THE EFFECT OF INTRAGANGLIONIC INJECTION OF GABA AND GABA AGONISTS ON PERIPHERAL SENSORY TRANSMISSION

by

Maryam Ranjbar Ekbatan

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

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submitted by Maryam Ranjbar Ekbatan in partial fulfillment of the requirements for

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Examining Committee:

Brian E. Cairns, Professor, Faculty of Pharmaceutical Sciences, UBC
Supervisor

Ernie Puil, Professor Emeritus, Department of Anesthesiology, Pharmacology and Therapeutics, UBC
Supervisory Committee Member

Ujendra Kumar, Professor, Faculty of Pharmaceutical Sciences, UBC
Supervisory Committee Member

Karla Williams, Assistant Professor, Faculty of Pharmaceutical Sciences, UBC
Additional Examiner

Additional Supervisory Committee Members:

Jacquelyn Cragg, Assistant Professor, Faculty of Pharmaceutical Sciences, UBC
Supervisory Committee Member

Abstract

Due to the lack of chemical synapses in the trigeminal ganglion, it is widely believed that it does not play a role in transmission of sensory signals. However, it has been shown that neurons and satellite glial cells in trigeminal ganglion express neuroreceptors such as γ-aminobutyric acid $(GABA)_A$ and $GABA_B$ receptors and can release neurotransmitters like GABA. Increasing the levels of GABA in the trigeminal ganglion was showed to have anti-nociceptive effects, while blockade of GABA receptor expression in the trigeminal ganglion was demonstrated to increase pain behaviors. However, the neuronal mechanism underlying these effects remains to be examined. In the current study, the expression of GABA receptors in the trigeminal ganglion neurons that innervate labial skin and masseter muscle was evaluated by immunohistochemistry. Using single unit recording, trigeminal brainstem and ganglion neuron responses to stimulation of labial skin and/or masseter muscle were evaluated after intraganglionic injections of GABA receptor agonists in rats. The mean frequency of expression of $GABA_A$ and $GABA_B$ receptors by masseter and labial skin ganglion neurons was 62.5% and 92.7%, and 55.4% and 20.3%, respectively. In both skin and muscle ganglion neurons, the expression of $GABA_A$ was higher than $GABA_B$. There was a higher frequency of $GABA_A$ as well as $GABA_B$ receptor expression in ganglion neurons that innervated the skin compared with those that innervated muscle. In ganglion neurons that innervated the skin, there was a higher expression of $GABA_A$ receptors and $GABA_B$ receptors in males compared to females. Masticatory muscle evoked brainstem trigeminal neuron responses were attenuated by intraganglionic injection of muscimol ($GABA_A$), but not baclofen ($GABA_B$). Compared to Phosphate Buffered Saline (PBS), all substances reduced mechanical threshold 30 minutes post injection. Further, GABA (500 mM) and baclofen 10 mM decreased mechanical threshold (MT)

compared to PBS for the entire recording period. All these changes were statistically significant. The mechanical sensitivity of slow and fast conducting masticatory muscle afferent fibers was decreased and increased, respectively, by intraganglionic injection of both muscimol and baclofen. This study suggests that activation of peripheral GABA receptors may exert a selective gating effect on sensory input passing through trigeminal ganglion.

Lay Summary

The aim of this study was to find an option for treating pain in the face like jaw, tooth and skin pain. Nerve fibers transmit information about pain from the face to the brain where it is perceived. I investigated whether injection of a substance called GABA into the trigeminal nerve can reduce pain signals. GABA is a chemical messenger that inhibit signals between brain cells by acting on molecules called GABA receptors. I found that there are GABA receptors on nerve fibers coming from skin and jaw muscle. I found that two drugs that act like GABA, baclofen and muscimol, decrease the transmission of information about pain from the face to the brain. I also found that the responses to these drugs are different in males and females. Specifically, the drugs work better on males. These findings indicate these drugs could treat pain by acting outside of the brain.

Preface

I performed all the work in the current study with assistance from my supervisor, Dr. Cairns. The experiments were conceived by Dr. Cairns and me. The University of British Columbia Animal Care Committee reviewed and approved all animal procedures (Animal Care Protocol Number: A17-0153). All the Figures were created by me. I have permission to publish images that I made using BioRender.

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List of Abbreviations

APV	2-amino-5-phosphonovalerate
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
GABA	Gamma-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GDP	Guanosine diphosphate
GIRK	G protein-coupled inwardly-rectifying potassium
GTP	Guanosine triphosphate
MT	Mechanical Threshold
NMDA	N-methyl-D-aspartate
NMDA	N-methyl-D-aspartate
PAD	primary afferent depolarization
PBS	Phosphate Buffered Saline
PNS	Peripheral Nervous System
RGS	Regulators of G protein Signaling
SGC	Satellite Glial Cells
SpVc	Spinal trigeminal subnucleus caudalis
TG	Trigeminal Ganglion
TMD	Temporomandibular Disorders
VGAT	Vesicular GABA Transporter

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Chapter 1: Introduction

1.1 Background

Orofacial pain, in conditions like temporomandibular disorders (TMD), headache and trigeminal neuralgia is thought to have a peripheral component. It is known that GABA receptor agonists can have anti-nociceptive effects, and a number of publications have related these effects of GABA to actions in the central nervous system (CNS) (McGowan and Hammond 1993; Malcangio and Bowery 1996; Yaksh 1989). However, many studies have also reported that activation of GABA receptors in peripheral nervous system (PNS) led to a reduction of pain (Bravo-Hernández et al. 2014; Lee et al. 2018; Naik, Pathirathna, and Jevtovic-Todorovic 2008). These studies support the idea that peripheral GABA receptors might have a significant function in reducing manifestations of pain.

GABAergic drugs are used to treat craniofacial pain conditions. Baclofen, a $GABA_B$ agonist is used clinically to reduce muscle pain in temporomandibular disorders (TMD) and decrease attacks of trigeminal neuralgia (Baker, Taylor, and Lilly 1985; Cairns 2010). Also, benzodiazepines, like diazepam, which modulate $GABA_A$ receptor function, are used to reduce muscle pain in TMD (Cairns 2010).

There is evidence from animal models that some of the effects of GABA receptor agonists may be mediated in the PNS. Administration glutamic acid decarboxylase (GAD) by adenoviral vectors (AdGAD65) to the trigeminal ganglion (TG) of rodents increases GABA synthesis by TG neurons, and significantly decreases inflammatory orofacial pain (Vit et al. 2009); however, there is no evidence from this study that GABA actually affects transmission of action potentials through ganglion (Vit et al. 2009). Also, in vivo administration of GABA or GABA reuptake inhibitors which increase GABA levels in the dorsal root ganglia significantly

alleviated nocifensive behaviors, and improved inflammatory and neuropathic pain (Du et al. 2017). Activation of $GABA_A$ receptors leads to depolarization of afferent fibers arising from tissues in dorsal horn which results in presynaptic inhibition and a decrease in signal transmission through reduction of neurotransmitter release (Malcangio and Bowery 1995). Additionally, GABA, through $GABA_B$ receptors, decreases the release of neurotransmitters such as glutamate, substance P and calcitonin gene-related peptide from primary afferent terminals caused by reduction in Ca^{2+} influx to these terminals (Kerr et al. 1987). Therefore, regulation of GABA levels is important in PNS as well as CNS for sensory transmission.

However, very little is known about the role of GABA in modulating sensory transmission through the trigeminal ganglion or how this might contribute to the actions of GABA-ergic drugs used to treat craniofacial pain disorders. Furthermore, it is believed that the consequences of nociceptive input from skin and muscle differ. One example of this is that, compared with noxious cutaneous afferent input, noxious muscle afferent input produces more prolonged and widespread sensitization to nociceptive stimuli (Wall and Woolf 1984; Woolf and Wall 1986; Xu, Ge, and Arendt-Nielsen 2010). Therefore, in this study, I first examined how GABA agonists affects transmission of sensory input through the trigeminal ganglion from the muscle and skin to the caudal trigeminal sensory nucleus, and then I investigated the role of γ-aminobutyric acid (GABA) receptors on sensory properties of masticatory muscle afferent fibers.

1.2 Trigeminal Anatomy

The trigeminal nerve is the fifth and largest cranial nerve. The trigeminal ganglion (TG) is a sensory ganglion comprised of cell bodies of the trigeminal ganglion neurons surrounded by satellite glial cells (SGCs). Trigeminal ganglion neurons are unipolar and project both to the

specific craniofacial tissue that they innervate and to the trigeminal sensory nuclear complex of the brainstem. Peripherally, after exiting from the trigeminal ganglion, their axons are divided into three branches: V1 (ophthalmic branch), V2 (maxillary branch), and V3 (mandibular branch) (Prasad and Galetta 2007). These divisions innervate the dermatomes as well as the deep and superficial structures of their innervation location (Figure 1.1). The V1 and V2 branches consist of sensory fibers; whereas, the V3 branch is comprised of both sensory and motor fibers (Prasad and Galetta 2007). Post-ganglionic sympathetic fibers and pre- or postganglionic parasympathetic fibers also join the peripheral trigeminal nerves to supply blood vessels and other glands(Liu 2005).

Satellite glial cells are also located in the trigeminal ganglion and are a type of glial cell that surrounds the ganglion neuron. They regulate the ganglion neuron microenvironment (Jasmin et al. 2010). SGCs are linked to each other via gap junctions (Ohara et al. 2009), and they are involved in ganglion neuron communication (Takeda, Takahashi, and Matsumoto 2009). Although there is no synaptic transmission in primary sensory ganglia, activation of neighboring neurons causes cross-excitation in the affected neuron (Takeda et al. 2011). It has been suggested that ATP released from a TG neuron and its associated SGC activates ATP receptors on the SGC to release more ATP. This neurotransmitter acts on the neighboring SGC and triggers the release of ATP to act on the ganglion neurons they surround to pass the signal to the next neurons (Goto et al. 2016). Trigeminal ganglion neurons and their associated SGCs also contain glutamic acid decarboxylase (GAD) 65 (Hayasaki et al. 2006). This enzyme maintains physiological production of GABA by decarboxylation of glutamate (Hayasaki et al. 2006). GAD65 is involved in rapid GABA release and provides most of the GABA for neurotransmitter release (Pandya et al. 2019). It has been suggested that SGCs may play a role in storing and releasing

GABA. In the case of continuous firing of action potential there is an increase of K^+ ions in the space between the neuron and SGC, which may cause GABA to be released from SGCs to affect ganglion neurons (Hayasaki et al. 2012).

Centrally, after exiting the trigeminal ganglion, the axons enter the brainstem at the level of the pons, where they separate and distribute caudally or rostrally. Trigeminal primary afferent fibers synapse in the spinal trigeminal nucleus (Vsp), the principal sensory nucleus (Vp), and the mesencephalic nucleus (Vmes) (Prasad and Galetta 2007). The spinal trigeminal nucleus (Vsp) receives input from cutaneous and nociceptive fibers and consists of three subnuclei; the caudalis (Vc), interpolaris (Vi) and oralis (Vo). Tactile and pressure stimuli are conveyed to the Vp, while kinesthetic face sensations are transferred to Vmes. (Prasad and Galetta 2007).

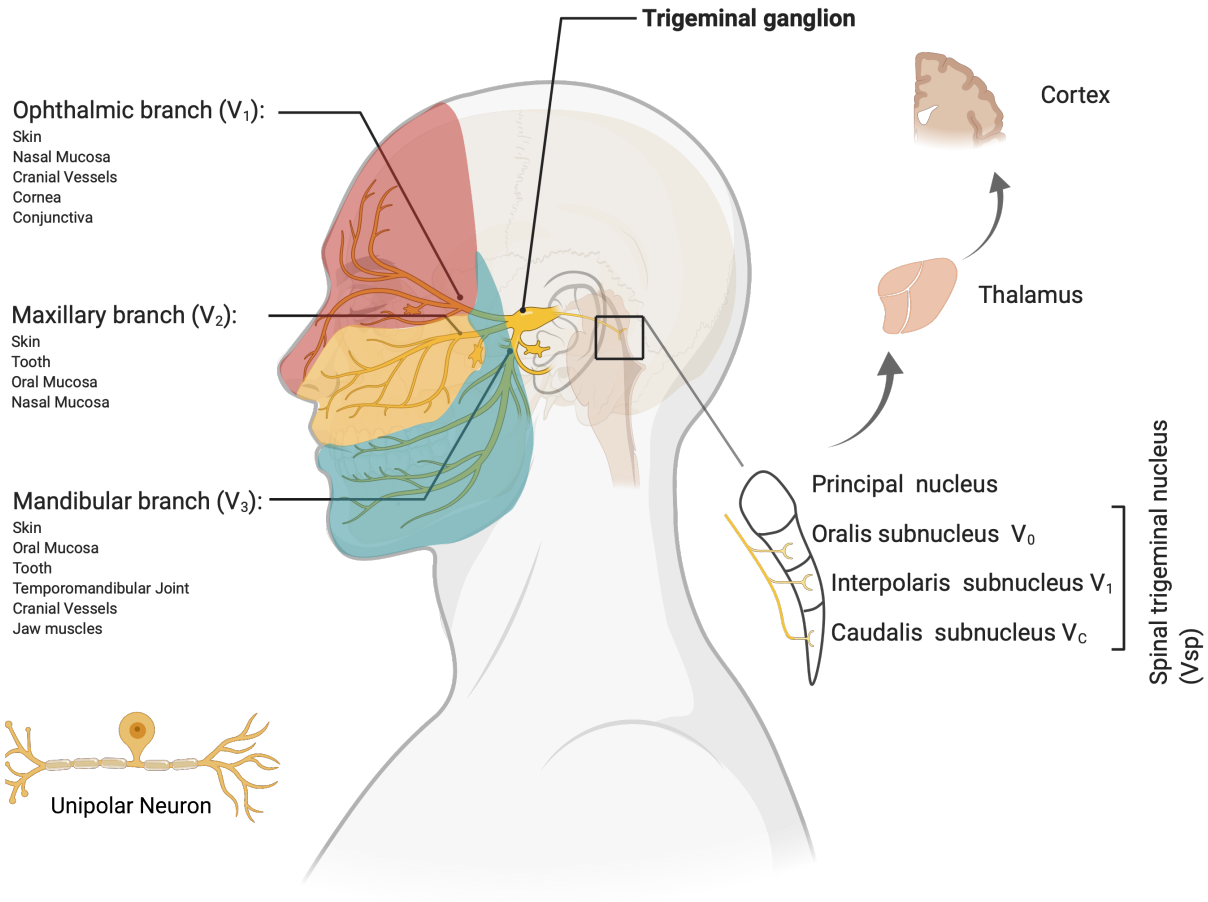

Figure 1.1 Main trigeminal somatosensory organization of orofacial region.

Primary afferent neurons get sensory input from different tissues and project to the second order neurons in the brainstem trigeminal sensory nuclear complex via trigeminal ganglion. (*Created with BioRender.com*)

1.3 The Spinal Trigeminal Nucleus Caudalis

The brainstem trigeminal sensory nuclear complex consists of the spinal trigeminal nucleus (Vsp) and the principal sensory nucleus (Vp), and receives both innocuous and nociceptive input from the trigeminal afferent fibers (Shigenaga and Yoshida 2007). It has been reported that the subnucleus caudalis (SpVc) is the main subnuclei that conveys facial noxious stimuli to higher levels of the brain (Amano, Hu, and Sessle 1986; Hayashi, Sumino, and Sessle

1984; Lisney 1983; Sessle and Greenwood 1976). Due to the similarity of the subnucleus caudalis (SpVc) to the spinal dorsal horn in physiological characteristics and laminar structure, it is also known as medullary dorsal horn (Gobel 1981; Messlinger, Dostrovsky, and Strassman 2006).

Central axons of the trigeminal nerve as well as axons of descending brainstem pathways project to the medullary dorsal horn (Dubner and Bennett 1983). Some of the neurons in subnucleus caudalis (SpVc) project directly to the ventral posteromedial thalamic nucleus (VPM) (Bruce, McHaffie, and Stein 1987; Fukushima and Kerr 1979; Kemplay and Webster 1989; Sessle 2000) while others use reticular formation and adjacent brainstem areas through poly-synaptic paths to project to the thalamus (Sessle 2000). There are also ipsilateral projections from SpVc to other regions of the brainstem trigeminal sensory nuclear complex (Hockfield and Gobel 1982; Ikeda, Tanami, and Matsushita 1984; Sessle 2000). Projections from SpVc to other areas of the brain such as lateral periaqueductal grey (PAG) matter (Noseda et al. 2008), the cerebellar cortex(Huerta, Frankfurter, and Harting 1983; Magnusson et al. 1987; Matsushita, Ikeda, and Okado 1982), superior colliculus (Bruce et al. 1987; Huerta et al. 1983) and the parabrachial nucleus (PB) (Cechetto, Standaert, and Saper 1985) have been also reported.

1.4 Neurotransmitter receptors

Neurotransmitter receptors can be categorized into two main classes: ionotropic and metabotropic receptors. Both of these receptor categories are activated by neurotransmitters. Ionotropic receptors are ion channels which allow the flow of ions (e.g. K^+, Na^+ or Cl^-) into and out of neurons (Smits et al. 2012), whereas activation of metabotropic receptors leads to the

modulation of intracellular signaling cascades that involve G protein coupled receptors and cause indirect effects on ion channels and metabolic function (Eric et al. 2000).

1.5 γ -aminobutyric acid (GABA)

GABA is the major inhibitory neurotransmitter in CNS of mammals (Möhler 2006). GABA exerts its inhibitory effects through $GABA_A$ and $GABA_B$ receptor subtypes. The function of GABA in the peripheral nervous system is not completely apparent (Magnaghi 2007); however, it is known that GABA plays a key role in modulating sensory transmission from primary afferent terminals to the brainstem trigeminal sensory nuclear complex and spinal cord dorsal horn (Malcangio and Bowery 1996). GABA does not cross blood brain barrier; therefore, the changes in the sensory transmission in this study after injection of GABA to the trigeminal ganglion are due to the peripheral effects of GABA, not its central effects (Kakee et al. 2001) . As GABA does not easily penetrate the CNS, it is peripherally restricted and can affect both $GABA_A$ and $GABA_B$ receptors on nerve fibers and ganglion neurons (Jasmin, Wu, and Ohara 2004). On the other hand, $GABA_B$ receptors are uniformly inhibitory, and the effects of $GABA_A$ receptor can be either inhibitory or excitatory. Due to the effects of GABA on both $GABA_A$ and $GABA_B$ receptors, it might not be a good candidate for attenuating pain; therefore, in this study we used $GABA_A$ and $GABA_B$ agonists as well as GABA itself (Hasbargen et al. 2010).

1.5.1 $GABA_A$ receptor

The $GABA_A$ receptor is a ionotropic ligand-gated chloride channel that increases Cl^- conductance upon activation and causes inhibition of central neuronal firing under normal

physiologic conditions (Figure 1.2) (Magnaghi 2007). However, activation of Cl^- chloride channel can be inhibitory or excitatory depending on the concentration of Cl^- on each side of the membrane. $GABA_A$ receptors are composed of 2 α (α1-3 postsynaptic, α4-6 extra synaptic), 2 β (1-3) and one γ2 (one γ more common for extra synaptic) subunit, many of which have several isoforms (Jyoti Puri et al. 2012). Functional $GABA_A$ receptors consist of at least an α and a β subunit (Schofield et al. 1989).

In primary afferent fibers, it is believed that synthesized $GABA_A$ receptor subunits in cell bodies are carried to the central terminals where they function to modulate neurotransmitter release. However, based on in-vitro studies, cell bodies in the TG neurons express functional $GABA_A$ receptors, and trigeminal satellite glial cells may have the role of storing and releasing GABA (Hayasaki et al. 2006). Trigeminal primary afferent fibers terminate in the spinal trigeminal nucleus caudalis (Pfaller and Arvidsson 1988) and have active presynaptic $GABA_A$ receptors (Grudt and Henderson 1998). Therefore, blocking $GABA_A$ receptors at spinal trigeminal nucleus caudalis could lead to the lower levels of inhibitory signals associated with $GABA_A$ function (Han and Youn 2008). Intracellular levels of Cl^- are controlled by NKCC1 and KCC2 channels which transport Cl^- ions in and out of the neuron, respectively.

Using in-vitro electrophysiological techniques, it was observed that GABA application to TG slices depolarizes TG neurons that was associated with an increase of their membrane conductance (Puil and Spigelman 1988). In some TG neurons, the depolarization resulted in increased firing and excitation, while in other neurons the firing frequency decreased because of the increased conductance (Puil and Spigelman 1988). Therefore, GABA-evoked depolarization can produce either excitation or inhibition of TG neuron action potential firing depending on where in the neuron the conductance increased. The GABA-induced depolarization was

diminished by bicuculline, a $GABA_A$ receptor antagonist, indicating that depolarization was due to $GABA_A$ receptor activation (Puil and Spigelman 1988).

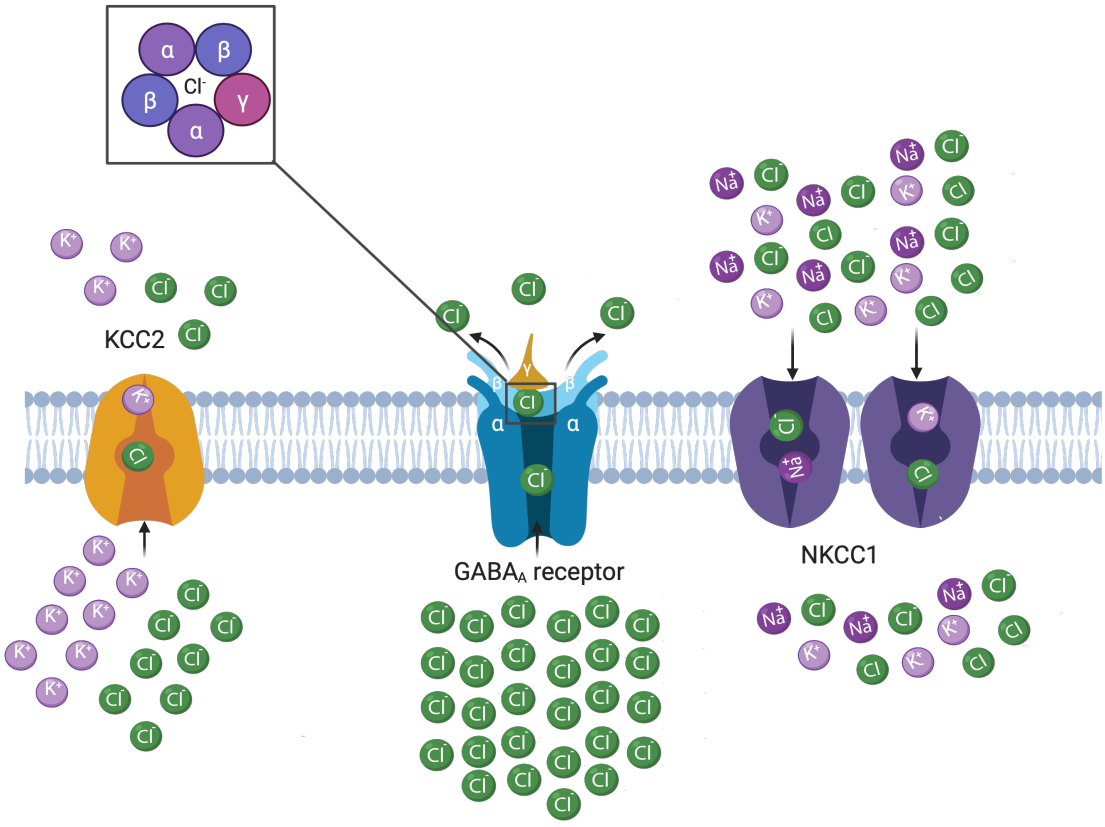

Figure 1.2 Schematic presentation of $GABA_A$ receptor in PNS.

Intracellular levels of Cl- are controlled by NKCC1 and KCC2 channels which transport Cl- ions in and out of the neuron, respectively. NKCC1 is expressed more than KCC2 in primary afferent neurons; therefore, Cl- ion is more inside the cell. Activation of $GABA_A$ receptor results in the efflux of Cl- ion and consequently depolarization of the neuron. (*Created with BioRender.com)*

1.5.2 $GABA_B$ receptor

$GABA_B$ receptors are metabotropic G protein coupled receptors that regulate Ca^{2+} and K^+ channels and are extensively expressed in dorsal root ganglia and spinal cord (Engle et al. 2012). $GABA_B$ receptors modulate intracellular G protein-coupled signaling cascades upon activation. $GABA_B$ receptors are comprised of two subunits: $GABA_{B1}$ subunits ($GABA_{B1A}$ and $GABA_{B1B}$) and $GABA_{B2}$ subunits (Bettler et al. 2004). Functional $GABA_B$ receptors consist of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits (Kaupmann et al. 1998). The main agonist binding site is located in the $GABA_{B1}$ subunit. $GABA_{B1}$ and $GABA_{B2}$ subunits have different roles. When a ligand binds at the $GABA_{B1}$ subunit, the $GABA_B$ receptor becomes activated; however, the $GABA_{B2}$ subunit activates the G-proteins that inhibit adenylyl cyclase. It has been reported that the cells that do not have $GABA_{B2}$ subunits accumulate $GABA_{B1}$ receptors intracellularly (Kaupmann et al. 1998). $GABA_{B2}$ receptor subunit might be involved in enabling the transferring of $GABA_{B1}$ receptor subunit to the cell membrane (Kaupmann et al. 1998). Due to the inability of the $GABA_{B2}$ subunit to bind to ligands, it cannot function on its own when inserted into the cell membrane (Couve et al. 1998). $GABA_{B1A}$ and $GABA_{B1B}$ are the two different subtypes of the $GABA_{B1}$ subunit. The $GABA_{B1A}$ subunit is expressed in axonal terminals and mediates pre-synaptic inhibition; however, $GABA_{B1B}$ subunits are expressed in dendritic spines and mediate postsynaptic inhibition (Pinard, Seddik, and Bettler 2010).

The endogenous neurotransmitter GABA and the selective $GABA_B$ receptor agonist baclofen are the main $GABA_B$ receptor agonists and are used for the identification of presence of $GABA_B$ receptors (Bowery et al. 1980). The activation mechanisms of $GABA_B$ receptors for these two substances are different. In order to activate $GABA_B$ receptors by GABA, Ca^{2+} should be present; however, baclofen doesn't require the presence of Ca^{2+}(Galvez et al. 2000).

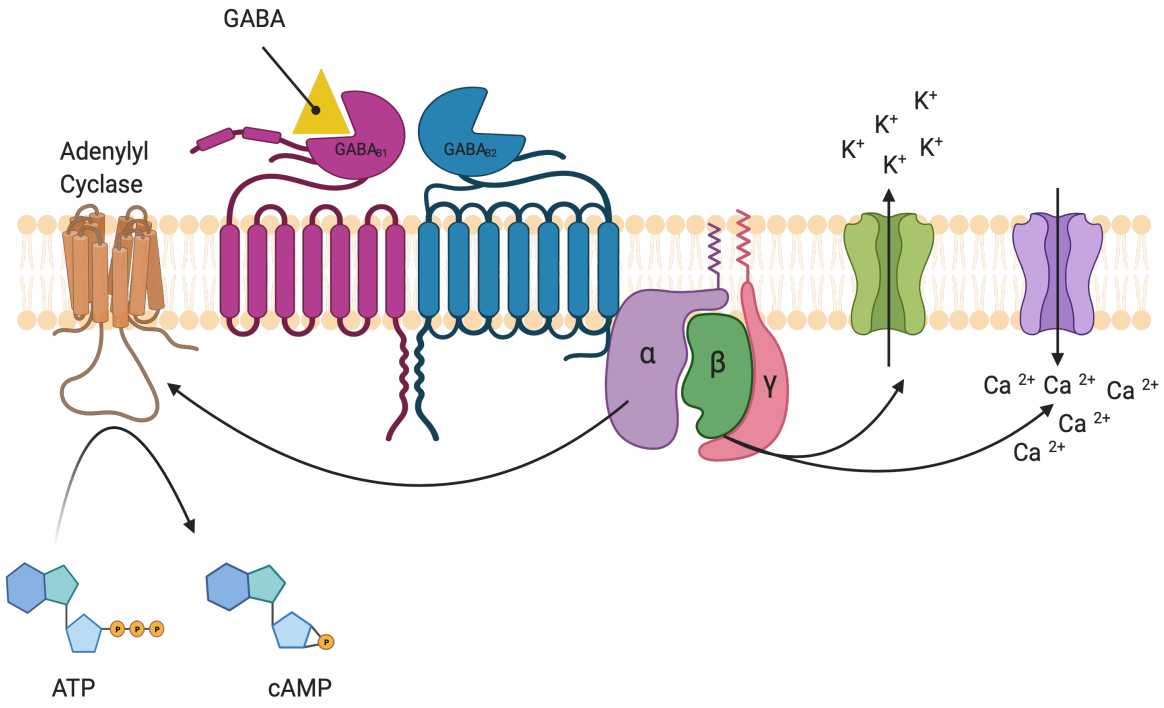

Figure 1.3 Schematic presentation of $GABA_B$ receptor.

Ligand binding of a $GABA_B$ agonist such as GABA or Baclofen to the $GABA_{B1}$ subunit leads to a conformational shift that modifies the binding properties of G-protein coupled receptors resulting in an exchange of a GTP for a GDP at the Gα subunit. Gα inhibits adenylyl cyclase causing lower levels of cAMP. Also, Gβγ is released and activates K^+ currents and inhibits Ca^{2+} channels. Regulators of G-protein signaling proteins (RGS) control the activity of Gα subunit. Reassociation of Gα and Gβγ results in the termination of receptor activity (Benarroch 2012). (*Created with BioRender.com)*

1.6 Peripheral effects of GABA and selective GABA receptor agonists

Preclinical studies suggest that GABA may be a promising peripherally acting analgesic. GABA injection into the temporomandibular joint of rats exerts antinociceptive effects via activation of peripheral $GABA_A$ receptors (Cairns, Sessle, and Hu 1999). Specifically, the

magnitude of evoked jaw muscle electromyographic (EMG) activity was decreased more by the co-administration of GABA with glutamate compared to glutamate alone in temporomandibular joint region, and this effect was reversed by application of $GABA_A$ antagonist, bicuculline but not the $GABA_B$ receptor antagonist phaclofen, indicating that GABA mediated inhibition of noxious responses is regulated by peripheral $GABA_A$ receptors (Cairns et al. 1999). 2-amino-2-methylbutanoic acid (isovaline) is a $GABA_B$ agonist without the ability to cross blood brain barrier; consequently, it cannot cause CNS effects, and its mechanisms are through actions in the PNS. It has been reported that isovaline decreased allodynia induced by prostaglandin administration into mouse hind paw by cutaneous $GABA_B$ receptors without any CNS effects (Whitehead et al. 2012). Moreover, isovaline restored limb function in mouse model of osteoarthritis during forced exercise to baseline values (Whitehead et al. 2012). Therefore, it can be concluded that activation of peripheral $GABA_A$ and $GABA_B$ receptors might play a critical role in mediating the transmission of nociceptive information.

Few studies have been conducted so far to try to translate findings in preclinical models into humans. However, the effect of a GABA oral rinse has been studied in a human model of burning mouth syndrome, where burning tongue pain is generated in healthy individuals by topical application of capsaicin. Rinsing the mouth with GABA solutions was reported to decrease this burning pain almost as much as rinsing with the local anesthetic lidocaine (Zhang et al. 2018). In contrast, injection of GABA into the masseter muscle of healthy men and women was found to induce a low level of muscle pain that was increased by co-injection of the $GABA_A$ receptor allosteric modulator lorazepam (Meijs et al 2019). Further, when GABA was co-injected with glutamate, which is used to induce moderate masseter muscle pain, the combination produced more pain in men, but not in women. This suggests that both analgesia

and pain can be produced by local administration of GABA to various craniofacial tissues in humans.

1.6.1 Local GABAergic system in Trigeminal ganglion

GABA binding to the GABA receptor in CNS can induce inhibition that causes a decrease in nociception. However, in the peripheral nervous system and trigeminal ganglion the effects of GABA might be either inhibitory or excitatory (Carr et al. 2010; Levy 1977; Price et al. 2009). $GABA_A$ receptors demonstrated excitatory actions via two important cotransporters NKCC1 and KCC2, which accumulate and disperse Cl^- in and out of the cell, respectively (Price et al. 2009). NKCC1 is expressed more than KCC2 in primary afferent neurons like dorsal root ganglion and trigeminal ganglion; therefore, the concentration of $[Cl^-]$ inside the cell remains high, and the activation of $GABA_A$ receptors will result in primary afferent depolarization (PAD), by permitting the flow of Cl^- out of the cell (Alvarez-Leefmans et al. 2001; Kanaka et al. 2001; Price, Hargreaves, and Cervero 2006; Toyoda et al. 2005). Activation of $GABA_B$ receptors in trigeminal ganglion decreased the excitability of neurons by potentiation of K^+ currents (Takeda et al. 2004). A suggested theory of the role of local GABAergic system in TG was proposed by Hayasaki et al, (Hayasaki et al. 2012). Based on their theory, GABA is synthesized by GAD 65 within the neuronal cell body, and released into the intercellular space between the cell body and glial cell (Hayasaki et al. 2012). Satellite glial cells store the GABA and release it in response to the release of K^+ ions into the interspace due to frequent firing of action potentials (Hayasaki et al. 2012). The released GABA binds to the $GABA_A$ and $GABA_B$ receptors, and increases Cl^- conductance via opening $GABA_A$ receptors, and activates $GABA_B$ mediated signaling pathways increasing K^+ conductance (Hayasaki et al. 2012).

1.6.2 Effects of $GABA_B$ receptor in Trigeminal ganglion

The physiology and distribution of $GABA_B$ receptors in TG is unclear. In-vitro studies have demonstrated that $GABA_B$ receptors are present within TG. These studies have detected mRNAs of all $GABA_B$ subunits as well as protein expression of $GABA_B$ receptor subunits in both small and large TG neuronal cells (Hayasaki et al. 2012).

There is some uncertainty regarding the effect of $GABA_B$ receptor activation on trigeminal ganglion neurons. It was demonstrated that $GABA_B$ receptors exert inhibitory effects in rat trigeminal ganglion neurons by increasing voltage-dependent K^+ currents (Takeda et al. 2004). Application of baclofen, a $GABA_B$ receptor agonist resulted in a variable number of action potentials dependent on the individual neuronal cell examined via bath perfusion in whole-cell current clamp experiments (Hayasaki et al. 2012). Baclofen 'increased', decreased', or did not change the generation of action potentials with the population ratio of 58%, 25%, and 16%, respectively (Hayasaki et al. 2012). Therefore, it can be concluded that GABA affects the excitability of the neuronal cell bodies in trigeminal ganglia heterogeneously via the $GABA_B$ receptors.

1.7 Experimental Hypothesis

Based on the previously mentioned animal and human experimental studies, I hypothesize that sensory transmission through the rat trigeminal ganglion is attenuated by GABA receptor activation. I have employed the following research aims to examine this hypothesis:

Aim 1: To determine the level of expression of $GABA_A$ and $GABA_B$ receptors in the trigeminal ganglion neurons that innervate masseter muscle and labial skin.

Within this aim there are two experimental questions being proposed:

1. Is there any difference in the expression of GABA receptors between the ganglion neurons that innervate masseter muscle and labial skin?
2. Is there any difference in the expression of GABA receptors in male and female rats?

Aim 2: To determine if intraganglionic administration of GABA agonists produces a net inhibitory effect on sensory transmission through the TG in rats.

Aim 3: To determine if intraganglionic injections of GABA, $GABA_A$ and $GABA_B$ agonists into the trigeminal ganglion change the mechanical threshold of afferent fibers innervating with masseter muscle.

Chapter 2: Method

2.1 Animals

Sprague-Dawley rats (n=90) were housed in groups of two with a 12-h light/dark cycle with free access to food and water. All animal procedures were reviewed and approved by the University of British Columbia Animal Care Committee (A17-0153).

2.2 Tissue processing and immunohistochemistry

Female (250–330 g, n=6) and male (260–590 g, n=6) Sprague–Dawley rats (Charles River, Canada) were briefly anesthetized with isoflurane 2-2.5% in oxygen 97-98%. Next, a microinjection of 5µl rhodamine fluorescent dye to both sides of lower lip and a microinjection of 10 µl fast blue dye (Polysciences, USA) into the right and left masseter muscle were done using Hamilton syringe connected to a 27 gauge needle. Seven days later, rats were anesthetized with isoflurane 2-2.5% in oxygen 97-98%. An incision was made to open the chest cavity, a needle which was connected to the saline was inserted into the left ventricle and then the atrium was cut to permit perfusion. Rats were perfused with cold saline followed by paraformaldehyde 4% to fix the tissues. The trigeminal ganglion was extracted carefully bilaterally, and left for 3 days in 20% sucrose, and another 3 more days in 40% sucrose to dehydrate the tissue. The dehydrated ganglion was embedded in OTC (optimal tissue cutting compound) and frozen (-20°C). The trigeminal ganglion was sliced into 10µm sections with a cryostat and mounted on poly-lysine-coated glass slides. Afterwards, tissue slices were treated with 5% normal goat serum (NGS) for 1 hour, and then washed in PBS (phosphate buffered saline). All sections were incubated for 24 hours with primary antibodies against GABA receptors; the α1 subunit of

$GABA_A$ receptors (rabbit monoclonal 1:500, Abcam; ab33299) and B1 subunit of $GABA_B$ receptors (mouse monoclonal 1:500, Abcam; ab55051). The next morning, slices were washed with PBS several times to remove the primary antibodies solution, and then incubated for 1 hour at room temperature in the presence of fluorescence- conjugated secondary antibodies (anti-rabbit 488 nm 1:700, anti-mouse 635nm 1:700, Invitrogen) in the dark. Next, slides were washed several times with PBS, and then they were mounted by AquaPerm mounting medium. As a control for selectivity, primary antibodies were omitted from the staining procedure. Sections were visualized using a Leica TCS SPE high resolution spectral confocal microscope, and noise to signal ratio was be adjusted using ImageJ (Figure 2.1).

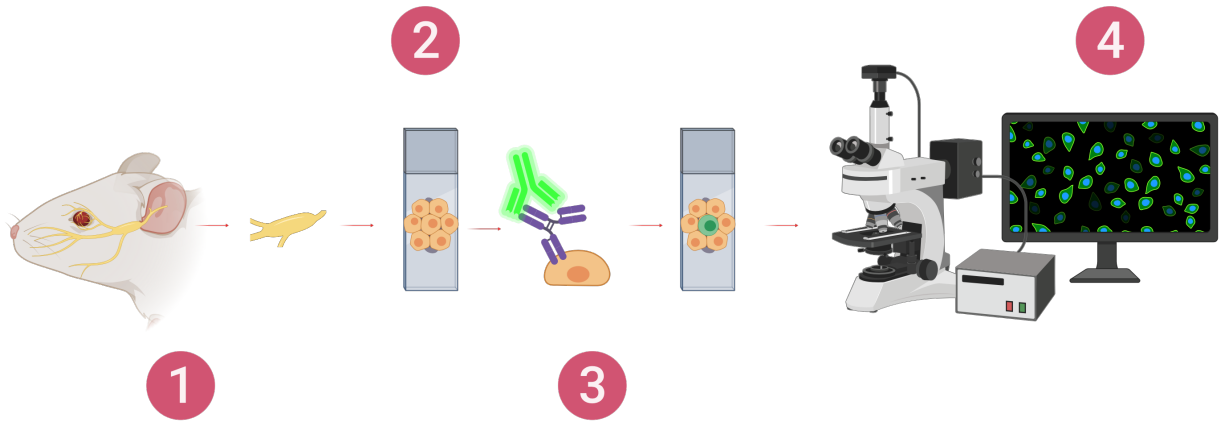

Figure 2.1 Immunohistochemistry procedure.

1) The trigeminal ganglion was extracted. 2) It was mounted on glass slides after slicing into sections. 3) Tissue slides were washed with primary antibodies and incubated in the presence of secondary antibodies. 4) Sections were visualized by confocal microscope. (*Created with BioRender.com)*

2.2.1 Data analysis

Trigeminal ganglion neurons that innervate skin and muscle labeled positively with rhodamine and fast blue, respectively were assessed for expression of $GABA_A$ and $GABA_B$ receptors. A positively labeled neuron had an intensity of staining at least two standard deviations above the baseline intensity of the surrounding tissue (an estimate of the 95% confidence interval). The positive cells were counted visually. The percentage of cells having $GABA_A$ or $GABA_B$ receptors in total and for each sex was calculated. The Mann Whitney rank sum test was used for determination of differences in the expression of GABA receptor subtypes between skin and muscle tissue as well as potential differences between GABA subtype expression by each sex. P-values less than 0.05 were considered statistically significant.

2.3 Electrophysiological Neuronal Recording

2.3.1 Animals

Female (250–330 g, n=9) and male (260–590 g, n=9) Sprague–Dawley rats (Charles River, Canada) were used for the evaluation of the effects of $GABA_A$, $GABA_B$ agonists and vehicle control (phosphate buffered saline) on sensory transmission.

2.3.2 Drugs

Muscimol and RS-baclofen were purchased from Sigma-Aldrich (MO, USA). Drugs were prepared in PBS (Sigma) for injection to achieve 10 mM concentrations of muscimol and baclofen. Three (3) µl of muscimol, baclofen and PBS were stored in microcentrifuge tubes and were frozen at -20°C until use. The experimenter was blinded to the treatments being

administered. Pentobarbital (Euthanyl; Bimeda-MTC Animal Health Inc. ON, Canada) was stored at room temperature (15°-30° C).

2.3.3 Surgical procedure

Sprague-Dawley rats were deeply anesthetized with 2-2.5% isoflurane in 97-98% oxygen. To record the heart rate, two wires connected to the ECG electrode incised near either side of the heart. After making an incision and isolating the trachea, a tracheal tube was inserted for artificial ventilation. Next, the carotid artery was identified and isolated, and a catheter was inserted to monitor the blood pressure. The depth of anesthesia was adjusted to maintain heart rate between 300-350 beats per minute and blood pressure between 60-90 mmHg through the experiments. Body temperature was measured using a rectal thermometer and was maintained at 37.0 ± 0.2 °C using an electric heating pad.

After shaving the masticatory muscle area and positioning the head prone in a stereotaxic frame, the stimulating needle electrodes were inserted into the masticatory muscle. Also, a loop wire was inserted into the skin of the lip to be connected to the electrodes that stimulate the skin. Electrical stimulation of the lip and muscle was done with stimulating electrodes inserted into these tissues.

The rat's head was shaved and the skin of the skull over the dorsal surface was reflected by a longitudinal incision. A trephination was made in the right side of the parietal bone of the skull using a drill to permit the insertion of a 27-gauge needle attached to a 10µl Hamilton syringe vertically into the trigeminal ganglion using a Kopf electrode manipulator. The needle was passed through the rat's brain until reaching the base of the skull. Microinjection of 3 µl per injection was done into the trigeminal ganglion using this catheter. Another incision was made to

allow the insertion of a microelectrode into the caudal brain stem. In order to permit electrode access, the overlying neck skin and muscle was dissected along the midline, and after removing the dura covering the brainstem, a recording parylene-coated tungsten microelectrode (0.010", 2 MΩ, A-M Systems Inc., Carlsborg, WA, USA) was placed in contact with the caudal brain stem to record trigeminal sensory neurons (Figure 2.2).

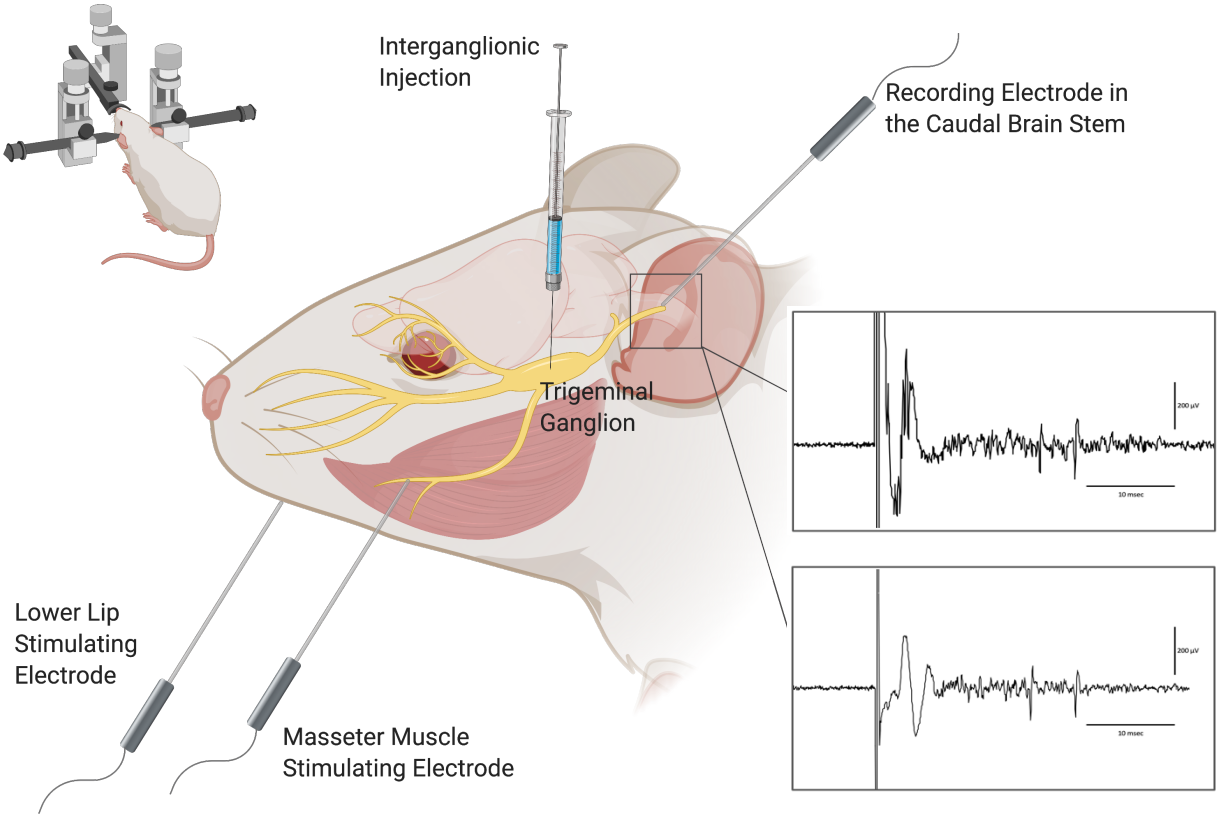

Figure 2.2 Schematic diagram of the neuronal recording setup.

Two stimulating electrodes was inserted in the masseter muscle and skin of lower lip. The recording electrode was inserted into the caudal brain stem, and the needle connected to the Hamilton syringe was inserted vertically into trigeminal ganglion. The stimulating electrode stimulated the skin and muscle every 6 seconds and after 600 seconds, the test substance was injected. The evoked response to the electrical stimulus was recorded pre and post injection for a duration of 2400 seconds. (*Created with BioRender.com)*

2.3.4 Stimulation and recording:

The minimum threshold to stimulate the muscle and skin neurons was identified. The stimulation intensity was 0.9-1.1 mA and 0.3-0.5 mA (duration 100 μs) for skin and muscle respectively and stimulation was applied at a frequency of 0.333 Hertz. Trigeminal sensory neurons that responded to the electrical stimulation of both the muscle and skin were used for the experiments. The intensity of the stimulation current was adjusted to produce at least one action potential per stimulation (see example, Figure 2.2).

2.3.5 Experimental design

Experiments to evaluate the effect of interaganglionic injection of muscimol (3 μl, 10 mM), baclofen (3 μl, 10 mM) or vehicle (3 μl, PBS) on sensory transmission were done on female and male rats prepared as per surgical procedure mentioned above. After identification of trigeminal sensory neurons that responded to electrical stimulation of the skin and muscle, the threshold for activation was determined. Threshold electrical stimulation was applied every 3 seconds alternating between muscle and skin stimulation for the duration of the experiment. The baseline (pre-injection) responses to the electrical stimulation were measured for 600 seconds (10 minutes), and the mean baseline response per stimulus was calculated as the average of 100 evoked responses from 0 to 600 seconds. At 600 seconds, a dose of one of the test substances was injected to trigeminal ganglion. The neuronal response to electrical stimulation after injection was recorded for 1800 seconds (30 minutes). The rats were euthanized at the end of the experiments by administration of 100 mg/kg pentobarbital through carotid artery catheter. The average response per electrical stimulus after the injection of a test substance was calculated using the sum of responses divided by the number of stimuli (100 stimuli per 600 seconds) for

each 10 minute post-injection time epoch (first time epoch (600-1200s), second time epoch (1200-1800s) and third time epoch (1800-2400s)) (Figure 2.3).

In order to confirm that the needle was inside the trigeminal ganglion, at the end of some experiments, Evans blue dye was injected to trigeminal ganglion in the same stereotaxic position as the test substance was injected. The rat was euthanized, and the trigeminal ganglion was dissected from brain tissue. The existence of dye in TG confirmed that the needle was injected to the trigeminal ganglion.

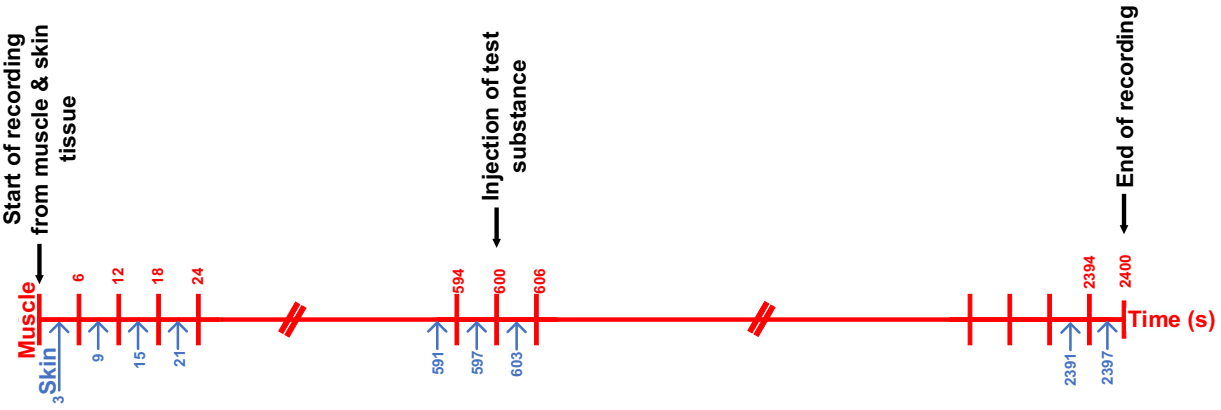

Figure 2.3 Timeline of electrical stimulation and injection of test substance.

The muscle and skin tissue were stimulated every 6 seconds. Therefore, neuron was stimulated every 3 seconds. After 10 minutes, test substance was injected to the ganglion, and the electrical stimulation continued for 1800 seconds.

2.3.6 Data analysis

Spike 2 (Cambridge Electronic Devices, UK) was used to sort the action potentials. Mean response per stimulus was calculated by dividing the sum of the evoked action potentials by the number of stimuli (100) applied in each 10 minute period. To calculate the relative response per stimulus, the mean response for each 10 minute period after injection was divided by the baseline

response. Data were analyzed with a two-way repeated measures ANOVA with treatment and time as factors. A Holm-Sidak test was employed for post hoc comparisons in case of significant ANOVAs. Data were analyzed using SigmaPlot for Windows (Systat Software Inc., San Jose, CA, USA). A P value less than 0.05 was considered as statistically significant. Analysis was done blinded to the test substances.

2.4 Electrophysiological Afferent Fibers Recording

2.4.1 Animals

Female (250–330 g, n=30) and male (260–590 g, n=30) Sprague–Dawley rats (Charles River, Canada) were used for the evaluation of the effects of GABA (500 mM and50 mM), muscimol (10 mM), baclofen (10 mM) on afferent mechanical threshold. Animals were kept in a 12-hour light/ dark cycle. All procedures were approved by the University of British Columbia Animal Care Committee.

2.4.2 Drugs

GABA, Muscimol and baclofen were purchased from Sigma-Aldrich (MO, USA). Drugs were prepared in PBS for injection. Drugs were prepared in PBS for injection to achieve 10 mM concentrations of muscimol and baclofen. Two concentrations of GABA were prepared 50 and 500 mM and the pH was adjusted to pH 7.0 with NaOH (Sigma). Three µl of muscimol 10 mM, baclofen 10mM, GABA 50 mM, GABA 500 mM and PBS were stored in microcentrifuge tubes and were frozen at -20°C until use. The experimenter was blinded to the treatments being administered. Pentobarbital (Euthanyl; Bimeda-MTC Animal Health Inc. ON, Canada) was stored at room temperature (15°-30° C).

2.4.3 Surgical procedure

Sprague-Dawley rats were deeply anesthetized with 2-2.5% isoflurane in 97-98% oxygen. To record the heart rate, two wires connected to the ECG electrode incised near either side of the heart. A rectal thermometer was used to measure and maintain the temperature at 37±0.5°C throughout the procedure by a heating pad. After making an incision and isolating the trachea, a tracheal tube was inserted for artificial ventilation. An Ugo Basile ventilator with the rate 50-60 ventilations per min was used. Next, the left carotid artery was identified and isolated, and a catheter was inserted to monitor the blood pressure. The depth of anesthesia was adjusted to maintain heart rate between 300-350 beats per minute and blood pressure between 60-90 mmHg through the experiments. Body temperature was measured using a rectal thermometer and was maintained at 37.0 ± 0.2 °C using an electric heating pad.

After shaving the head and positioning the rat prone in a stereotaxic frame (Kopf, USA), the skin of the skull over the dorsal surface was reflected by a longitudinal incision. A trephination was made in the right side of the parietal bone of the skull using a hand drill to permit the insertion of a 27-gauge needle attached to a 10µl Hamilton syringe and to lower a microelectrode for recording using a Kopf electrode manipulator (model number, Kopf, USA). The needle was passed through the rat's brain with 30° angle until reaching the base of the skull (Laursen et al. 2014). Since the goal was to insert the needle into the trigeminal ganglion, Evans blue dye was injected to confirm the aim with the same angle and stereotaxic position as the needle was first inserted. This procedure is described in detail in the Experimental Design section. Microinjection of 3 µl per injection was done into the trigeminal ganglion using this

catheter. A stimulating electrode was inserted into the caudal brainstem to permit antidromic identification of masseter muscle afferent fibers that project to the trigeminal subnucleus caudalis; an important relay of craniofacial pain (Cairns et al. 2002; Laursen et al. 2014). In order to permit electrode access, the overlying neck skin and muscle were dissected along the midline, and after removing the dura covering the brainstem, a stimulating parylene-coated tungsten microelectrode (0.010", 2 MΩ, A-M Systems Inc., Carlsborg, WA, USA) was placed in contact with the caudal brain stem (Figure 2.4). All the surgical procedure stated above was previously developed in the lab (Cairns et al. 2002, 2003).

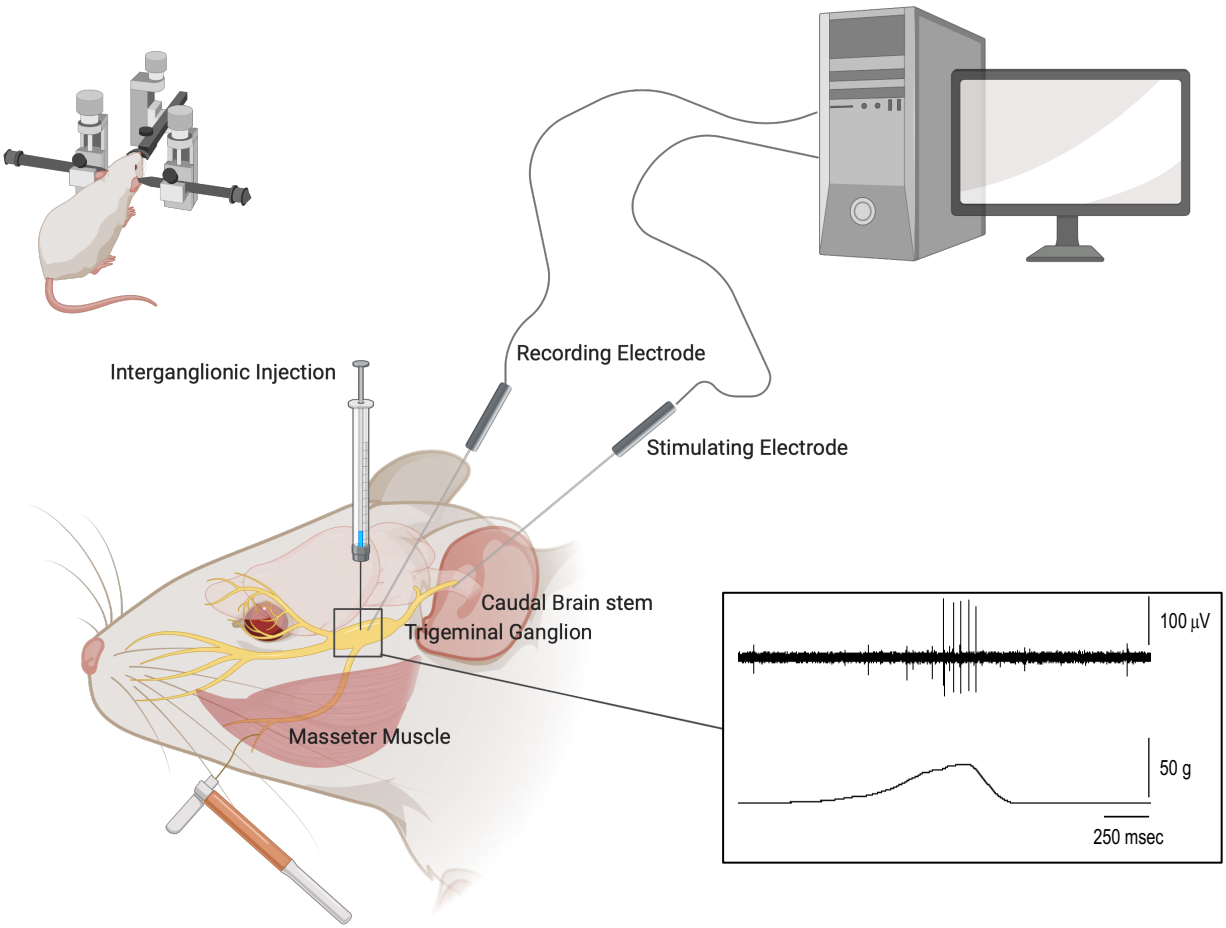

Figure 2.4 Schematic diagram of the afferent fibers recording setup.

The recording electrode was inserted into the trigeminal ganglion, and the needle connected to the Hamilton syringe was inserted into trigeminal ganglion with 30° angle until reaching the base of the skull. The mechanical force was applied to the muscle using electronic von Frey hair. The action potential discharges are shown in the upper trace and the mechanical force being applied to the afferent fiber receptive field of this muscle afferent fiber with the electronic von Frey hair is shown in the lower trace. (*Created with BioRender.com)*

2.4.4 Stimulation and recording

In order to determine the position of recording electrode in the part of ganglion that innervates the mandibular nerve territory, a cotton swab was brushed over the V3 innervated area of the face (whisker pad, chin and side of face). Trigeminal ganglion neurons that responded to mechanical stimulation of the muscle were used for the experiments. Afferent fibers that responded to the overlying skin of the masseter muscle were excluded from the experiment. The skin was pinched and poked to provide evidence that the afferent fibers did not innervate the skin. Since nociceptors that innervate the orofacial region project to the caudal brain stem, antidromic collision was used to verify the projection of TG neurons to the caudal brain stem (Cairns et al. 2002; Laursen et al. 2014). The conduction velocity of the afferent fibers was calculated by dividing the distance between the recording and stimulating electrode by the antidromic latency (Cairns et al. 2002). An electronic von Frey hair (model 1601C, Life Science, USA) was used to evaluate the mechanical sensitivity in the masseter and temporalis muscle afferent fibers. Mechanical activation threshold (grams of force) was recorded as the minimum force by von Frey hair needed to obtain an action potential. Von Frey hair was applied at 1 min intervals for recording of mechanical activation threshold of baseline and post injection of drugs.

2.4.5 Experimental design

Experiments were undertaken to evaluate the effect of GABA (500 mM and50 mM), muscimol (10 mM), baclofen (10 mM) (Sigma-Aldrich) and vehicle (phosphate buffered, isotonic saline) on the excitability and mechanical activation threshold of afferent fibers. The baseline mechanical activation threshold was recorded for 10 minutes. An individual threshold determination was made each minute, and the mean baseline activation threshold was calculated

as the average of the 10 individual thresholds from 0 to 10 minutes. Following a 10 minute baseline recording to determine spontaneous afferent discharge, a dose of test substance was injected into the trigeminal ganglion. Afferent discharge evoked from this injection was recorded for 10 minutes after the injection. Next, the mechanical activation threshold was recorded for 30 min at 1 min intervals using the electronic von Frey hair. The rats were euthanized at the end of the experiments by administration of 100 mg/kg pentobarbital through carotid artery. In order to confirm that the needle was inside the TG, following the end of some experiments, Evans blue dye was injected to trigeminal ganglion in the same stereotaxic position as the test substance was injected. The trigeminal ganglion was dissected from brain tissue. The presence of dye in trigeminal ganglion confirmed that the needle injected into the trigeminal ganglion.

2.4.6 Data analysis

Cumulative discharge was calculated by subtracting the sum of action potentials which occurred for the 10 minute period after injection from the sum of action potentials which occurred during the 10 minute pre-injection baseline period. One way ANOVA was used to compare cumulative discharge evoked by the vehicle control with that evoked by muscimol, baclofen and GABA. The average response per mechanical stimulus for each 10 min was calculated using the sum of mechanical thresholds divided by the number of stimuli (10 stimulus) for each 10 min. To calculate the relative mechanical activation threshold, the mean mechanical activation threshold for each 10 minute epoch after injection was divided by the baseline mechanical activation threshold. Relative mechanical activation threshold data was analyzed with a two-way repeated measures ANOVA with treatment and time as factors. A

Holm-Sidak test was employed for post hoc comparisons in case of significant ANOVAs. SigmaPlot for Windows (Systat Software Inc., San Jose, CA, USA) was used to run the statistical tests. Spearman correlation coefficient analysis was used to assess the association between conduction velocity and mechanical threshold. A P value less than 0.05 was considered as statistically significant. Data analysis was done without knowledge of the content of the injections (blinded).

Chapter 3: Results

3.1 Expression of GABA receptors

The expression of $GABA_A$ and $GABA_B$ receptors by trigeminal ganglion neurons that innervate both labial skin and masseter muscle was investigated using immunohistochemistry. The trigeminal ganglion neurons that innervated the labial skin were labeled with rhodamine and those that innervated the masseter muscle were labeled with fast blue. Analysis demonstrated that $GABA_A$ and $GABA_B$ receptors are expressed by the somas of afferent fibers that innervate both skin and muscle.

A total of 706 masseter muscle ganglion neurons and 1420 labial skin ganglion neurons with a median cell body diameter of 24.7 μM (Range 4.8-78.3 μM) and 26.1 μM (Range 9 - 149 μM), respectively, were identified. The mean frequency of expression of $GABA_A$ and $GABA_B$ receptors by masseter ganglion neurons was 62.5% (N=441) and 20.3% (N=143), respectively (Figure 3.2). In skin ganglion neurons, the frequency of expression of $GABA_A$ and $GABA_B$ receptors was 92.7% (N=1317) and 55.4% (N=786), respectively (Figure 3.1). The co-expression of $GABA_A$ and $GABA_B$ receptors in masseter and skin ganglion neurons was 18.4% (N=130) and 54.8% (N=778), respectively. Almost all the ganglion neurons that were positive for $GABA_B$ receptors also expressed $GABA_A$ receptors. In both skin and muscle ganglion neurons, the expression of $GABA_A$ was significantly higher than $GABA_B$ (P value 0.001 and <0.001, respectively). There was a significantly higher frequency of $GABA_A$ as well as $GABA_B$ receptor expression in ganglion neurons that innervated the skin compared with those that innervated muscle (both P value< 0.001) (Figure 3.4).

In males, 310 masseter muscle ganglion neurons and 750 labial skin ganglion neurons with a median cell body diameter of 24.9 μm (Range 4.8-78.3 μm) and 27.2 μm (Range 9-58.7 μm), respectively, were identified from trigeminal ganglion sections of male rats. In females, 396 masseter muscle ganglion neurons and 670 labial skin ganglion neurons with a median cell body diameter of 24.6 μm (Range 9.3-50.9 μm) and 25.1 μm (Range 10.9-149 μm), respectively, were identified. There was no significant difference in the average diameter of these ganglion neurons when males and females were compared. While there was a wide range of neurons with different diameters, the highest expression of $GABA_A$ and $GABA_B$ receptors was in ganglion neurons with a diameter of 12-30 μm in both sexes.

In male masseter ganglion neurons, the mean frequency of $GABA_A$ and $GABA_B$ receptor expression was 52.9±7.5% (N=161) and 28.8±9.3% (N=71), respectively. In male skin ganglion neurons, the mean frequency of $GABA_A$ and $GABA_B$ receptor expression was 98.2±1.1% (N=740) and 71.3±11% (N=750), respectively. The mean frequency of co-expression of $GABA_A$ and $GABA_B$ receptors in masseter and skin ganglion cells of male rats were 26.1±9.7% (N=64) and 71.3±11% (N=581), respectively.

In females, the mean frequency of $GABA_A$ and $GABA_B$ receptor expression by masseter ganglion neurons was 68.2±4.9% (N=280) and 18.6±6.1% (N=72), respectively. In skin ganglion neurons of females, the mean frequency of $GABA_A$ and $GABA_B$ receptor expression by masseter ganglion neurons was 85.5±5% (N=577) and 28.2±7.8% (N=205), respectively. The mean frequency of co-expression of $GABA_A$ and $GABA_B$ receptors in masseter and skin ganglion cells of female rats were 16.1±6.3% (N=66) and 27.1±7.8% (N=197), respectively.

There was a sex related difference in both $GABA_A$ and $GABA_B$ expression in skin ganglion neurons. In ganglion neurons that innervated the skin, there was a significantly higher

expression of $GABA_A$ receptors (P value = 0.015) and $GABA_B$ receptors (P value = 0.026) in males compared to females.

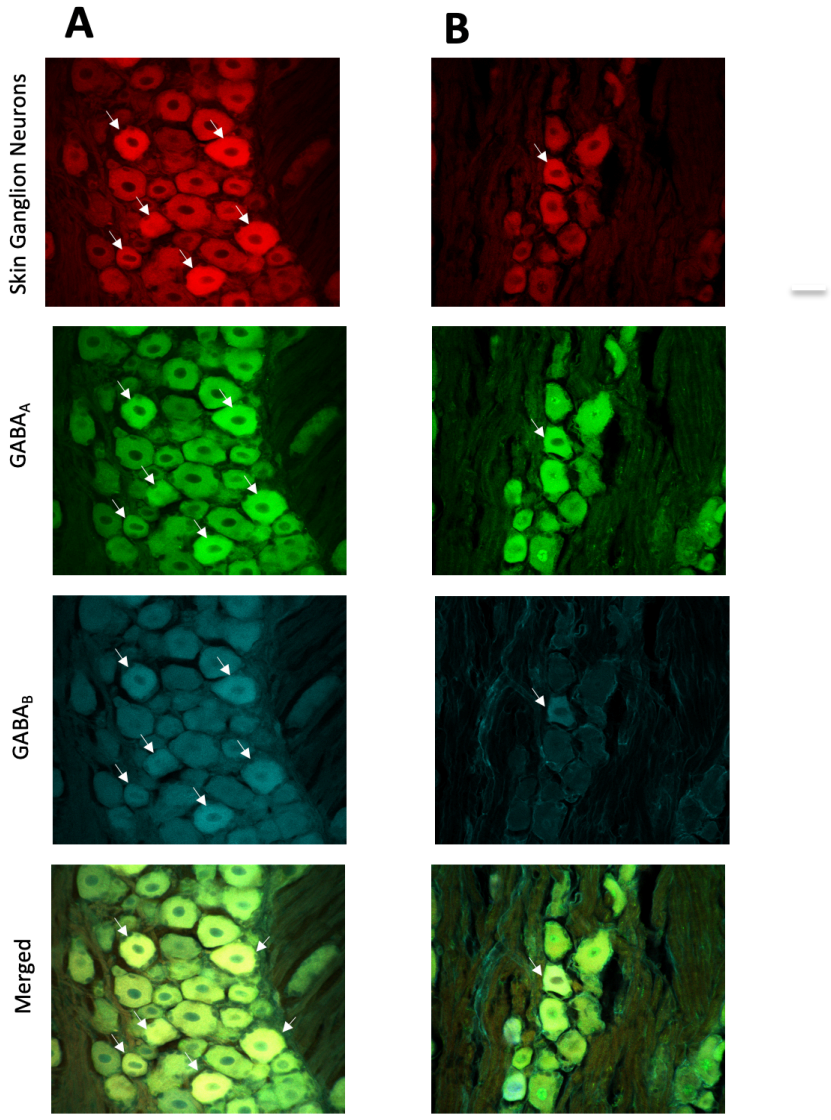

Figure 3.1 GABA receptor expression in labial skin ganglion neurons in male and female rats.

A. Ganglion neurons that innervate skin in male rats (arrows). B. Ganglion neurons that innervate skin in female rats (arrows). There was a significantly higher expression of $GABA_A$ receptors (P value = 0.015) in males compared to females in skin ganglion neurons. Also, the expression of $GABA_A$ was significantly higher than $GABA_B$ (P value 0.001 and <0.001, respectively) in skin afferent fibers (P value 0.001). The white calibration bar on the upper right photo indicates 25 µm.

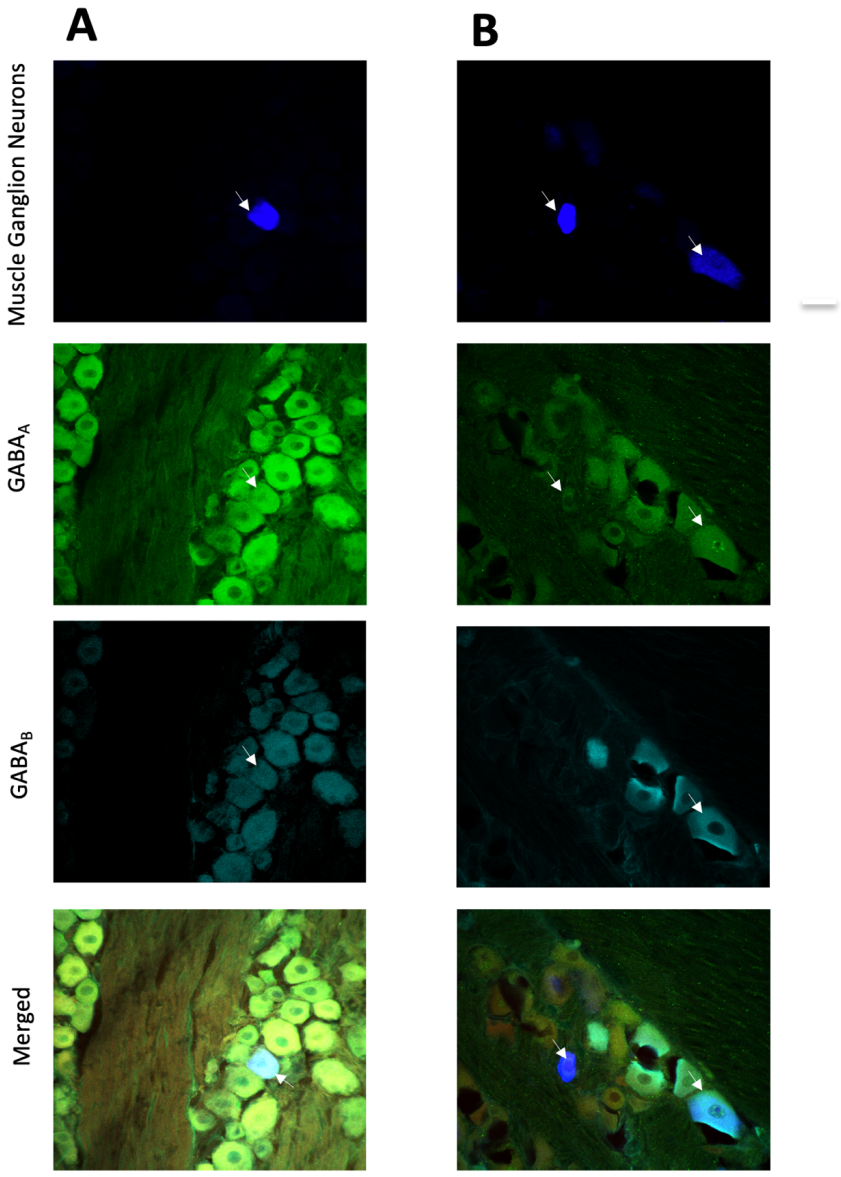

Figure 3.2 GABA receptor expression in masseter ganglion neurons in male and female rats.

A. Ganglion neurons that innervate masseter muscle in male rats (arrows). B. Ganglion neurons that innervate masseter muscle in female rats (arrows). The expression of GABA$_A$ receptors was significantly higher than GABA$_B$ receptors in muscle ganglion neurons (P value <0.001). The white calibration bar on the upper right photo indicates 25 µm.

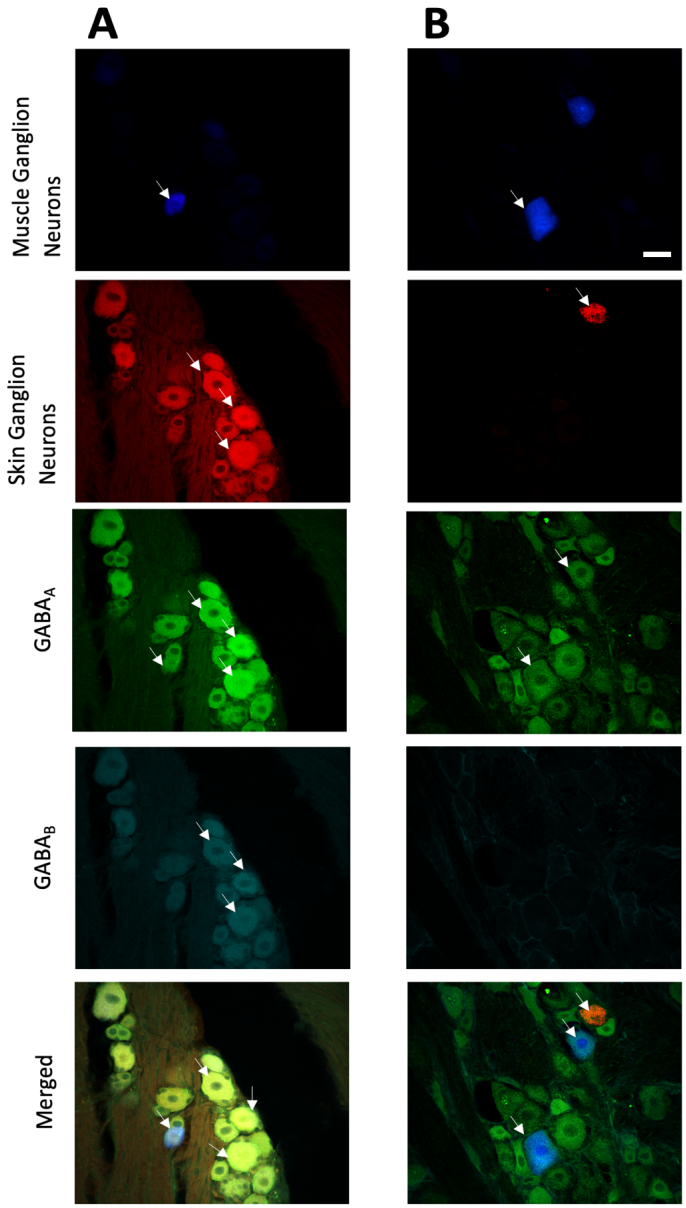

Figure 3.3 GABA receptor expression in labial skin and masseter ganglion neurons in male and female rats. A. Ganglion neurons that innervate masseter muscle (blue with arrow) and skin in male rats (red with arrow). B. Ganglion neurons that innervate masseter muscle (blue with arrow) and skin in female rats (red with arrow). Almost all the ganglion neurons that were positive for $GABA_B$ receptors also expressed $GABA_A$ receptors. There was a significantly higher mean frequency of $GABA_A$ as well as $GABA_B$ receptor expression in ganglion neurons that innervated the skin compared with those that innervated muscle (both P value< 0.001). The white calibration bar on the upper right photo indicates 25 µm.

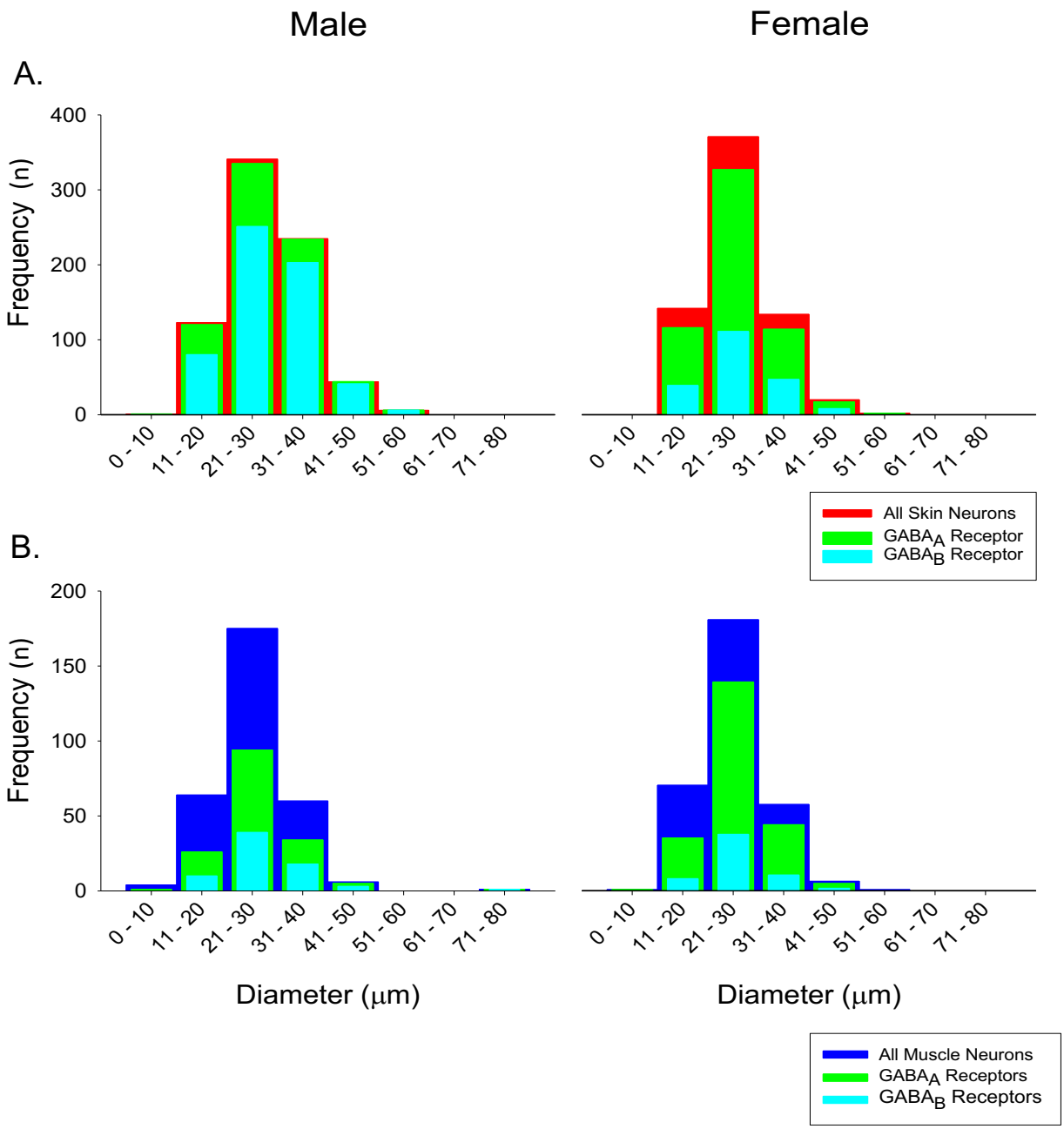

Figure 3.4 Frequency of expression of GABA receptors based on neuronal diameter.

In all groups, neurons with 21-30 μm diameter had the highest expression of both $GABA_A$ and $GABA_B$ receptors. The expression of $GABA_A$ was significantly greater than $GABA_B$ in both ganglion neurons that innervated skin and muscle. Also, there was a higher expression of both $GABA_A$ and $GABA_B$ receptors in skin compared to muscle afferent fibers. In skin, there was significantly higher expression of both $GABA_A$ and $GABA_B$ receptors in males compared to females.

3.2 Effect of GABA agonists on transmission through the trigeminal ganglion

Electrophysiological recordings of brain stem trigeminal neurons were performed to evaluate the effects of intraganglionic injections of $GABA_A$ and $GABA_B$ agonists on the transmission of sensory signals from skin and muscle to the brain stem. Histograms that show the responses of individual trigeminal sensory neurons to electrical stimulation of the labial skin and muscle before and after vehicle, baclofen and muscimol (Figures 3.5, 3.6 and 3.7). The histograms were built from responses recorded from 10 msec before to 100 msec after the electrical stimulation of the muscle and skin. At 0 msec, lower labial skin and masseter muscle were electrically stimulated.

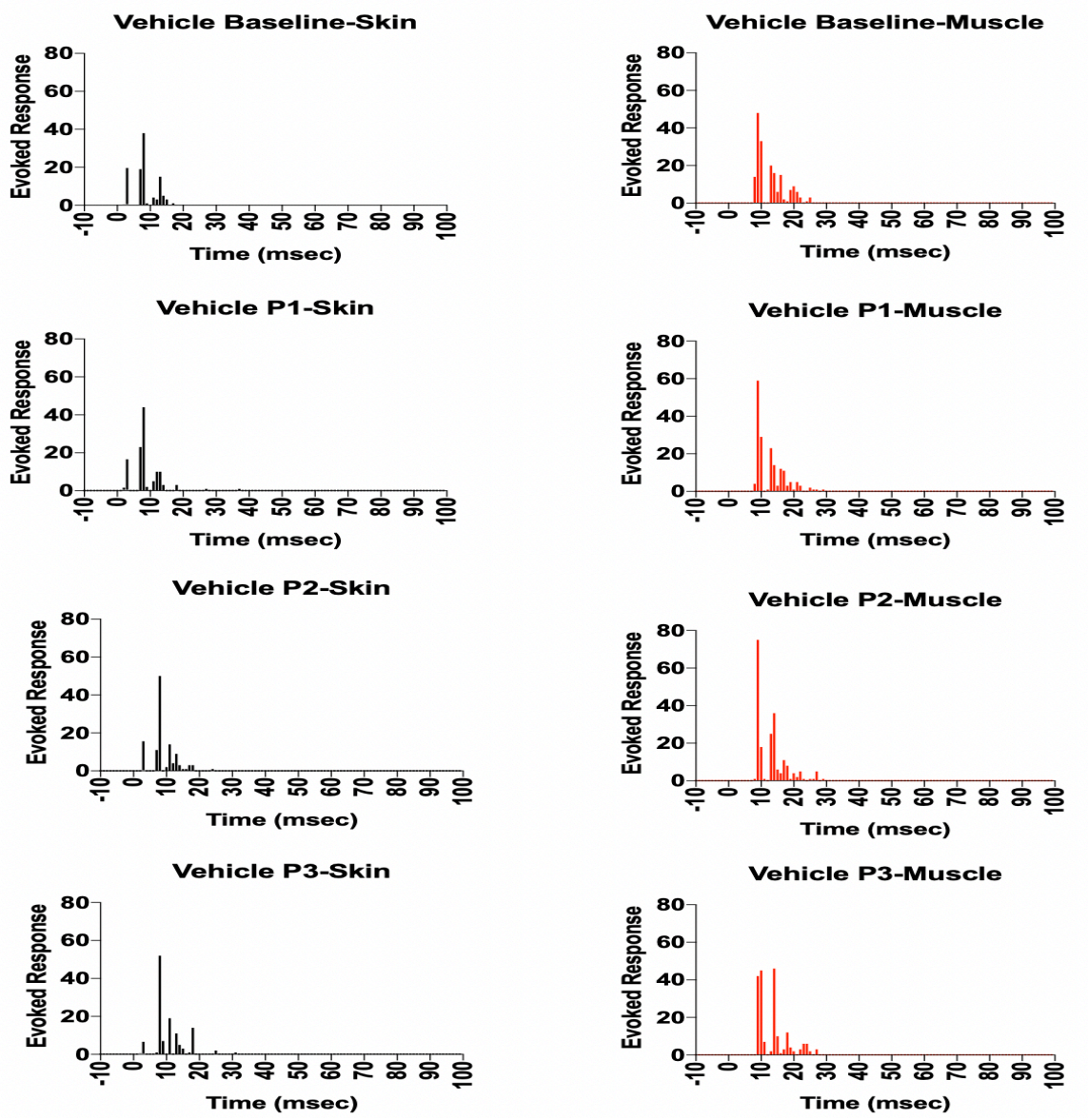

Figure 3.5 An example of histograms illustrating evoked responses from a single trigeminal sensory neuron before and after the intraganglionic injection of vehicle.

The Y axis shows the number of evoked responses and X axis shows time in msec. The evoked responses were recorded for a total of 40 min. 10 min before (baseline) and 30 min after (P1, P2 and P3) the injection of test substances. P1, P2 and P3 refers to 0-10, 10-20, 20-30 min, respectively after the injection of test substance. The histograms were built from 100 evoked responses from 10 msec before to 100 msec after the electrical stimulation of skin and muscle, respectively. In the graphs, at 0 msec the neurons that innervate skin and muscle were electrically stimulated.

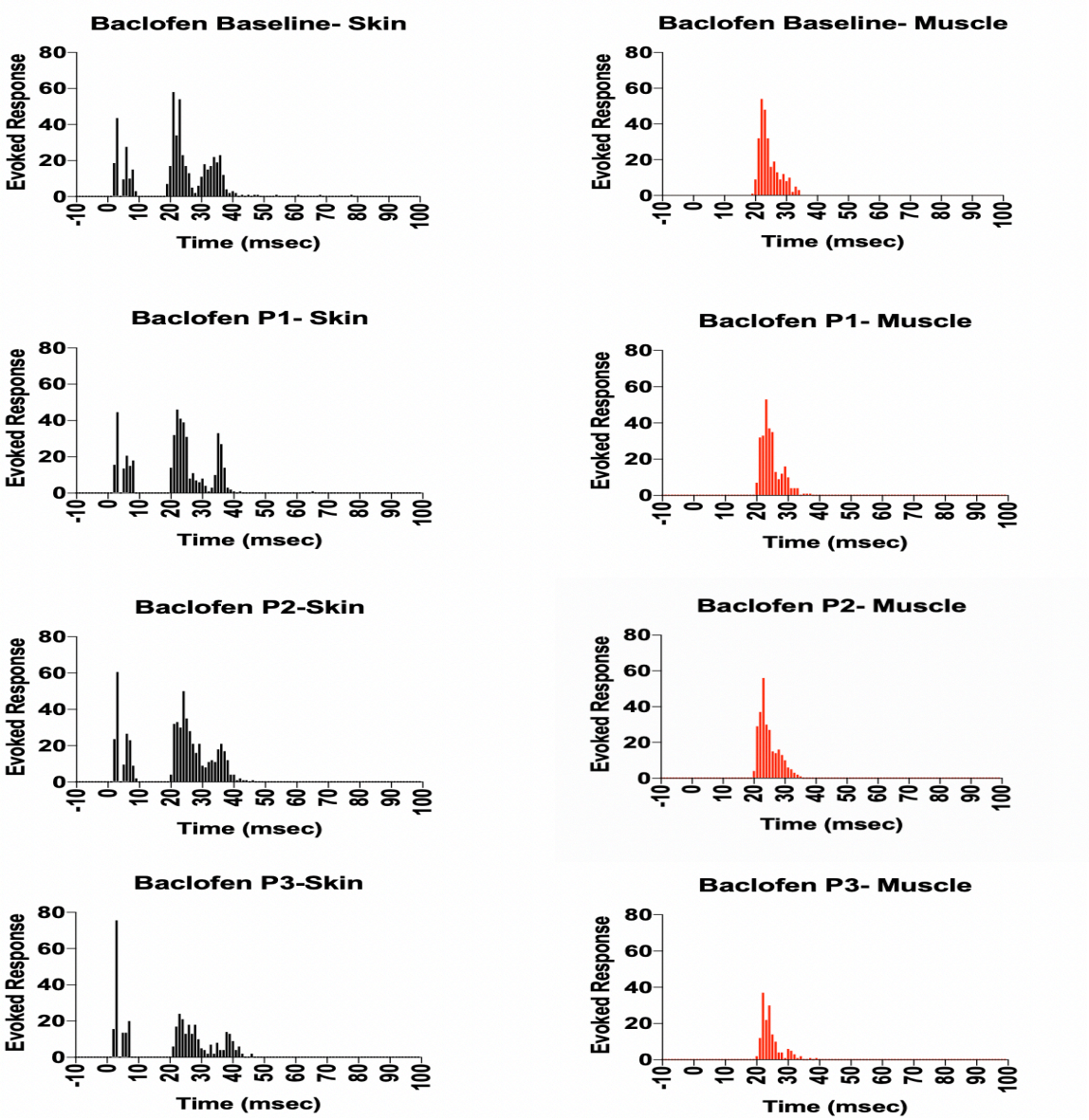

Figure 3.6 An example of histograms illustrating evoked responses from a single trigeminal sensory neuron before and after the intraganglionic injection of baclofen.

The Y axis shows the number of evoked responses and X axis shows time in msec. The evoked responses were recorded for a total of 40 min. 10 min before (baseline) and 30 min after (P1, P2 and P3) the injection of test substances. P1, P2 and P3 refers to 0-10, 10-20, 20-30 min, respectively after the injection of test substance. The histograms were built from 100 evoked responses from 10 msec before to 100 msec after the electrical stimulation of skin and muscle, respectively. In the graphs, at 0 msec the neurons that innervate skin and muscle were electrically stimulated.

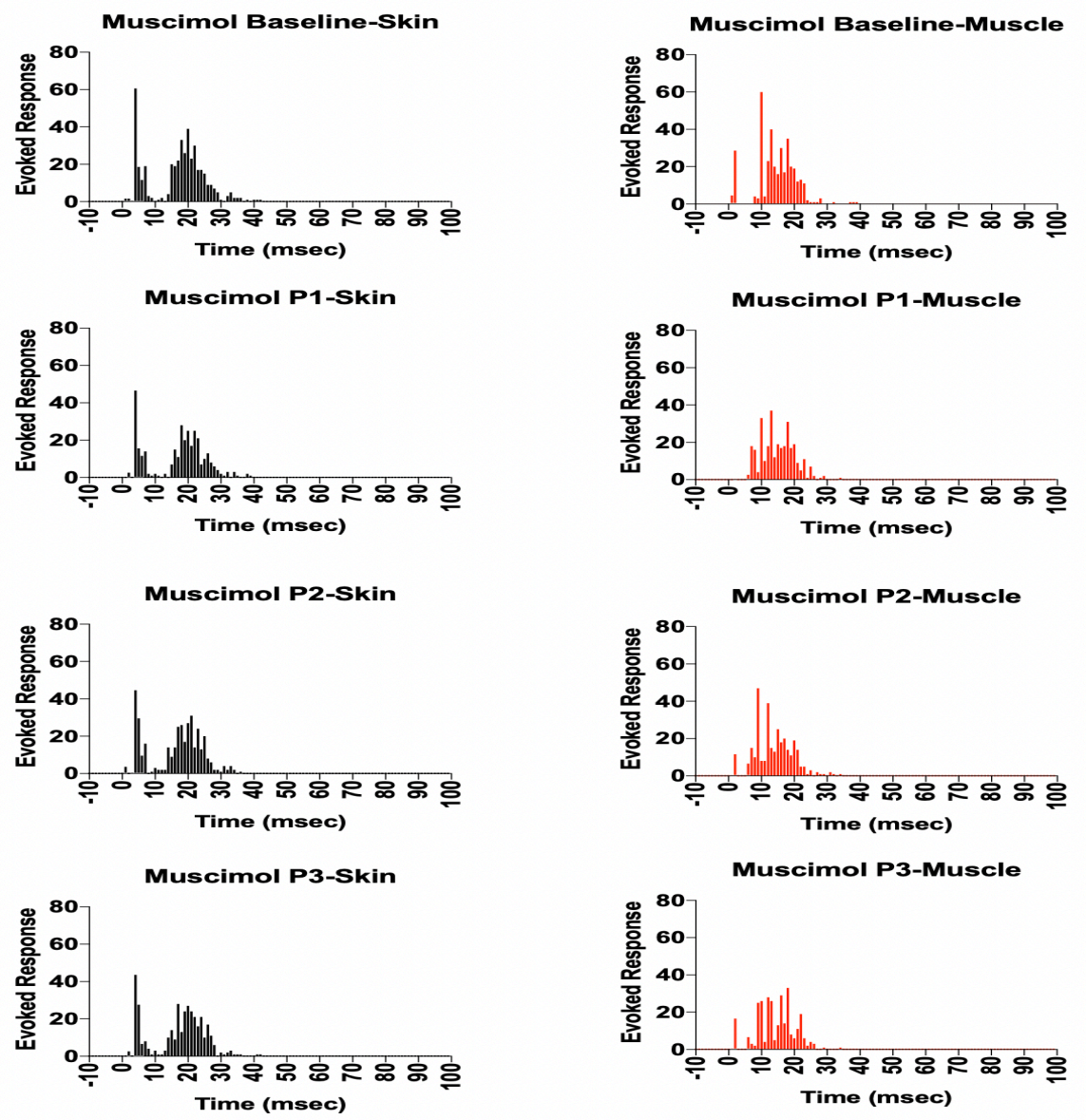

Figure 3.7 An example of histograms illustrating evoked responses from a single trigeminal sensory neuron before and after the intraganglionic injection of muscimol.

The Y axis shows the number of evoked responses and X axis shows time in msec. The evoked responses were recorded for a total of 40 min. 10 min before (baseline) and 30 min after (P1, P2 and P3) the injection of test substances. P1, P2 and P3 refers to 0-10, 10-20, 20-30 min, respectively after the injection of test substance. The histograms were built from 100 evoked responses from 10 msec before to 100 msec after the electrical stimulation of skin and muscle, respectively. In the graphs, at 0 msec the neurons that innervate skin and muscle were electrically stimulated.

Analysis of overall neuronal responses (n=6) before and after intraganglionic injections showed that muscimol inhibited transmission of sensory signals through the trigeminal ganglion compared with vehicle (Figure 3.8). The relative neuronal response from stimulation of the masseter muscle was significantly decreased between 20 and 30 minutes post intraganglionic injection of muscimol when compared with vehicle.

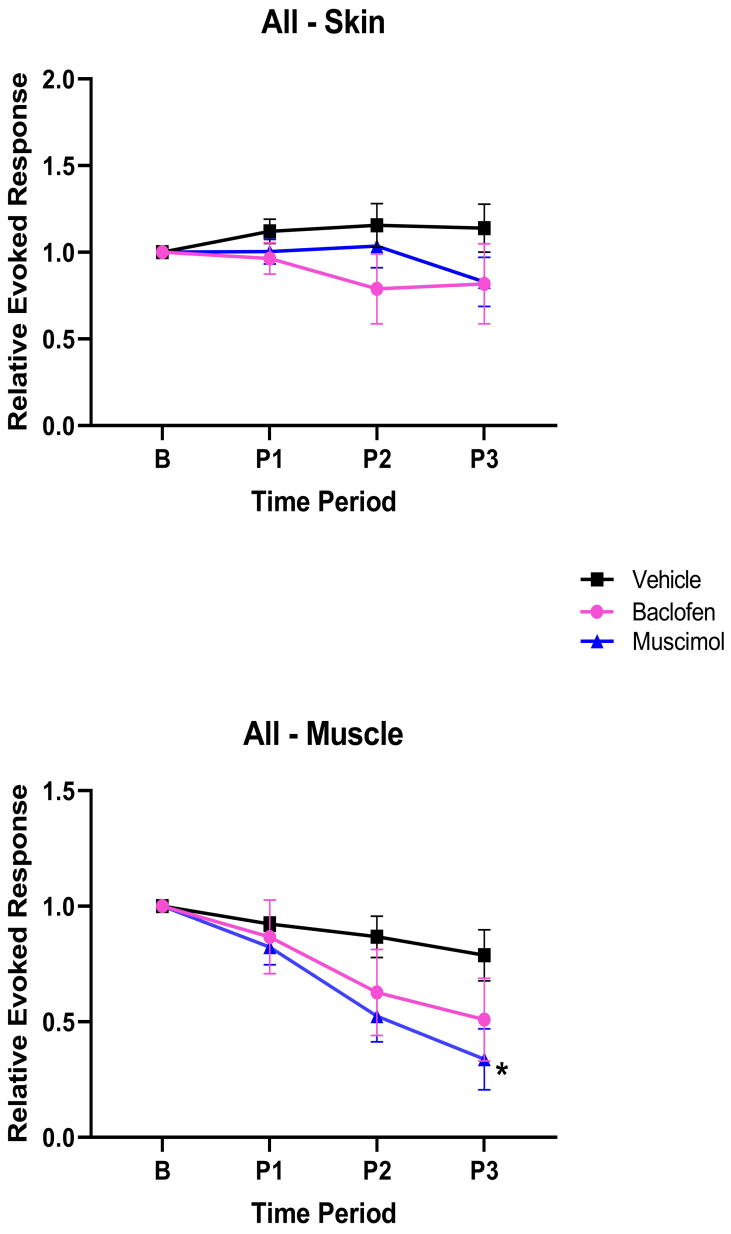

Figure 3.8 Effects of GABA agonists on Relative Evoked Responses in all neurons.

These line and scatter plots show the effect of intraganglionic injection of muscimol and baclofen on the relative evoked response in in trigeminal sensory neurons in response to electrical stimulation of the skin and masseter muscle. The only significant difference between treatment and vehicle was for muscle evoked responses between 20 and 30 minutes post injection of muscimol (n= 6 per group, P values <0.005). B= baseline, P1 = 0-10 minutes, P2 = 10-20 minutes, P3 = 20-30 minutes.

Male and female responses were also assessed separately. In male rats, it was found that both baclofen and muscimol significantly decreased the number of action potentials evoked in trigeminal neurons by stimulation of the labial skin, between 10 and 30 minutes post injection when compared with vehicle. Baclofen significantly decreased the number of action potentials evoked in trigeminal neurons by stimulation of the masseter muscle between 10 and 20 minutes post injection when compared with vehicle (Figure 3.9).

In female rats, there was no significant effect of either treatment on the number of action potentials evoked in trigeminal neurons by stimulation of the labial skin. However, muscimol significantly decreased the number of action potentials evoked in trigeminal neurons by stimulation of the masseter muscle between 10 and 30 minutes post injection when compared with vehicle (Figure 3.9).

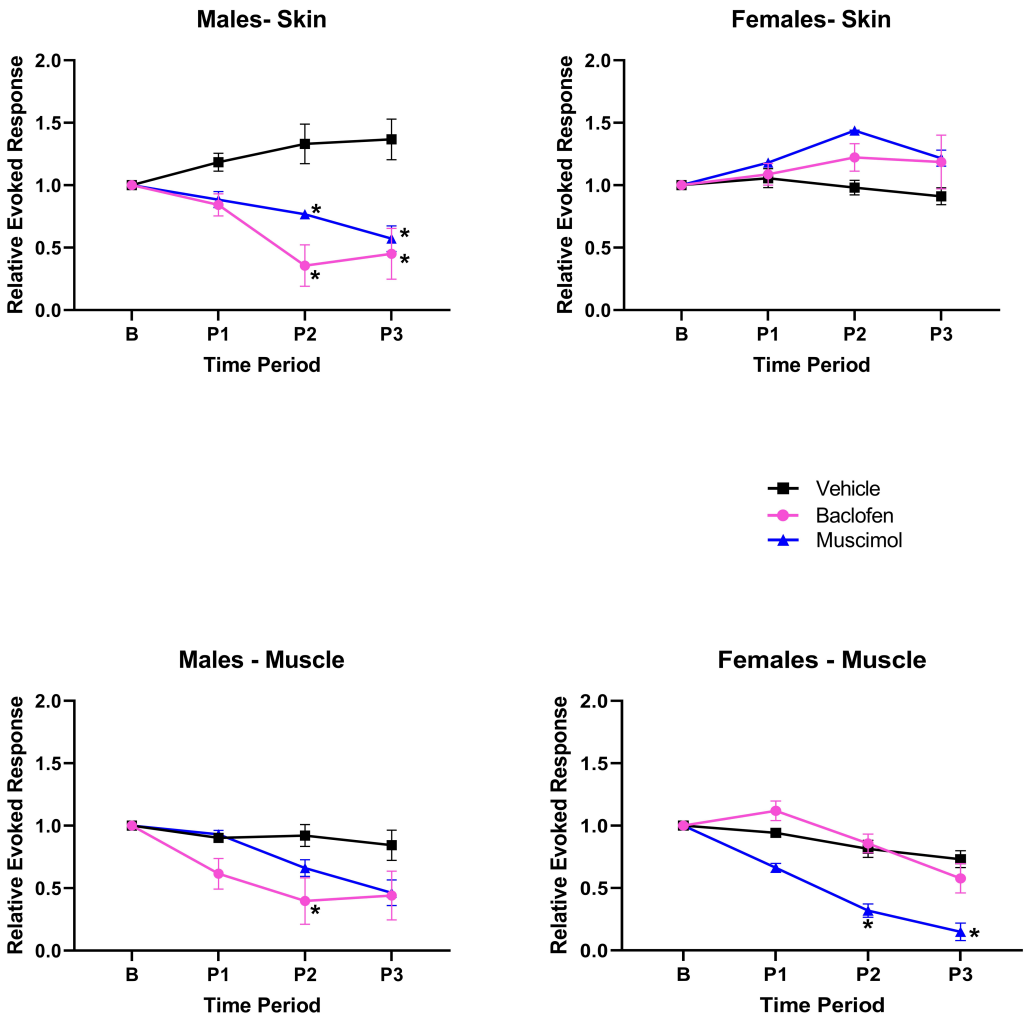

Figure 3.9 Effects of GABA agonists on Relative Evoked Responses in males and females.

The line and scatter plots illustrate the change in relative neuronal relative evoked response over time after intraganglionic injection of vehicle, muscimol and baclofen in males and females. In males, both baclofen and muscimol significantly decreased labial skin evoked neuronal response at P2 and P3, while baclofen significantly decreased masseter muscle evoked neuronal response at P2, when compared with vehicle. In contrast, in females none of the treatments significantly altered labial skin evoked neuronal responses. However, muscimol significantly decreased masseter muscle evoked neuronal discharge at P2 and P3 compared to vehicle control. *: 2-way repeated measures ANOVA, Holm-Sidak post-hoc test, n= 3 per group, P<0.05. Error bars indicate the standard error of the mean. B: -10-0 minutes before injection, P1, P2 and P3, 0-10, 10-20 and 20-30 minutes post injection, respectively.

To assess whether the effects of muscimol and baclofen were different for the short and long latency components of trigeminal neuron response to skin and muscle stimulation, the effect of intraganglionic injection on relative evoked response from 0-10msec (short latency), and 11-100 msec (long latency) (Figure 3.10) were plotted separately. The short latency relative evoked response of neurons to skin and muscle stimulation remained stable over time in the vehicle, muscimol and baclofen groups. The long latency relative evoked response of neurons to skin and muscle stimulation was decreased in the muscimol and baclofen groups when compared with vehicle. However, the differences did not reach statistical significance at any time point.

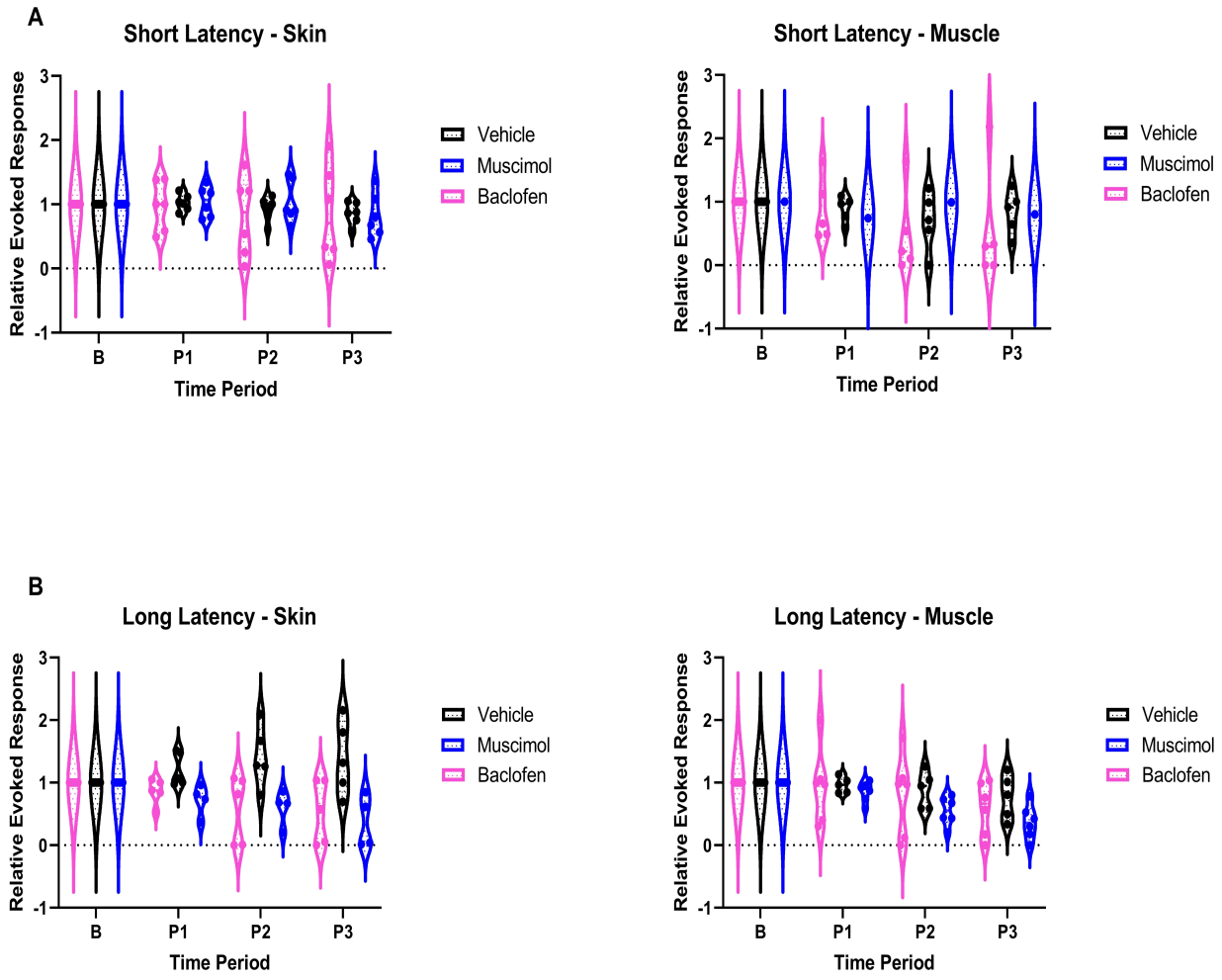

Figure 3.10 Short and long latency responses in skin and muscle ganglion neurons

A. violin plots showing the short latency responses (0-10msec after electrical stimulation) to the skin (left graph) and masseter muscle (right graph) electrical stimulation. The short latency responses did not change over time after injection of GABA agonists compared to the vehicle. B. violin plots showing the long latency responses (11-100msec after electrical stimulation) to the skin (left graph) and masseter muscle (right graph) electrical stimulation. Both muscimol and baclofen decreased the relative evoked response of skin and muscle ganglion neurons compared to the vehicle; however, their decrease compared to the control group did not reach statistical significance at any time point. Kruskal-Wallis ANOVA, Dunn's multiple comparisons test, $P<0.05$. B: -10-0 minutes before injection, P1, P2 and P3, 0-10, 10-20 and 20-30 minutes post injection, respectively.

3.3 Effect on muscle afferent fibers

Data has been collected to examine the effect of intraganglionic injection of GABA (50 and 500 mM), GABA receptor agonists and vehicle on the excitability of trigeminal ganglion neurons that innervate masticatory muscles (masseter and temporalis). Intraganglionic injection of GABA 500 mM, 50 mM, muscimol and baclofen did not evoke action potential discharges in the ganglion neurons that were greater than the vehicle control. However, compared to PBS, all substances significantly reduced MT 30 minutes post injection (Figure 3.11). Further, GABA (500 mM) and baclofen significantly decreased MT compared to PBS for the entire recording period.

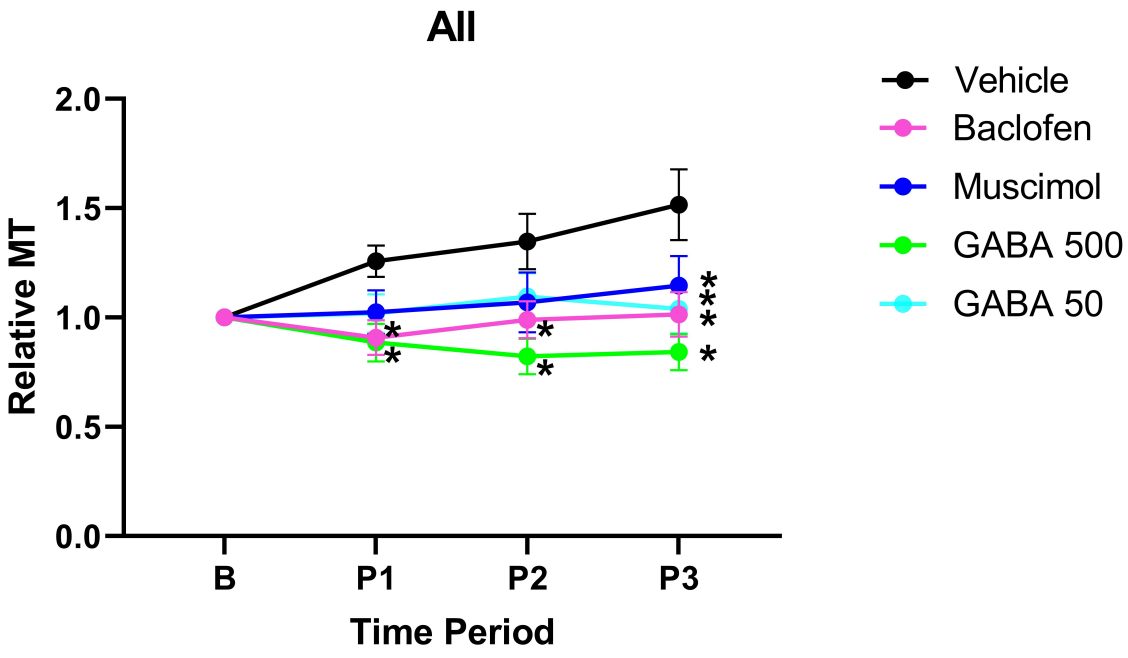

Figure 3.11 Effects of GABA and GABA agonists on MT in all the neurons.

The line and scatter plot shows the effect of intraganglionic injection of GABA 50 and 500 mM concentrations, baclofen 10mM and muscimol 10mM into the TG on masticatory muscle mechanical activation threshold. All test substances changed the mechanical activation threshold of afferent fibers compared to the vehicle control at 30 minutes post-injection. GABA 500mM and baclofen 10mM significantly reduced mechanical activation threshold compared to vehicle at all post-injection time points and all treatments significantly reduced MT compared to vehicle at P3. *: 2-way repeated measures ANOVA, Holm-Sidak post-hoc test, n= 12 per group, P<0.05. Error bars indicate standard error of the mean. B: -20-10 minutes before injection, P1, P2 and P3,10-20, 20-30 and 30-40 minutes post injection, respectively.

After plotting the data separately for each sex, it was observed that in female rats, MT decreased significantly for all GABA receptor agonists 30 to 40 minutes after the injection. However, in male rats, there were not any significant effects of any of the GABA receptor agonists found.

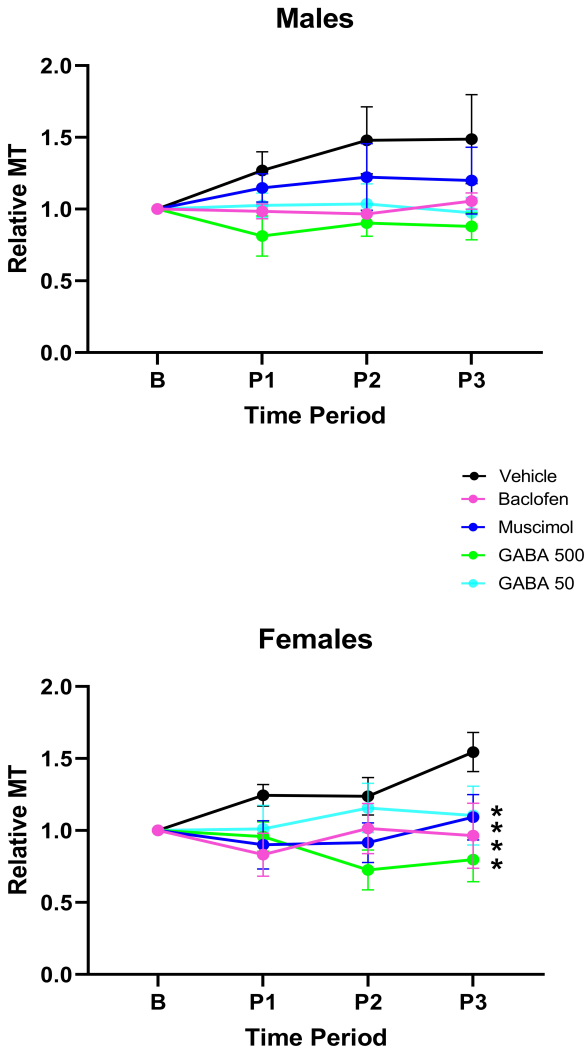

Figure 3.12 Effects of GABA and GABA agonists on MT in males and females.

The effect of intraganglionic injection of GABA, muscimol, baclofen and PBS on the mechanical activation threshold of masticatory muscle afferent fibers is illustrated. The line and scatter plots show how mean relative MT was altered after intraganglionic injection in all masseter ganglion neurons as well as those recorded in males only and females only. In males, there was no significant effect of any of the treatments compared with vehicle. In females, all treatments were significantly different from vehicle at P3. *: 2-way repeated measures ANOVA, Holm-Sidak post-hoc test, n=6 per group, P<0.05. Error bars indicate standard error of the mean. B: -20-10 minutes before injection, P1, P2 and P3,10-20, 20-30 and 30-40 minutes post injection, respectively.

Sixty afferent fibers with conduction velocity between 0.2- 17 m/s were used to evaluate the effect of intraganglionic injection of baclofen and muscimol to the TG. The association between conduction velocity and relative mechanical activation threshold and evoked discharge was assessed (Figure 3.13). For GABA 500 mM, a significant inverse relationship between conduction velocity and relative MT was identified 10 to 20 minutes post injection ($r= -0.580$, P $P=0.045$; Figure 3.13). For muscimol, a significant inverse relationship between conduction velocity and relative MT was identified 30 to 40 minutes post injection (Figure 3.13). No significant relationship between conduction velocity and relative MT at any time point was found for GABA 50 mM, baclofen 10 mM or PBS. These results indicate that after intraganglionic injection of GABA 500 mM or muscimol 10 mM, slowly conducting afferent fibers became less sensitive while faster conducting afferent fibers became more sensitive to mechanical stimulation.

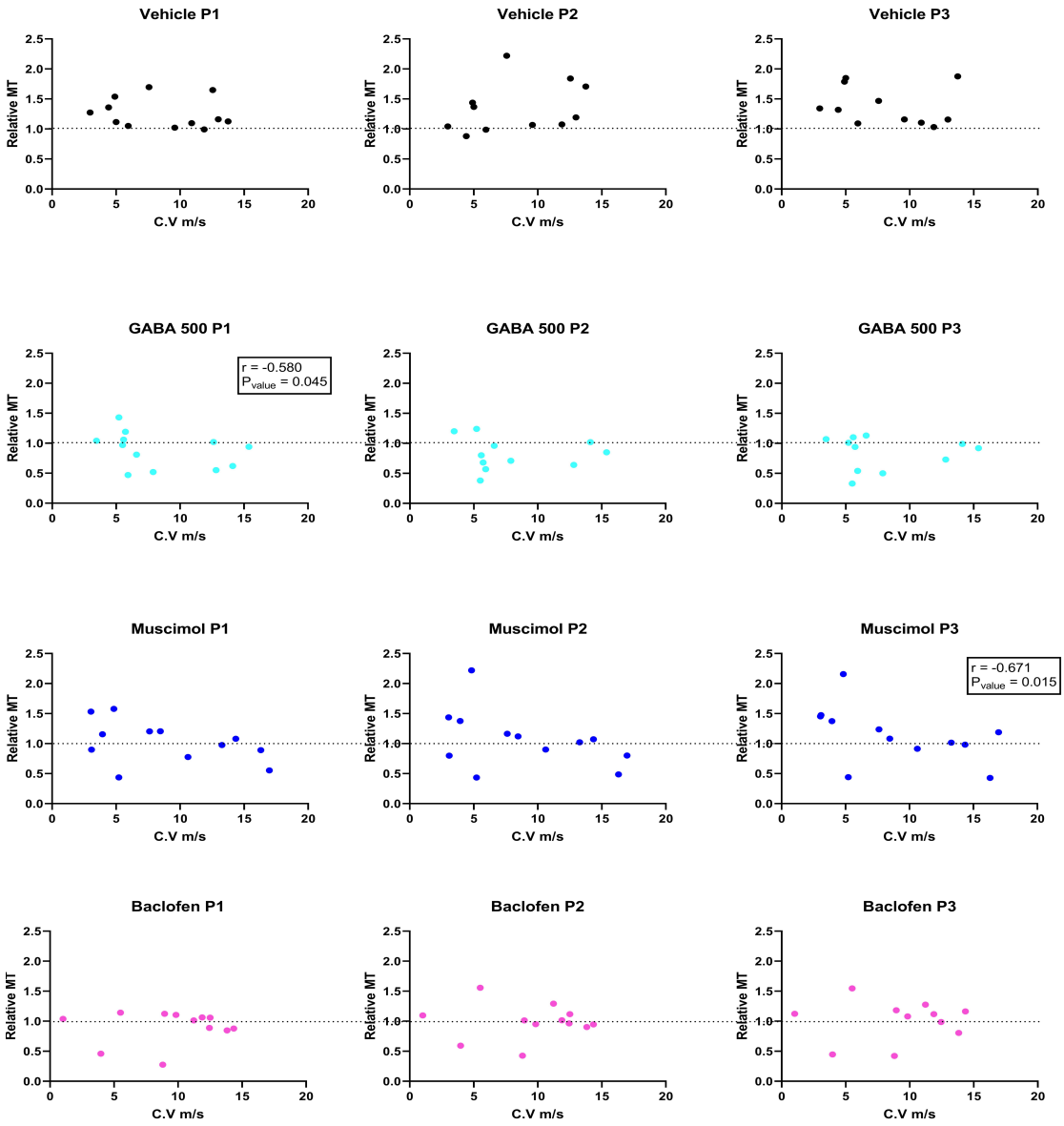

Figure 3.13 Correlation between MT and C.V in all rats.

The scatter plots show the relationship between overall change in relative mechanical activation threshold 30 min post-injection and conduction velocity for vehicle, GABA 500 mM, muscimol 10 mM and baclofen 10 mM injections. The dotted A linear regression analysis indicated that there was a significant inverse relationship between the mechanical activation threshold and conduction velocity for muscimol 10 mM and GABA 500 mM. However, there was no significant relationship seen for baclofen 10 mM. (n=12 per group). The dotted lines indicate no change in mechanical activation threshold relative to baseline. P1, P2 and P3,10-20, 20-30 and 30-40 minutes post injection, respectively.

To explore the effect of sex on the relationship between mechanical activation threshold and conduction velocity, the relationship between conduction velocity and MT was further examined separately for male and female rats. In male rats, a significant inverse relationship was found for muscimol 20 to 40 minutes post injection, and for baclofen 10 to 20 minutes post injection. There was also a significant positive correlation for GABA 50 mM at 20 to 30 minutes post injection. In female rats, no significant correlations were found between conduction velocity and MT after any of the treatments.

Treatment	Male			Female		
	P1	P2	P3	P1	P2	P3
Control	r=-0.08 (0.92)	r=-0.1 (0.95)	r=-0.14 (0.8)	r=-0.6 (0.24)	r=0.49 (0.36)	r=0.37 (0.5)
GABA 50	r=0.8 (0.10)	**r=0.9 (0.03)**	r=0.8 (0.1)	r=0.2 (0.6)	r=-0.3 (0.56)	r=0.4 (0.5)
GABA 500	r=-0.2 (0.71)	r=-0.37 (0.50)	r=-0.43 (0.42)	r=-0.2 (0.71)	r=-0.4 (0.52)	r=-0.2 (0.78)
Muscimol 10	r=-0.77 (0.1)	**r=-0.89 (0.033)**	**r=-0.94 (0.017)**	r=-0.54 (0.3)	r=-0.14 (0.8)	r=-0.43 (0.42)
Baclofen 10	**r=-0.943 (0.017)**	r=-0.657 (0.18)	r=-0.371 (0.5)	r=0.09 (0.92)	r=0.26 (0.66)	r=0.1 (0.95)

Table 3.1 Correlation between MT and C.V in females and males. The table shows the Spearman correlation coefficients and probability value (in brackets) for the relationship between conduction velocity and MT for after each treatment in male and female rats. Significant correlations are in bold. n= 6 per group, P1, 10-20 minutes post injection, P2, 20-30 minutes post injection, P3,30-40 minutes post injection.

3.4 Blood pressure Recordings:

Blood pressure was recorded to assess systemic consequence of the injection of $GABA_A$ and $GABA_B$ agonists into the trigeminal ganglion (Figure 3.13). Muscimol injection caused a drop in BP after injection; however, it came back to baseline 15 mins after injection.

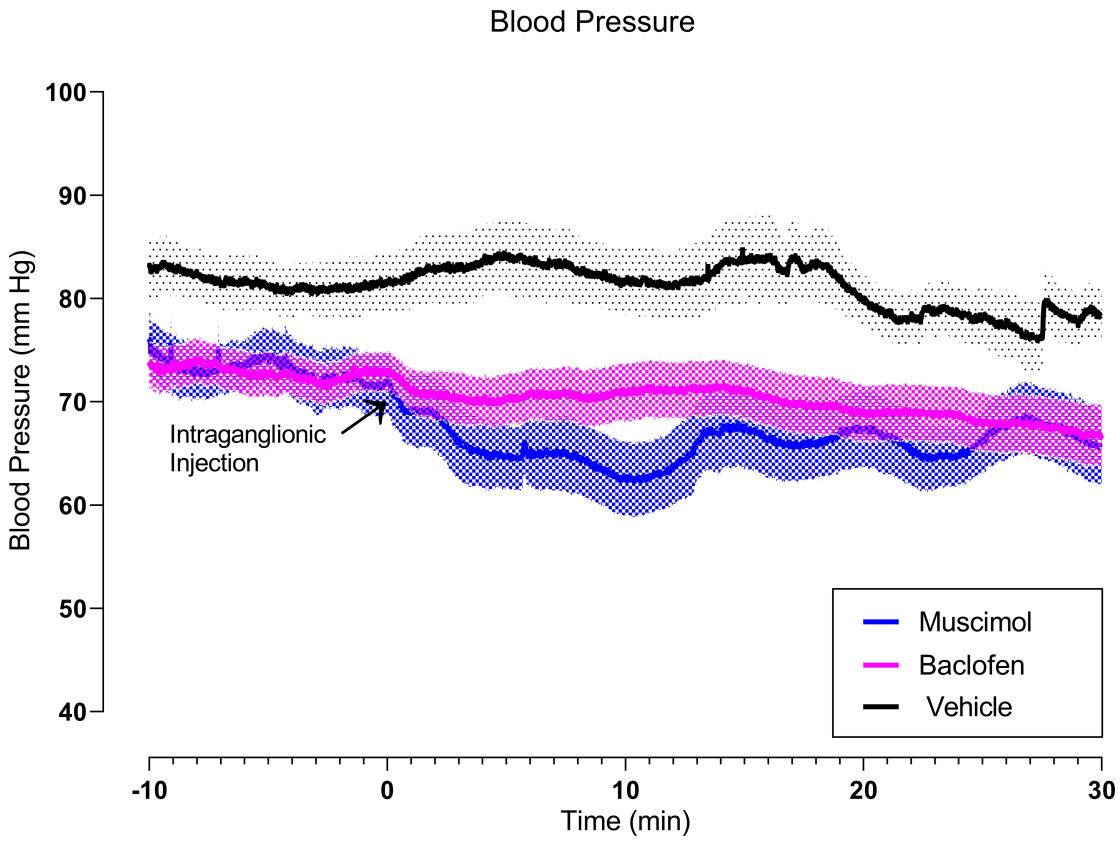

Figure 3.14 BP changes after administrations of GABA agonists.

The line plot indicates the mean blood pressure before and after injection of muscimol (n=10), baclofen (n=11) or vehicle (n=13). The shaded areas indicate Standard Error of the mean. Only muscimol decreased the blood pressure after the intraganglionic injection; however, the blood pressure returns to the baseline approximately 15 minutes after the injection. Baclofen and vehicle did not change the blood pressure compared to their baseline before injection.

Chapter 4: Conclusion

Immunohistochemistry results demonstrated that there was a higher expression of $GABA_A$ and $GABA_B$ receptors in the ganglion neurons with a diameter of 12-30 μm in both sexes. Almost all the ganglion neurons that were positive for $GABA_B$ receptors also expressed $GABA_A$ receptors. There was a significantly higher expression of $GABA_A$ compared to $GABA_B$ receptors by ganglion neurons that innervate both skin and muscle. There was also a significantly higher expression of both GABA receptors in ganglion neurons that innervated the skin compared with muscle. In ganglion neurons that innervated the skin, there was a significantly higher expression of $GABA_A$ and $GABA_B$ receptors in males compared to females. Electrophysiological recordings of brain stem trigeminal neurons were performed to evaluate the effects of intraganglionic injections of $GABA_A$ and $GABA_B$ agonists on the transmission of sensory signals from skin and muscle to the brain stem. The analysis showed that intraganglionic injection of both muscimol and baclofen inhibited transmission of sensory signals through the trigeminal ganglion; however, only intraganglionic muscimol injections significantly decreased neuronal discharge in response to muscle stimulation between 20-30 minutes post injection. Short latency neuronal discharge evoked by skin or muscle (0-10 msec post stimulus) remained stable over time in all treatment groups. Long latency neuronal discharge (11-100 msec post stimulus) to skin and muscle stimulation was decreased by both muscimol and baclofen; however, there were no significant decrease compared to the vehicle in any time point. To further understand where sensory transmission was inhibited, electrophysiological recordings of trigeminal ganglion neurons were performed to evaluate the effects of intraganglionic injections of GABA and GABA agonists on the mechanical stimulation of masseter and temporalis muscle. GABA, muscimol and baclofen did not evoke increased afferent discharge when compared with

the vehicle control. Compared to vehicle, GABA 50 and 500 mM, muscimol and baclofen decreased the mechanical threshold significantly 20-30 min post injection. Also, GABA 500 mM and baclofen decreased the mechanical threshold compared to vehicle at all time points P1 (0-10 min), P2 (10-20 min) and P3 (20-30 min) post injection. There was a significant inverse relationship between conduction velocity and relative mechanical threshold 20-30 min post injection of muscimol and GABA 500 mM.

It has been shown that main proteins required for GABA synthesis and release such as GAD and vesicular GABA transporter (VGAT) are found in sensory neurons of dorsal root ganglion(Du et al. 2017). Also, almost all neuronal cell bodies in trigeminal ganglion contain α1, α5, β2/3, and γ1/2/3 subunits; however, no immunoreactivity was detected for α2 subunit (Hayasaki et al. 2006). α1, α5 subunits were detected in %100 of the ganglion neurons. In this study, to detect all of the $GABA_A$ receptors in trigeminal ganglion, we used alpha 1 primary antibody. Many trigeminal ganglion neurons express glutamate decarboxylase (GAD), which synthesizes GABA from glutamate, and thus contain GABA (Hayasaki et al. 2006; Nakagawa, Hiura, and Kubo 2003; Stoyanova 2004) Also, the immunoreactivity of $GABA_{B1}$ and $GABA_{B2}$ subunits of $GABA_B$ receptors were seen in the trigeminal ganglion (Hayasaki et al. 2012; Takeda et al. 2013). Both $GABA_{B1}$ and $GABA_{B2}$ are necessary for a functional expression of the GABAB receptor (Hayasaki et al. 2012). Therefore, the existence of both subunits suggests that in the trigeminal ganglion neurons the $GABA_B$ receptors could be functional. Both $GABA_A$ and $GABA_B$ receptors are expressed in trigeminal ganglion; however, the present study provides the first demonstration of $GABA_A$ and $GABA_B$ receptors in the ganglion neurons that innervate skin and muscle (Kondo et al. 1994; J. Puri et al. 2012; Takeda et al. 2004, 2013; Vit et al. 2009). We found that the expression of both GABA receptors is greater in skin ganglion neurons than

muscle ganglion neurons which might be due to the high cutaneous innervation of neurons (Nolano et al. 2013). Our laboratory previously found that 95% of tongue epithelial nerve fibers expressed the γ subunit of $GABA_A$ receptor (Tan et al. 2014). In this study, we showed that a similar expression of $GABA_A$ receptors (93%) is found in skin ganglion neurons. In ganglion neurons that innervate muscle, the expression of $GABA_A$ receptor (62%) was substantially lower than that found previously in the tongue or in the labial skin ganglion neurons. Previous studies have reported that all neurons express $GABA_B$ receptors in trigeminal ganglion, which is not similar to my results of $GABA_B$ receptors (55%) in skin or $GABA_B$ receptors (20%) muscle. The possible reason behind this observation is that none of the mentioned studies done on the expression of $GABA_B$ receptors evaluated the expression of $GABA_B$ receptors based on their innervation.

There was significantly higher expression of both GABA receptors in males compared to females. One of the reasons for this observation might be because of sex hormones. Previously, differences in expression of receptors were seen in different sexes. For example, it has been shown that estrogen increased the expression of NMDA receptors in the masseter muscle nociceptors (Dong et al. 2007). In another study, it has been demonstrated that 5-HT3 expression by trigeminal ganglion neurons that innervate muscle was significantly higher in female (66%) than in male (39%)(Sung et al. 2008). Also, female rats showed significantly greater expression of CB_1 and CB_2 in amygdala, hippocampus, prefrontal cortex, and hypothalamus compared to males (Xing et al. 2014). However, in hippocampus and amygdala of rats, expression of serotonin-1A receptor was greater in males compared to females (Zhang et al. 1999). Indeed, it has been found that GAD 65 expression is significantly higher in the sensory cortex of men compared to women (Pandya et al. 2019). A significantly higher expression of the α1, α2, α5 and

β3 subunit of $GABA_A$ receptor was seen in human male superior temporal gyrus compared to female (Pandya et al. 2019); however, in another study, gonadectomy in male rats significantly increased the expression of serotonin-1A in cortex and hippocampus and all gonadectomy mediated changes reversed by concomitant administration of testosterone (Zhang et al. 1999). Therefore, perhaps the higher expression of both $GABA_A$ and $GABA_B$ receptors in skin ganglion neurons of male rats is due to testosterone.

Due to the expression of $GABA_A$ and $GABA_B$ receptors in the skin and muscle ganglion neurons, we performed electrophysiological experiments to see if intraganglionic injections of $GABA_A$ and $GABA_B$ agonists inhibit sensory transmission through the trigeminal ganglion from skin and muscle tissue. Focal application of GABA, muscimol or baclofen into the dorsal root ganglion decreased nocifensive behaviors produced by hind-paw injection of bradykinin, and focal application of $GABA_A$ (bicuculline) and $GABA_B$ (CGP35348) antagonists exacerbated the pain behaviors (Du et al. 2017). Also, application of muscimol into the dorsal root ganglion in the rat sciatic nerve injury model inhibited thermal and mechanical hypersensitivity (Naik et al. 2012). Increasing the levels of GABA synthesis by transfecting satellite glial cells in trigeminal ganglion with glutamic acid decarboxylase decreased the cutaneous pain behaviors in orofacial formalin test by activation of $GABA_A$ receptors (Vit et al. 2009). Reducing the expression of axonal $GABA_A$ receptors increased nocifensive behaviors in models of masseter muscle or temporomandibular joint inflammation (Kramer and Bellinger 2013, 2014). In the present study we found that intraganglionic injection of muscimol and baclofen decreased the sensory transmission through trigeminal ganglion from both masseter and skin tissue; however, only muscimol showed a significant decrease in evoked responses of masseter ganglion neurons compared to the vehicle group. Therefore, we conducted further experiments to evaluate the

effect of GABA and GABA agonists on sensory transmission from the masticatory muscle to the trigeminal ganglion.

Spontaneous afferent discharge did not change significantly after the injection of GABA or selective GABA receptor agonists to the trigeminal ganglion. Muscimol increased discharge in 4 cells out of the 12 cells, and baclofen in 2 cells out of 12 cells. There have been some studies on the evoked activity of afferent neurons after injection of different substances such as glutamate and potassium to the TG. Glutamate exerts its excitatory actions through metabotropic and ionotropic glutamate receptors acting through G-protein coupled secondary messenger systems or ligand-gated ion channels, respectively. N-methyl-D-aspartate (NMDA) receptors are a type of ionotropic receptors that upon their activation, the influx of sodium and calcium ions and efflux of potassium ion increases which will result in depolarization (Akkuratov et al. 2015). Bath application of glutamate caused depolarization of some of the neurons of the trigeminal root ganglion (Puil and Spigelman 1988). Intraganglionic injection of glutamate, the major excitatory neurotransmitter in the nervous system, evoked afferent discharge and this effect was diminished by the NMDA receptor antagonist 2-amino-5-phosphonovalerate (APV) (Laursen et al. 2014). Furthermore, injection of 500 mM potassium chloride to the trigeminal ganglion evoked discharge, and this effect was greater than the cumulative discharge compared to the equivalent concentration of glutamate (Laursen et al. 2014). Therefore, some substances such as glutamate and potassium can lead to the discharge by depolarization of ganglion neurons. In this study, the amount of depolarization produced by GABA is likely less than these substances, because GABA did not produce ganglion neuron discharge.

I showed that intraganglionic injection of GABA and muscimol to the trigeminal ganglion in rats did not evoke activity in most neurons. It has been found that application of

GABA to slices of guinea-pig trigeminal ganglia depolarized a majority of ganglion neurons due to the activation of $GABA_A$ receptors but was not reported to evoke action potential discharge (Puil and Spigelman 1988). Injection of GABA into the rat masseter muscle also did not evoke afferent discharge (Cairns et al. 2001). In my study, there might be the possibility that the depolarizations that occurred after application of GABA did not decrease the membrane potential of the neuron below the threshold to generate action potentials. Furthermore, it should be noted that it was not expected that application of baclofen would evoke discharge due to the inhibitory effects of $GABA_B$ receptor activation.

Injection of other substances to the trigeminal ganglion such as glutamate or potassium changed the mechanical threshold. It has been reported that mechanical threshold decreased significantly after the intraganglionic injection of glutamate and KCl (Laursen et al. 2014). The mechanisms involved in the effects of glutamate might be due to the effects of NMDA receptors since addition of 2-amino-5-phosphonovalerate (APV) to the glutamate injection decreased the mechanical sensitization (Laursen et al. 2014). In a model of burning mouth pain, it has been shown that bath application of muscimol decreased the mechanical sensitivity of tongue afferent fibers compared to control group; however, GABA itself did not show such effects (Tan et al. 2014). In my study, a significant reduction in the mechanical threshold was seen 20-30 min after the injection of 50 and 500 mM GABA, muscimol and baclofen to the trigeminal ganglion compared to the control (PBS) group. GABA 500 mM and baclofen sensitized the afferent endings at all time points after the injection compared to vehicle group. I did not expect to see a decrease in the mechanical threshold after administration of baclofen due to its inhibitory effects. The reduction in the MT after the injection of baclofen might be because of irritation of the tissue.

However, apart from the mechanisms that might be involved due to the pharmacological effects of substances injected to the TG, there are some mechanisms that might explain the sensitization of afferent fibers after the injection of substances. These mechanisms might also be involved in the slight sensitization seen after the injection of muscimol. Injection might depolarize the axons in trigeminal ganglion neurons, and this causes the release of glutamate and neuropeptides like, CGRP and substance P. It has been reported that CGRP released from the peripheral terminals results in the initiation of a cascade of events such as higher synthesis of nitric oxide and sensitization of afferent fibers (Iyengar et al. 2019). Also, studies have shown substance P increases nociceptive sensitization by participation in nociceptive signaling as a neurotransmitter during peripheral sensitization (Sahbaie et al. 2009). The other mechanism that might be involved in the sensitization of afferent fibers is neurogenic inflammation which results in plasma protein extravasation and vasodilation due to release of calcitonin G-related peptide, substance P and glutamate from the trigeminal afferents (Ramachandran 2018). Glutamate can depolarize the membrane potential and thus lower the activation threshold for action potential generation in the ganglion neurons.

Studies did not find significant correlation between conduction velocity and change in mechanical threshold in facial cutaneous and masseter muscle nerve fibers in female rats (Gazerani et al. 2010; Mann et al. 2006). Also, no correlation between conduction velocity and mechanical threshold in tongue afferent fibers after bath application of GABA and muscimol was found (Tan et al. 2014); however, these results were seen in female rats only. In male rats, a significant inverse correlation between conduction velocity and change in mechanical threshold was found in tongue afferent fibers (Tan et al. 2014). These data are consistent with the present study; I found that there was no significant correlation between conduction velocity and change

in mechanical threshold in females in muscle ganglion neurons for the muscimol and baclofen groups; however, there was a significant inverse correlation in male rats in the groups that received intraganglionic injections of muscimol and baclofen. The data from both female and male rats showed that mechanical threshold decreased as the conduction velocity increased in the GABA and muscimol groups in masticatory afferent fibers. In another words, slow conducting afferent fibers were more likely to be desensitized and fast conducting afferent fibers were more likely to be sensitized to mechanical stimulation. In the rat neonatal optic nerve that has slow conduction velocity due to their lower levels of myelination, GABA and isoguvacine, a $GABA_A$ agonist, increased the latency and decreased the amplitude of the compound action potential, resulting in reduction in nerve excitability (Sakatani, Hassan, and Ching 1991). Therefore, the increase in relative mechanical threshold of slowly conducting fibers after injection of GABA and muscimol I found in this study might be because of an increase in the latency of action potentials. In adult rat optic nerve that has faster conducting fibers due to the more myelination, GABA and isoguvacine application resulted in a decrease in latency (Sakatani et al. 1991). Therefore, the decrease in relative mechanical threshold of fast conducting fibers I found might be due to the increase in conduction velocity in these fibers after intraganglionic injection of GABA and muscimol. On the other hand, there was no significant relationship between conduction velocity and mechanical activation threshold of fibers in the rats that received baclofen injections. Therefore, perhaps activation of $GABA_A$ receptor might have a selective effect on afferent fibers innervating masticatory muscle.

The source of GABA in the trigeminal ganglion is still unknown. GABA might come from satellite glial cells (SGC) in the trigeminal ganglion. Trigeminal ganglion neurons and their associated SGCs contain glutamic acid decarboxylase (GAD) 65 (Hayasaki et al. 2006). This is

enzyme maintains physiological production of GABA by decarboxylation of glutamate (Hayasaki et al. 2006). It has been suggested that SGCs may have the role of storing and releasing GABA. In the case of continuous firing of action potential and K+ ions will increase in the space between ganglion neurons and SGCs. This is proposed to result in the release of GABA from SGCs to affect ganglion neurons (Hayasaki et al. 2012). Satellite glial cells express inward rectifying K+ (Kir) channel subunit which is specific to glial cells (Tang et al. 2010). Immunohistochemical studies showed that Kir channel subunit and $GABA_B$ receptors are co-expressed in SGCs of TG (Takeda et al. 2015). Using in vivo patch clamp technique, it has been demonstrated that potentiation of $GABA_B$ receptors by application of baclofen increases Kir current in SGCs in the absence of nerve injury or inflammation, and these effects were eliminated via co-application of saclofen, a $GABA_B$ antagonist (Takeda et al. 2015). GABA released from neuronal cell bodies causes modifications in extracellular K^+ concentration after excitation of TG neurons and decreases trigeminal nociceptive transmission (Takeda et al. 2015). It is suggested that $GABA_B$ receptors on SGCs of TG may be promising targets for attenuation of trigeminal pain (Takeda et al. 2015). However, based on the current study, it seems that $GABA_A$ receptors are more involved in the desensitization of nociceptors compared to $GABA_B$ receptors.

It is predicted if the endogenous GABA level increases in the trigeminal ganglion, the afferent fibers might become sensitized. Since GABA can work both on $GABA_A$ and $GABA_B$ receptors, the action of drugs that affect the $GABA_A$ and $GABA_B$ receptors with regard to a peripheral mechanism of action could be different. It has been shown that activation of $GABA_A$ receptors in orofacial tissues results in antinociceptive actions in animal models of acute craniofacial pain (Cai et al. 2001; Cairns et al. 2001, 1999). For example, reflux jaw muscle activity evoked by painful stimulation of temporomandibular was suppressed by intra-articular

injection of GABA (Cairns et al. 1999). Local application of bicuculine, a $GABA_A$ antagonist, reversed the GABA mediated inhibition of nociceptive responses showing that these effects were mediated through peripheral activation of $GABA_A$ receptors (Cairns et al. 1999; Carlton, Zhou, and Coggeshall 1999). Based on the obtained data from this study it seems like in the TG muscimol made nociceptors less sensitive to mechanical activation, while fast conducting fibers showed an increase in mechanical sensitivity. However, $GABA_B$ did not appear to regulate the afferent fibers based on their conduction velocity. This suggests that $GABA_A$ activation could differentially modulate sensory input.

4.1 Limitations

Due to the possible central effects of GABA receptor agonists, blood pressure was recorded to assess any consequence of the injection of muscimol and baclofen into the trigeminal ganglion. Muscimol 10 mM lowered the mean blood pressure after the injection; however, the blood pressure lowering effects of muscimol did not last long and blood pressure came back to baseline within 10 minutes post injection. Also, baclofen did not change the blood pressure compared to baseline. These results suggest that $GABA_A$ receptors are probably involved in lowering the blood pressure. Another mechanism that might be involved in these effects is vasodilation as the result of release of neuropeptides like CGRP. This neuropeptide could be released as the result of depolarization of ganglion neurons caused by activation of $GABA_A$ receptors. The decline in blood pressure might be as the result of neurogenic inflammation and plasma protein extravasation and vasodilation due to the release of neuropeptides into the blood stream. A significant drop in blood pressure was seen after the injection of KCL to the TG of rats. In other studies, it was reported that ex vivo administration of KCL or a selective

excitotoxin such as capsaicin increased the CGRP release from trigeminal ganglion (Labastida-Ramírez et al. 2020)(Flores et al. 2001). Therefore, CGRP might be the reason for the significant decrease in blood pressure after the intraganglionic injection of GABA and muscimol. Previous studies have shown that very large decreases in blood pressure or blood flow can increase afferent mechanical threshold (Sung et al, 2008; Benbow et al 2020), and the changes in blood pressure produced by muscimol in addition to being transient, were not very large. Since intraganglionic injections of muscimol, baclofen slightly decreased afferent mechanical activation threshold, I conclude that blood pressure changes did not contribute to the results obtained in my study.

4.2 Future studies

Based on the results of this thesis, future work could include testing the correlation of conduction velocity and mechanical threshold in afferent fibers innervating the labial skin. To further understand the sex related differences in the GABA receptor performance and possible association with testosterone and estradiol, orchiectomized male and ovariectomized female rats could be used and their sensory transmission in the presence or lack of testosterone and estradiol could be evaluated, respectively. The effects of $GABA_A$ and $GABA_B$ agonists on the primary afferent endings that innervate muscle and skin could also be investigated. It would be interesting to evaluate the sensory transmission in $GABA_A$ and/or $GABA_B$ knockout animals.

Trigeminal ganglion neurons or SGCs surrounding them could be the potential sources for GABA in the trigeminal ganglion. Since we identified changes in the sensory transmission through trigeminal ganglion after injection of GABA, the next step could be to look for the possible sources of GABA in the trigeminal ganglion.

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