 minutes on an orbital shaker in primary

diluent: 0.01M PBS (pH 7.4), 0.3% Triton x-100 and 5% normal donkey serum (Cat.

#S30, Chemicon, Temecula, CA). The sections were incubated overnight at 4°C on an

orbital shaker in primary rabbit antiserum against *c-fos* (1:15000, Ab-5, Cat. #PC05,

Oncogene Science, Manhasset, NY). Following incubation, sections were rinsed in

0.01M PBS for 20 minutes. Sections were then incubated in 2° donkey biotinylated

antibodies against rabbit IgG (Chemicon, Temecula, CA) diluted 1:300 in diluent for 90

minutes and then rinsed twice for 20 minutes in 0.01M PBS. Sections were incubated

sequentially in ABC (Avidin-Biotin Complex, PK-4000 series Vectastain kit, Vector,

Burlingame, CA) for 60 minutes, rinsed in 0.01M PBS twice for 15 minutes and

NiDAB solution (SK-4100, Vector, Burlingame, CA) for 2-4 minutes. Following

staining, sections were rinsed in water for 2 minutes and then placed in a solution of

cold 0.01 PBS until mounting. C-fos positive cells were counted manually under 200X

magnification for all sections. Cell counts were determined for each section for laminae I/II (a relay center for pain nerve fibers) and laminae III-V (a relay center for both touch and pain) for both the ipsilateral and contralateral sides and 75 total sections per rat were averaged from the obex to 4mm caudal to the obex.

3.2.5   TrigeminAir device and behavior testing

The TrigeminAir device (Fig. 1) consists of a 15"x15" cage with a hole in one of the walls that allowed the rat to sip sweetened condensed milk (SCM). The sipper was surrounded by a polished concrete head guide ensuring the 15-psi air blows directly onto the right whisker pad. Food pellets and water were provided ad libitum and weighed daily during periods of behavioral testing. Rats were weighed daily to confirm their weight was not decreasing by >10%.

For measuring the number of sips, the metal floor of the cage was connected to a conductance meter on the sipper to detect a change in resistance when the rat contacts the sipper. Six TrigeminAir devices were constructed, each containing a conductance meter that consisted of a 2.6 V DC power source with a 470-ohm resistor in series with a DATAQ lead, which were in parallel with the sipper and metal floor. The conductance meters from each cage relayed its signal to a DATAQ analog-to-digital converter (DI-148U, Akron, OH), which was processed by a computer running DATAQ software.

Voltage information for each DATAQ lead was ported into MATLAB (Mathworks Inc., Natick, MA) where a custom-made program counted the number of sips per hour. A sip was an event where the voltage dropped below the baseline (voltage level when no sipping was occurring) by 10% and then rose above it, corresponding to the initial sipper contact and subsequent sipper disengagement, respectively. The data analysis program acquired data at 30 Hz and, based on adaptive threshold settings for voltage levels corresponding to sipper contact and disengagement, calculated a time-averaged sipping frequency. The correlation between voltage changes and sipping counts were confirmed using an infrared camera relayed to a screen in a separate room.

Rats were trained to sip from the sweetened condensed milk through acclimation in the TrigeminAir device prior to CGN treatment. Rats were acclimated to the device for ~1 month and sipping was monitored continuously. After whiskerpad injection, facial sensitivity was monitored with the TrigeminAir device continuously for 1 week. Facial swelling after CGN injection lasted for only ~3 days so after one week the rats were re-injected with CGN for a total of four times in one month. Sip rate for each rat at each hour of the day was determined by averaging the same hour in the same rat over each of the four weeks.

3.2.6 General behavioral tests

The sticker test, grip strength test, open field test, and balance beam test were performed both before and four hours after isoflurane anesthesia and intranasal

lidocaine administration. Pre- and post-treatment differences were used for each rat and

each rat received every treatment in a randomized presentation in a cross-over design

(N=15). Lidocaine treatment dose for each rat changed after each week. The week

before treatment, rats were trained by performing each test twice. The sticker test

measured sensory motor ability and quantified the amount of time it takes for a rat to

remove a neon orange sticker from its left front paw. The sticker was applied for a

maximum of 60 seconds and the three removal times were averaged. The grip strength

test measured the amount of force on the forearm and hand before the rat released from

a metal grid connected to a force meter (Columbus Instruments, Columbus, OH)

connected to a computer. Grip strength was measured six times for each rat and

averaged. The open field test measured normal investigative behavior and quantified

the distance traveled by a rat inside a 3'x3' box over 5 minutes. A camera recorded the

movements of the rat and the path distance was measured by Ethovision software

(Leesburg, VA). The balance beam test measured the amount of time and score of a rat

balancing on a 1" diameter rod suspended 1' above a padded surface. The rats were

removed from the balance beam after 60 seconds. Balance for the rats were scored as

follows: 1) balances with steady posture on the top of the beam the entire 60 seconds, 2)

grasps the side of the beam, 3) hugs the beam and one limb falls, 4) two limbs fall or rat

spins around on the beam but stayed on the beam the entire 60 seconds, 5) attempts to

balance on the beam but falls after 40 seconds, 6) attempts to balance on the beam but

falls after 20 seconds, 7) falls off with no attempt to balance.

3.2.7 Statistical analysis

Data was analyzed and graphed using Prism statistical software (version 5.0; GraphPad

Software Inc., La Jolla, CA). For TrigeminAir experiments, a two-way ANOVA with

repeated measures assuming equal variances compared the following factors: 1)

treatment and 2) each four-hour block. Each four-hour block was calculated by

averaging four one-hour sip rate time points. P-values were determined by a Bonferroni

post-test, which compared replicate means of sip rate for each four-hour block. Each

four-hour block was repeatedly measured on each rat once a week for a month.

Differences in c-fos cell counts among lidocaine treatment groups were analyzed by

one-way ANOVA and Tukey post-test was used to calculate p-values and confidence

intervals. For the sticker, grip strength and open field tests, a one-way ANOVA with

repeated measures assuming equal variances was used to compare the three lidocaine

doses on the three different days each dose was administered to each rat. The Tukey

post-test was used to compare factors (i.e. subjects, dose, time to remove sticker,

average grip strength, and distance traveled in open field) and determine p-values and

confidence intervals. For the balance beam test, due to ceiling effects of time and score

being a categorical factor, a non-parametric one-way ANOVA with repeated measures

(Friedman test) was used to compare the lidocaine treatment groups. The Dunn post-

test (significance alpha = 0.05, 95% confidence interval) was used to compare both

factors. Change in medians and interquartile ranges were calculated. All statistical

tests used corrections for multiple comparisons.

3.3   Results

3.3.1   *After effects of infraorbital injection of carrageenan on TrigeminAir device behavior.*

After one month of training in the TrigeminAir device (Fig. 1), sip rate followed a bell-shaped curve over the 12-hour awake/lights-off period with minimal sipping during the 12-hour lights-on period (data not shown). After infraorbital vehicle injection (0% carrageenan control), sip rate followed a similar pattern (Fig. 2A) peaking at 1,843 sips in the first 12 hours and 1,982 sips during the next 12-hour awake period. Infraorbital injection of 2% or 4% carrageenan (CGN) resulted in a sip rate conforming to a similar bell-shaped curve, but peaking at a significantly lower sip rate in the first 12 hours [2%: 1,029 sips, $p<0.05$ (Fig. 2B); 4%: 656 sips, $p<0.001$ (Fig. 2C)]. In the absence of the air puff, carrageenan-treated rats sip rate was similar to vehicle-treated rats (first 12 hour peak: 1,873 sips; second 12 hour peak: 1,934 sips). During the second 12-hour awake period, peak sip rate was similar across carrageenan doses (0%: 1,982 sips; 2%: 1,648 sips; 4%: 2,166 sips). There were no statistically significant weight changes between the three groups.

The greatest decrease in sip rate occurred 5 to 12 hours after CGN injection. The total number of sips over the first 12-hour awake period after CGN injection was significantly different in a dose dependent manner (0%: 11,473 sips; 2%: 5,636 sips, $p<0.05$, 4%: 5,136 sips, $p<0.01$). During the first four hours after CGN injection, sip rate was not significantly different between the three CGN doses: 0% (2,876 sips), 2%

(2,088 sips), and 4% (1,776 sips). However, 2% and 4% CGN groups sipped

significantly less than the control group 5 to 8 hours after injection (2%: 45.1% less,

$p<0.05$; 4%: 44.6% less, $p<0.05$) and at 9 to 12 hours post-injection (2%: 47.7% less,

$p<0.05$; 4%: 39.2% less, $p<0.01$). The total number of sips was similar between all

three CGN doses in the second 12-hour lights off period (0%: 14,941 sips; 2%: 12,188

sips; 4%: 14,844 sips).

3.3.2   *Effects of intranasal lidocaine on behavior*

Following intranasal lidocaine administration and CGN injection, sipping over a 24-

hour period displayed a similar pattern compared to CGN treatment alone (Fig. 3A).

However, over the first 12 hours the 10% lidocaine group had a total of 1,927 more sips

($p<0.05$) than the 0% lidocaine control group (N=6). Similar to the CGN only

experiment, in the first four hours post-treatment there was no significant difference

between the 10% lidocaine (1,707 sips) and 0% lidocaine (1,901 sips) groups (Figure

3B). From the 5th to the 8th hour after CGN injection, the 10% lidocaine group sipped

400% more (894 vs. 210 sips, $p<0.05$) than the 0% lidocaine control group, and 971%

more during the 9th to the 12th hour (1,605 vs. 159 sips, $p<0.01$). There was no

significant difference in sipping after 12 hours.

3.3.3   *Effect of intranasal lidocaine on c-fos positive cells*

Four hours after intranasal lidocaine and carrageenan injection, rats (N=6) administered

either 4% or 10% lidocaine had fewer ($p<0.001$) c-fos positive cells compared to the

0% lidocaine control group on both the ipsilateral and contralateral sides of laminae I/II and III-V of brainstem and cervical spinal cord (Figure 4, Table 2). There were no significant differences between the effects of 4% and 10% lidocaine. It was qualitatively noted using somatotopic maps of the brainstem and cervical spinal cord from previous studies[108-110], that the number of c-fos positive cells decreased in areas of the brainstem and cervical spinal cord that typically innervate the whiskerpad, snout, palate, and maxillary teeth.

3.3.4 *Sensory and motor behavior following intranasal lidocaine.*

Intranasal lidocaine did not significantly alter performance in sticker test, forearm grip strength, or open field activity (Table 2). Pre-treatment performance in each test was similar between lidocaine treatment groups. In the sticker test, four hours after intranasal lidocaine treatment, the time to remove the sticker increased overall in all groups (p< 0.01), but there were no significant differences between the 0%, 4%, and 10% treatment groups. In the balance test, there were no significant differences in performance before treatment between the lidocaine groups, but a significant decrease in pre-to-post performance with the 10% treatment (p<0.0001). Rat's use their whiskers for balance and qualitatively, the most notable observation was that rats receiving lidocaine had little whisker movement and/or intermittent uncoordinated movement.

3.4   Discussion

Using a new behavioral assessment device these experiments demonstrate that intranasal lidocaine decreases inflammatory-induced orofacial pain-like behavior with few side effects. The TrigeminAir device detected orofacial pain by measuring sip rate of sweetened condensed milk in the presence of an air puff stimulus on the whisker pad. Orofacial pain was detected 5-12 hours after carrageenan injection into the whisker pad of rats. A similar inflammatory orofacial pain model has been assessed in a thermal operant device, which demonstrated increased orofacial sensitivity in the first 0.5 hr of sipping, but the effect over longer time periods was not reported[80, 81]. The TrigeminAir device did not detect a change in sipping during the first four hours after carrageenan injection, yet the number of c-fos positive cells in trigeminal subnucleus caudalis were increased, a response that was reversed by intranasal lidocaine administration, consistent with previous studies showing bilateral hyperalgesia[111, 112]. The TrigeminAir device has several advantages for the mass screening of new orofacial pain models and treatments since animals can be measured repeatedly in a cross-over design despite the initial acclimation period. The TrigeminAir device could accelerate the development of orofacial pain therapeutics through streamlined pre-clinical testing methods.

Intranasal administration of lidocaine targeted and anesthetized trigeminal nerves with few behavioral side effects suggesting limited involvement of other CNS structures. Intranasal administration of 10% lidocaine and other therapeutics to rats has previously been demonstrated to target the trigeminal nerve ($147\mu M$) and maxillary dental

structures (476-803µM) compared to brain structures (16-33µM excluding olfactory bulb)[23, 24, 29]. Low lidocaine concentrations in CNS brain structures are consistent with the low number of behavioral side effects in this study[29]. The most pronounced side effect was inactive and drooping whiskers affecting the rats' proprioception, which may have impacted performance on the balance beam test without actually impacting balance. Intranasal administration of 10% lidocaine has been shown to result in 35±7.9µM in the cerebellum, which may be enough to affect cerebellar function. However, behavior tests of other brain regions containing similar lidocaine concentrations (i.e. cortex 33±4.5µM; lower cervical spinal cord 24±5.5µM) showed no functional effects. If the cerebellum was affected, sticker test performance, which requires complex balance on the hindpaws while removing the sticker on the forepaws, should have been hampered following intranasal lidocaine administration, but it was not. It is possible that a lower dose of lidocaine, such as 4%, which both significantly decreased c-fos expression and did not have side effects, would demonstrate efficacy as measured by the TrigeminAir device.

The duration of pain, topography of facial pain, and side effects of intranasal anesthetics measured in this rat model using the TrigeminAir device were consistent with clinical studies. When 8% intranasal lidocaine was administered clinically to treat TN stimulation of the trigger zone, intranasal lidocaine blocked the orofacial pain for as long as 12 hours with the only side effects being unpleasant odor and taste [34]. Similarly, the present study demonstrated that 10% intranasal lidocaine blocked orofacial

sensitivity for at least 12 hours when combining the c-fos and TrigeminAir results, with few side effects. Intranasal lidocaine is delivered throughout the trigeminal system and could affect the muscles of mastication or autonomic function, but so far clinically this has not been identified as a side effect of intranasal anesthetics [32-34]. Intranasal anesthetics have also been used clinically for treatment of maxillary tooth pain with few side effects [32, 33]. Similarly, our study found intranasal lidocaine blocked brainstem c-fos expression in areas that innervate the teeth and palate. These results suggest the combination of the TrigeminAir device with c-fos measurement is an effective method to pre-clinically screen orofacial pain models and treatments.

The TrigeminAir device has several advantages in measuring pre-clinical orofacial pain compared to other behavioral tests (Table 1). The TrigeminAir device objectively measures orofacial sensitivity over time by an air puff stimulus on the whiskerpad (a specific TN stimuli). This technique allows comparison of different treatments on the same rat after acclimation and requires little staff time. The fact that carrageenan dose-dependently decreases sip rate, while numbing the whisker pad with intranasal lidocaine or removal of the air puff restores sipping provides evidence suggesting that the TrigeminAir device assesses facial sensitivity versus the air puff simply being annoying. The device did not detect a change in orofacial pain in the first four hours following injection or treatment, which may have been due to nausea or drowsiness induced by the isoflurane used to sedate the rats during intranasal administration and carrageenan injection. Sedation was more frequent (daily) and longer (30 minutes) after

intranasal administration plus CGN compared to carrageenan alone (administered once for a few minutes) resulting in greater sip rate across all groups. Rats require sedation for intranasal administration but high concentration nitrous (60-70%) as a replacement may minimize the transient side effects. Alternatively scaling down the device for mice, which do not require sedation during intranasal administration would enable testing of genetic knockouts of TN, migraine, and headache. The disadvantages of the TrigeminAir device are minimal compared to other alternative behavior testing methods including using von Frey filaments on the face, measuring the formalin scratch test, or even a comparable thermal or bite force operant behavior device [77-81, 106, 107].

The TrigeminAir device is an efficient pre-clinical screening method for orofacial pain and may aid in the development of nasally administered formulations to treat orofacial pain. Current treatments for orofacial pain disorders such as TN (i.e. carbamazepine, baclofen, phenytoin, clonazepam, gabapentin, and lamotrigine) were originally developed to treat other disorders such as seizures and later adopted to treat orofacial pain disorders. Nasally administering these therapeutics may minimize their side effects and improve their effectiveness, but testing each clinically would be inefficient and costly. The TrigeminAir device could efficiently screen these various intranasal treatments to determine the most effective intranasal treatment to be tested clinically. Combining targeted intranasal delivery and the rapid screening of the TrigeminAir device provides the methods needed to accelerate development of the next generation of orofacial pain treatments.

3.5   Tables and Figures:

		Orofacial pain test				
		TrigeminAir device	Thermal operant device	C-fos	Formalin scratch test	Von Frey test
Advantages	Simple testing procedure	+	+	+	+	-
	Objective	+	+	+	+	-
	Capable of mass screening	+	+	-	-	+
	Screen multiple models	+	+	-	-	+
	Efficient testing procedure	+	+	-	-	-
	Measures pain over time	+	+	-	-	+
	Trigeminal neuralgia stimulus	+	-	-	-	+
	Short acclamation period	-	-	+	+	-
	Measures pain while sleeping	-	-	+	-	-

Table 1 – Comparison of pre-clinical orofacial pain tests. Each orofacial pain test has advantages (+) and disadvantages (-). The TrigeminAir device and c-fos test have complementary advantages and disadvantages in testing trigeminal neuralgia and other orofacial pain disorders.
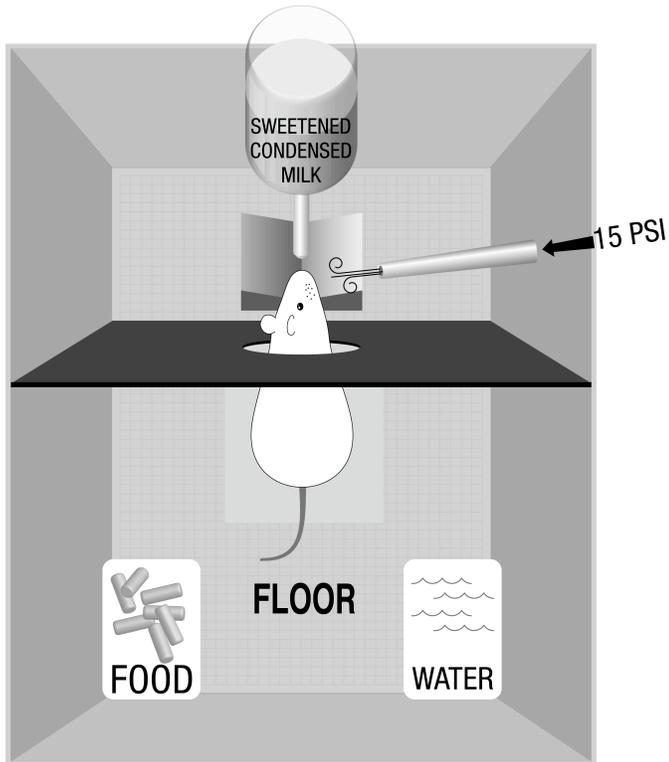
Figure 4 –TrigeminAir device. The TrigeminAir device was designed to test facial

sensitivity by measuring sip rate of sweetened condensed milk while blowing 15-psi air

onto the whisker pad. Sip rate was recorded via a change in conductance at the sipper,

which was relayed to a computer. Rats had ad libitum access to food pellets and water

during measurement periods.

A   0% carrageenan injection

B   2% carrageenan injection

C   4% carrageenan injection
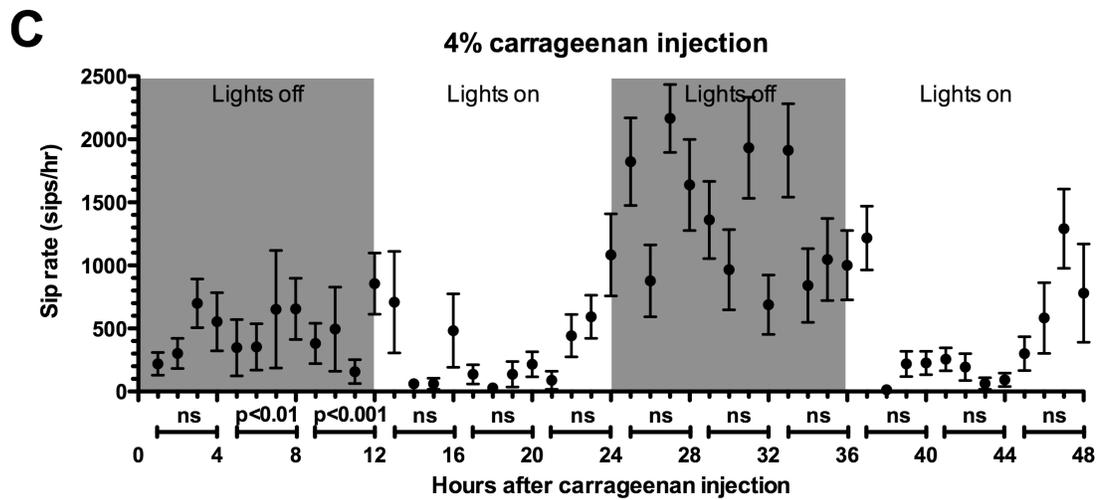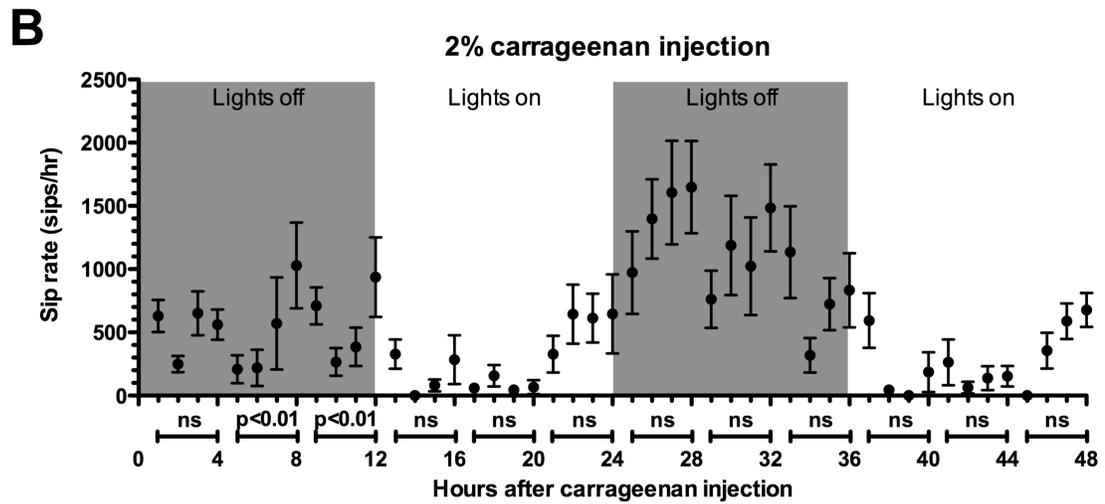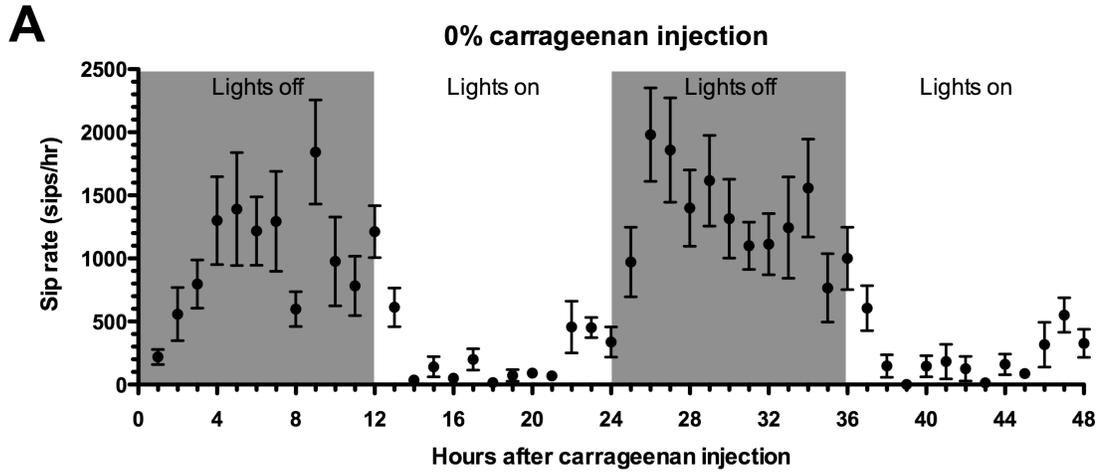
Figure 5 – Carrageenan (CGN) injection into the whisker pad reduced sip rate from 5 to 12 hours post-treatment. The points and error bars represent mean ± SEM of sip rate each hour post-CGN injection A) 0% CGN, B) 2% CGN, and C) 4% CGN (N=6). Higher sip rates were observed during awake/lights-out hours 0-12 and 24-36, and minimal sipping when the lights were on. Whisker pad injection of 2% ($p<0.01$) and 4% ($p<0.01$) carrageenan reduced the sip rate from 5 to 12 hours, as measured in the TrigeminAir device. No significant reduction in sip rate occurred after 12 hours. Significance was assessed using a repeated measures two-way ANOVA over each 4 hr bin in a non-crossover design

A

B
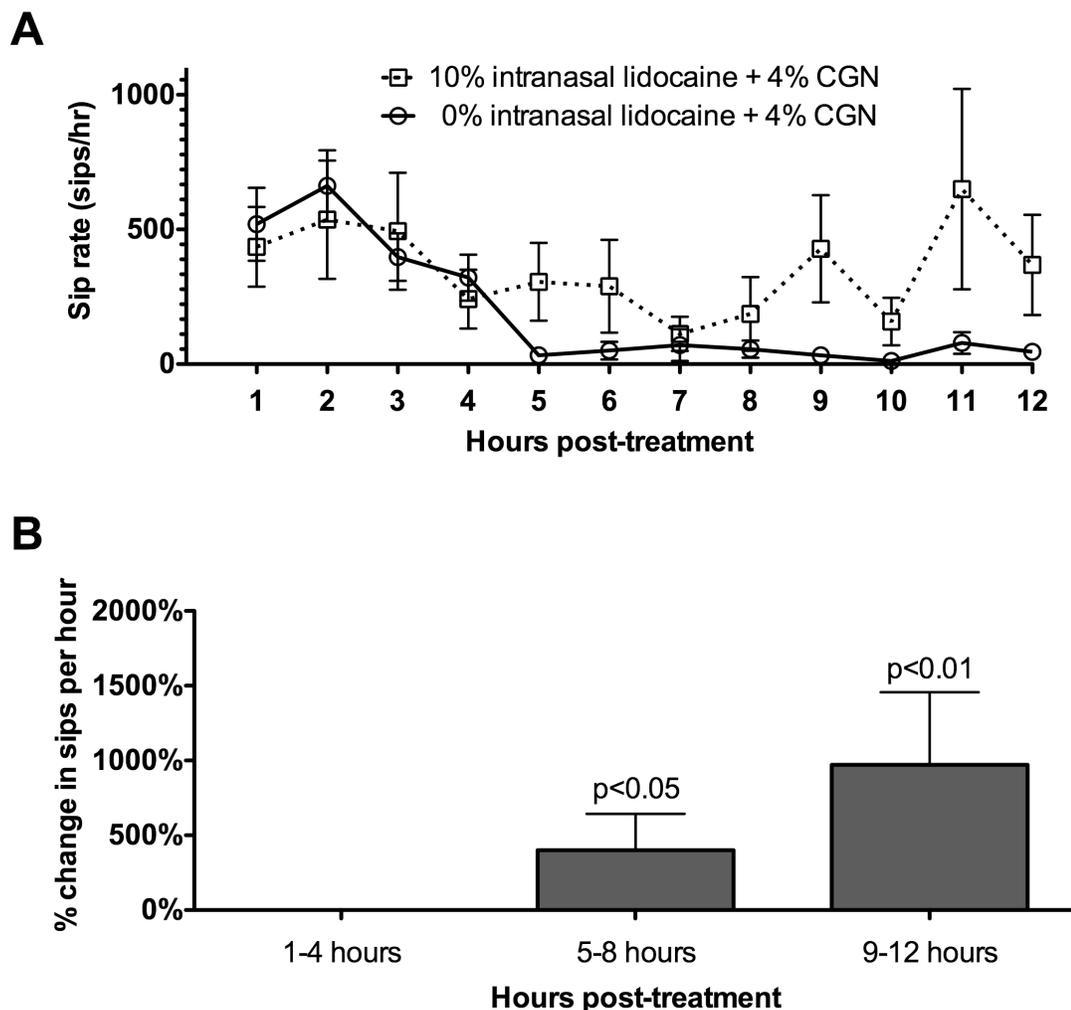
Figure 6 – Intranasal lidocaine increased sip rate during hours 5 to 12 after carrageenan injection into the whisker pad. A) Sip rate per hour (mean ± SEM) over a 12-hour period for intranasal lidocaine treated rats combined with a 4% carrageenan injection. B) Percent increase in mean ± SEM sip rate for the 10% lidocaine treated group compared to the 0% lidocaine control group after a 4% CGN injection (N=6). A repeated measures two-way ANOVA was used to calculate significance using a cross-over design where each rat was given each treatment on a different week.
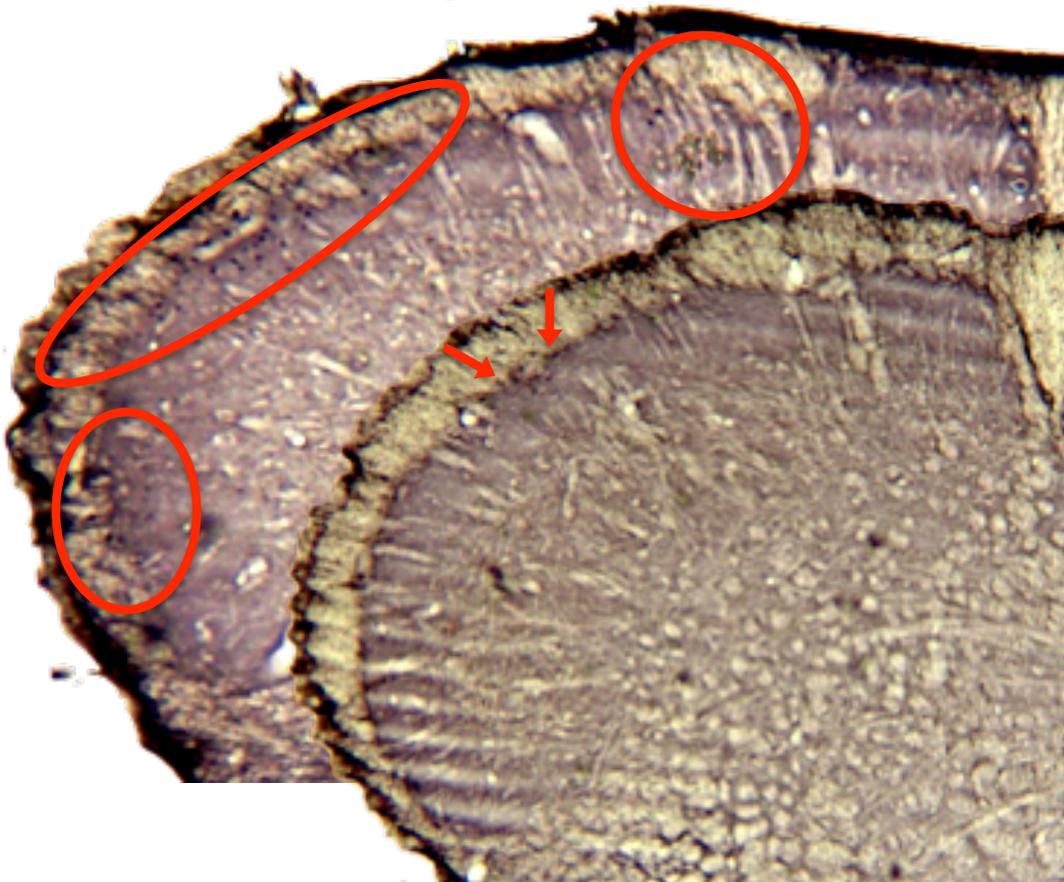
Figure 4 – Increased c-fos neuronal activity in brainstem areas that relay pain from the face. Rats injected with 4% carrageenan into the whisker pad expressed more c-fos positive cells (red circles) in laminae I/II compared to rats treated with 4% carrageenan + intranasal 10% lidocaine (arrows).

|	Lidocaine % dose	Laminae I/II (# of c-fos cells)		Laminae III-V (# of c-fos cells)	
		95% CI	p-value	95% CI	p-value
Ipsilateral	0% (0 mg)	41.4-48.5	-	6.4-7.6	-
	4% (32 mg)	16.6-20.6	p<0.001[a]	3.2-4.0	p<0.001[a]
	10% (80 mg)	20.7-24.8	p<0.001[a]	3.5-4.5	p<0.001[a]
Contralateral	0% (0 mg)	12.5-15.5	-	4.3-5.1	-
	4% (32 mg)	7.5-9.9	p<0.001[a]	2-2.6	p<0.001[a]
	10% (80 mg)	9.0-11.0	p<0.001[a]	2.3-2.9	p<0.001[a]

[a] – 0% group comparison using One-way ANOVA Tukey post-test

Table 2 – Intranasal lidocaine reduced c-fos positive cells in the brainstem following CGN whisker pad injection. C-fos positive cells in laminae I/II and III-V decreased as intranasal lidocaine dose increased (0%, 4%, 10%) in the first four hours following treatment despite an inflammatory CGN (4%) injection into the whisker pad (N=6). The 95% confidence interval (CI) is the range of c-fos positive cells for each dose and laminae (I/II or III-V). For both the ipsilateral and contralateral sides and both laminae I/II and III-V, 4% and 10% lidocaine treated groups had significantly (p<0.001) fewer c-fos positive cells compared to the 0% lidocaine group (one-way ANOVA with Tukey post-test).

Test	0% IN Lidocaine		4% IN Lidocaine			10% IN Lidocaine		
	ΔMean	95% CI	ΔMean	95% CI	p<0.05	ΔMean	95% CI	p-value
Sticker (sec)	13	(6 to 21)	12	(-2 to 25)	ns	16	(6 to 26)	ns
Forearm grip	51	(-17 to 119)	-31	(-164 to 101)	ns	-52	(-155 to 51)	ns
Open field (cm)	-429	(-811 to -46)	-986	(-1481 to -491)	ns	-987	(-1580 to -394)	ns
	ΔMedian	IQR	ΔMedian	IQR		ΔMedian	IQR	
Balance time (sec)	0	(0 to 0)	0	(0 to 3)	ns	5	(0 to 28)	<0.01
Balance score	0	(0 to 1)	1	(-1 to 2)	ns	2	(1 to 3)	<0.001

Table 3 – Following intranasal lidocaine there were no significant differences in the behavioral tests except reduced balance beam performance. Each rat received each dose of intranasal lidocaine in a randomized order, and each dose was separated by a minimum of one week. Performance was measured before and after each of the three intranasal doses and data analyzed by comparing the difference in performance pre- vs. post- treatment in each rat (N=15). Median and IQR were used for some measures because of non-Gaussian distributions due to ceiling effects of those tests. Change (Δ). Interquartile range (IQR). Confidence Interval (CI). Intranasal (IN).

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