ABSTRACT

Recent human clinical evidence suggests a relationship between intramuscular (IM) serotonin (5-HT) levels and chronic masticatory muscle pain associated with temporomandibular disorders (TMDs) and fibromyalgia (FM). Further, human experimental pain studies have demonstrated that injection of 5-HT into masticatory muscle causes modest, short-lived pain and a longer-lasting mechanical sensitization that appear to be mediated by activation of peripheral 5-HT$_3$ receptors. However, it has not yet been clearly delineated what 5-HT$_3$ receptor types are expressed by sensory (afferent) fibres that innervate masticatory muscle. Also, it is not known what types of muscle afferent fibres 5-HT excites or sensitizes. In this study, immunohistochemistry techniques were used to determine the frequencies of expression of the 5-HT$_1$, 5-HT$_2$, and 5-HT$_3$ receptors by rat masticatory muscle ganglion cells. Electrophysiology experiments were carried out to determine the effects of repeated IM injection of 5-HT (0.1, 1, or 10 mM) on the excitability and mechanical thresholds of rat masticatory muscle afferent fibres. An electronic Von Frey Hair was used to measure afferent fibre mechanical thresholds before the initial and 10 min after the second IM injection. Our immunohistochemistry results suggest that 5-HT$_{1B}$ and especially 5-HT$_{2A}$ receptors, owing to their low frequencies of expression, may play minor roles in peripheral nociceptive transduction in uninflamed muscle tissue. In contrast, 5-HT$_3$ receptors, which are relatively highly expressed, likely play a more prominent role in peripheral nociception. Our electrophysiology results demonstrate that IM injection of 5-HT evokes rapid, relatively reproducible and concentration-dependent increases in afferent fibre excitability that are mediated, at least in part, by a peripheral receptor mechanism...
involving the 5-HT₃ receptor. However, IM injection of 5-HT did not significantly sensitize putative muscle afferent fibres to mechanical stimuli. We unexpectedly found that IM injection of 5-HT resulted in a marked decrease in blood pressure, a phenomenon that has yet to be explained. These findings indicate that 5-HT excites putative nociceptive afferent fibres that innervate masticatory muscle through activation of peripheral 5-HT₃ receptors, which suggests that the peripheral 5-HT₃ receptor may be an important target for future analgesic drugs.
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<tr>
<td>5-HIAA</td>
<td>5-hydroxyindole acetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin or 5-Hydroxytryptamine</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>Aδ</td>
<td>A-delta fibre</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>C</td>
<td>C fibre</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CV</td>
<td>Conduction velocity</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>FB</td>
<td>Fast Blue</td>
</tr>
<tr>
<td>FM</td>
<td>Fibromyalgia</td>
</tr>
<tr>
<td>g</td>
<td>Gram(s)</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>H^+</td>
<td>Protons</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram(s)</td>
</tr>
<tr>
<td>L</td>
<td>Litre(s)</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre(s)</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre(s)</td>
</tr>
<tr>
<td>µV</td>
<td>Microvolt(s)</td>
</tr>
<tr>
<td>M</td>
<td>Moles per litre</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre(s)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimoles per litre</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MT</td>
<td>Mechanical threshold</td>
</tr>
<tr>
<td>m/s</td>
<td>Meters per second</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus raphe magnus</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Pk</td>
<td>Peak discharge</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phosphlipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PPT</td>
<td>Pressure pain thresholds</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBSNC</td>
<td>Trigeminal brainstem sensory nuclear complex</td>
</tr>
<tr>
<td>TMDs</td>
<td>Temporomandibular disorders</td>
</tr>
<tr>
<td>TMJ</td>
<td>Temporomandibular joint</td>
</tr>
<tr>
<td>V1</td>
<td>Ophthalmic nerve</td>
</tr>
<tr>
<td>V2</td>
<td>Maxillary nerve</td>
</tr>
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</table>
V3  Mandibular nerve
VAS  Visual analog scale
$V_D$  Volume of distribution
ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my supervisor and mentor, Dr. Brian Cairns for his inspiration, and unrelenting guidance and support. Brian, thank you for facilitating a challenging and enjoyable learning experience.

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INTRODUCTION

Chronic Myofascial Pain

Chronic myofascial (muscle) pain has been reported to affect a significant proportion (14.4%) of the American population [116]. Chronic myofascial pain can be manifested locally as in low back pain or in temporomandibular disorders (TMDs) of the craniofacial region, or more generally as in the widespread pain of fibromyalgia (FM). Of particular interest in the epidemiology of chronic myofascial pain conditions is the higher prevalence in women compared to men [140]. For instance, both FM and TMDs are at least twice as common in women than in men. The intuitive assumption has been made that reproductive hormones may explain the reported sex difference; however, in actuality the sex-related difference in the prevalence of myofascial pain is poorly understood, as is the etiology of myofascial pain conditions in general [140]. In addition, differences in coping mechanisms, pain report, and seeking of and response to treatment further contribute to the confounds between women and men in their perceived experiences of chronic myofascial pain [16].

Chronic myofascial pain imposes not only a physical burden on those it afflicts, but also has an emotional and social impact on sufferers and the persons closest to them [151]. The emotional impact is supported by the high rate of depression and other psychological disturbances among chronic pain sufferers [111]. In addition to the direct burden on pain sufferers, chronic myofascial pain takes a toll on society in terms of the associated economic costs (sick-leave days, health care services, etc.) [151]. As an illustration of the magnitude of this cost, in the United States alone, the annual cost over two decades ago was estimated at over 65 billion dollars, and this figure has only grown...
since [151]. The physical, emotional, and psychological impact, in addition to the economic costs to society, offer great incentive to gain a better understanding of chronic myofascial pain conditions.

Unfortunately, the neurobiological basis of chronic myofascial pain syndromes such as FM and TMDs is poorly understood. In part, this may be a consequence of the "problematic" study of pain transduction (nociception) primarily in cutaneous rather than muscle tissue, despite the fact that chronic myofascial pain is of greater clinical relevance than cutaneous pain [116]. The lack of understanding of the mechanisms underlying chronic myofascial pain conditions (such as FM and TMDs) is the main reason behind the poor responsiveness of chronic pain sufferers to conventional pharmacological interventions. However, progress is being made and in particular there is significant interest in the emerging role of a number of peripherally released neurochemicals, in particular the neurotransmitter serotonin (5-HT), in chronic myofascial pain. In the following sections, I will first review the neuroanatomical pathways associated with the transduction and transmission of pain from skeletal muscle, with a focus on the sensory innervation of the masticatory muscles on which the experiments presented in this thesis were conducted. This will be followed by a detailed review of the receptor pharmacology of 5-HT and the location of the various 5-HT receptors in the peripheral nervous system. Then, I will review two chronic myofascial pain conditions that often co-exist in the masticatory muscles, namely TMDs and FM, and discuss theories of their underlying cause, in particular the potential role of 5-HT in these pain syndromes.
Craniofacial Anatomy and Innervation

The trigeminal nerve, the largest of the cranial nerves, is a mixed nerve with both sensory and motor functions [161]. The sensory and motor branches of the trigeminal nerve emerge from the brain as two distinct roots, the sensory and motor trigeminal roots [37]. The sensory root enlarges in the periphery (ventral to the pons, but still within the skull) to form the bilateral trigeminal ganglia (or Gasserian or semilunar ganglia) containing the cell bodies of most of the sensory (afferent) fibres [37,161]. The axons of these fibres form three major divisions (hence the name *trigeminal*) within the skull: the ophthalmic (denoted V1), maxillary (V2), and mandibular (V3) nerves, which leave the skull through three distinct openings: the superior orbital fissure, the foramen rotundum, and the foramen ovale [37]. While the ophthalmic and maxillary branches are purely sensory, the mandibular branch is composed of both sensory and motor fibres [37,161]. The trigeminal nerve conveys nearly all sensory input from the craniofacial region, including the facial skin, conjunctiva, regions of the dura mater, and masticatory muscles [37]. In addition, the trigeminal nerve carries sensory input from the tooth pulp, tongue, and gingival periodontal membrane [37]. Although almost exclusive to the trigeminal, sensory transmission from the craniofacial region can also involve the upper cervical spinal nerves and other cranial nerves [151].

The primary afferents of the trigeminal nerve innervate craniofacial tissues. The peripheral ends of these primary afferents can be associated with receptors that sense light tactile stimuli (called low threshold mechanoreceptors), or proprioceptive stimuli such as stretch and tension (referred to as proprioceptors) [151]. Some primary afferents, however, terminate without receptors, and therefore have poorly differentiated
terminals called free nerve endings [64,151]. Free nerve endings detect painful (noxious) stimuli and are hence termed nociceptors [151]. Nociceptive primary afferents consist of A\(\delta\) fibres, which are small diameter, thinly myelinated, and slowly conducting, and C fibres, which are even smaller diameter, unmyelinated and slower conducting [151]. A\(\delta\) and C fibres have conduction velocities (CVs) of 2 – 20 m/s and < 2 m/s, respectively [64]. Although nociceptive by classification, it is noteworthy to mention that some A\(\delta\) and C fibres also mediate the transmission of non-noxious stimuli, such as cooling or warming, or even tactile stimuli [151].

From the periphery, sensory input is projected via the trigeminal nerve to the trigeminal brainstem sensory nuclear complex (TBSNC), which is best described as "...a bilateral, multinucleated structure in the dorsolateral brainstem that extends from the pons to the upper cervical spinal cord" [151]. It is the part of the brainstem responsible for relaying sensory information from craniofacial tissues, including the masticatory muscles, to higher brain regions such as the thalamus [151]. The TBSNC is comprised of four subnuclei that relay sensory information: the principalis (or main), the oralis, the interpolaris and the caudalis [37,151,161].

In the brainstem, primary afferent fibres travel along the trigeminal spinal tract and make synaptic connections with second order neurons located in the TBSNC or adjacent sites [151]. Primary afferents may also form collaterals that project to one or more subnuclei within the TBSNC before terminating on second order neurons, or may even project to entirely different regions of the brainstem (e.g., the reticular formation, supratrigeminal nucleus, solitary tract nucleus) [151]. The TBSNC receives the majority of its sensory input from the trigeminal primary afferents, but may also receive inputs
from upper cervical nerves and other cranial nerves (e.g. VII, IX, X, XII) [151]. Of particular importance to the relay of noxious stimuli from the craniofacial region is the subnucleus caudalis, which is, as its name implies, the most caudal subnucleus in the TBSNC [151]. It is a laminated structure bearing resemblance to the dorsal horn of the spinal cord, and is fused to the cervical end of the spinal cord [151]. Most of the nociceptive (Aδ and C) afferent fibres are known to terminate in laminae I, II, V, and VI of the subnucleus caudalis [5,151], implying that caudalis neurons are important in the relay of craniofacial pain [5]. Indeed, both anatomical [121,138,153] and electrophysiological [23,28,137] evidence indicates most, if not all nociceptive afferent fibres that innervate masticatory muscle project to this (i.e. the caudal) region of the TBSNC.

As mentioned earlier, nearly all primary afferent fibres that innervate craniofacial tissues have their cell bodies in the trigeminal ganglion in the peripheral nervous system [151]. The trigeminal ganglion is unique in not having cell bodies of muscle proprioceptors, thus allowing for easier recording of nociceptive primary afferents [151]. In contrast to this, the primary afferent cell bodies of proprioceptive (non-nociceptive) fibres such as jaw muscle spindle afferents are located in the CNS within the trigeminal mesencephalic nucleus [151]. Trigeminal mesencephalic fibres project to the trigeminal motor nucleus and adjacent regions such as the supratrigeminal nucleus or trigeminal subnucleus oralis, where they synapse onto interneurons and initiate craniofacial reflexes [151].
Transmission of pain

Peripheral Mechanisms

Tissue injury or inflammation causes the production and release of a wide range of neuroactive mediators from nerve fibres, vascular endothelial cells, blood cells, mast cells and other immune cells, that can activate the peripheral sensory nerve endings of local primary A\(\delta\) and C fibres [89,147]. Some of these mediators act indirectly by sensitizing primary afferent nerve endings to other stimuli, as evidenced by prostaglandins, prostacyclin, and the cytokines tumor necrosis factor-\(\alpha\), interleukin-1\(\beta\) (IL-1\(\beta\)), IL-2, IL-6, and IL-8 [147]. Other mediators, however, may activate the sensory nerve directly, as observed with H\(^+\), ATP, glutamate, bradykinin (BK), histamine, and, in particular, 5-HT [147].

Peripheral inflammation can cause changes in the sensitivity or responsiveness of primary afferents, a phenomenon commonly seen in chronic nerve injury or inflammatory conditions [147]. Primary afferents may exhibit alterations in excitability and in the expression of certain receptors, neurotransmitters, enzymes, and ion channels [147]. Blood flow to the region can also be altered such that migration of immune cells, growth factors as well as trophic factors from the surrounding tissue are augmented [147]. These changes lead to the development of peripheral sensitization, a phenomenon marked by allodynia (excitation of nociceptors to non-noxious stimuli) and primary hyperalgesia (augmented response to noxious stimuli) [64]. Allodynia and hyperalgesia, being clinical terms, are more often used to describe the behavioral manifestation of peripheral sensitization. The extent of 5-HT involvement in these phenomena, however, has yet to be determined.
Central Mechanisms

In addition, peripheral sensitization induces changes at the TBSNC level, decreasing the mechanical threshold (MT) and enlarging the peripheral receptive fields of TBSNC neurons, leading to a central sensitization termed secondary hyperalgesia [64]. Upon noxious stimulation, nociceptive primary afferents release certain amino acids and neuropeptides that act as excitatory neurotransmitters or neuromodulators in the transmission of nociceptive input to the TBSNC [151]. Myelinated and unmyelinated primary afferents release neurochemicals that act on wide dynamic range neurons in the TBSNC or the spinal dorsal horn. In particular, Aδ and C fibres release the excitatory amino acid glutamate and the peptide substance P, which exert their excitatory effects trans-synaptically on second order neurons or on interneurons which can in turn evoke excitatory effects on second order neurons [151]. Both NMDA (N-methyl-d-aspartate) (an excitatory amino acid receptor) and non-NMDA ionotropic receptors are implicated in the excitatory actions of these neurochemicals [151]. Other neuropeptides have been reported to be co-released with glutamate from primary nerve endings of Aδ and C fibres in response to noxious thermal, electrical or mechanical stimuli [64]. Among these neuropeptides are neurokinin A and calcitonin gene-related peptide (CGRP) [64]. The involvement of these neuropeptides is well established in central pain mechanisms associated with peripheral tissue inflammation, however the role these substances play in pain mechanisms associated with non-inflamed tissues such as the masticatory muscles in most TMD cases has not been well characterized.

In contrast to its effects in the periphery, 5-HT in the CNS and particularly in the spinal cord is believed to be involved in antinociception [139]. 5-HT-mediated
antinociception is thought to occur via inhibition of dorsal horn neurons by 5-HT released from descending nucleus raphe magnus (NRM) neurons located in the medulla [130,139]. This descending pathway is often referred to as the raphe-spinal pathway [130]. It was shown in rats that pharmacological antagonism of 5-HT\textsubscript{1A/1B} receptors but not the 5-HT\textsubscript{2A} receptor in dorsal horn neurons blocked the antinociceptive effects of medullary NRM stimulation, thereby suggesting the involvement of 5-HT\textsubscript{1A/1B} receptors in descending antinociception [46]. Nonselective antagonism of 5-HT receptors in the medial NRM with methysergide was shown to attenuate the antinociceptive effects of lateral hypothalamus stimulation, indicating 5-HT involvement in descending antinociception [3]. In addition to the 5-HT\textsubscript{1} receptor, the 5-HT\textsubscript{3} receptor has also been implicated in descending inhibition of dorsal horn neurons from higher CNS sites [130]. The effects of 5-HT on neurons within the caudal trigeminal nucleus showed both excitatory and inhibitory influences depending on the type of neuron studied [21].

5-HT Pharmacology

5-HT is a biogenic amine that acts as a neurotransmitter and neuromodulator [66]. Distributed throughout the body in the central and peripheral nervous systems, platelets, and smooth muscles, 5-HT acts mainly in these organs to modulate diverse neural, vascular, platelet, and smooth muscle functions [66]. It is present in high concentrations in enterochromaffin cells (hormone-secreting cells located in the epithelium of the digestive tract) and in the myenteric plexus of the intestine, in blood platelets, the cardiovascular system, and in the midbrain and spinal cord of the CNS.
Through its multiple receptor families, 5-HT has been linked to the etiology of wide-ranging disorders including psychological (depression, anxiety, schizophrenia, obsessive-compulsive disorder), cardiovascular (hypertension), gastrointestinal (irritable bowel syndrome, vomiting) and pain conditions such as migraine, and of particular interest to us, myofascial pain conditions such as TMDs and FM [76].

5-HT is found in many food products but is poorly absorbed from the gastrointestinal tract [65]. Thus, it is endogenously synthesized from the essential amino acid L-tryptophan by a two-step process [66,144]. L-tryptophan is initially hydroxylated in the 5-position by the enzyme L-tryptophan hydroxylase to form 5-hydroxytryptophan, which is subsequently decarboxylated by L-aromatic amino acid decarboxylase to yield 5-hydroxytryptamine (5-HT) (Figure 1.1) [56,66]. The metabolism of 5-HT occurs mainly through the actions of monoamine oxidase (MAO), which converts 5-HT to 5-hydroxyindole acetaldehyde. Of the two MAO subtypes, MAO-A is more selective for 5-HT than MAO-B [60]. 5-hydroxyindole acetaldehyde is subsequently metabolized by aldehyde-dehydrogenase to form 5-hydroxyindole acetic acid (5-HIAA), which is the main metabolite excreted in the urine [66,144]. An alternative route occurs by the reduction of the acetaldehyde via aldehyde-reductase to the alcohol 5-hydroxytryptophol, which is a minor product [66,144]. It is probable that neuronal 5-HT is mainly metabolized in serotonergic neurons (i.e. the same neurons that synthesize it) [60].

Despite what is already known about 5-HT, its modes and sites of action are not fully understood, and this may be in part due to the large number of receptor families that exist for this particular neurotransmitter [144]. To date, seven 5-HT receptor
Figure 1.1. The diagram illustrates the biosynthetic and metabolic pathways for 5-HT, and the major enzymes involved.
families (5-HT\textsubscript{1,2,3,4,5,6,7}) have been identified, among them a total of 14 subgroups [66]. The recent addition of two more subtypes to the 5-HT\textsubscript{3} receptor family boosts this number to 16 [76]. All but one of the 5-HT receptors are G-protein coupled; the exception is the ligand-gated 5-HT\textsubscript{3} receptor [66,73]. The G-protein coupled receptors, when activated, initiate a receptor-effector cascade involving multiple steps, and are thought to be involved in relatively slow cellular responses [66]. The 5-HT\textsubscript{3} receptors, on the other hand, are directly linked to fast cation-selective ion (Na\textsuperscript{+}/K\textsuperscript{+}) channels, and can thus mediate faster cellular responses (see Table 1.1) [66].

5-HT\textsubscript{1} Receptors

The 5-HT\textsubscript{1} receptor class is the largest of the 5-HT receptor families and consists of five subtypes, 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{1D}, 5-HT\textsubscript{1E}, and 5-HT\textsubscript{1F} [76,96]. In humans, the 5-HT\textsubscript{1} receptor subtypes are 40-63\% homologous, and couple mainly to G/Go, inhibiting adenylyl cyclase (AC) and cAMP formation [76,96]. The 5-HT\textsubscript{1} receptors are also characterized by their high affinity for the endogenous ligand, 5-HT [96].

The 5-HT\textsubscript{1A} receptor is comprised of seven transmembrane hydrophobic domains [96]. It is densely distributed in the CNS, particularly on the somatodendrites of 5-HT neurons in the mesencephalic raphe nuclei where it acts as an autoreceptor to inhibit action potential discharge [12,76]. It is also highly expressed in limbic brain regions such as the hippocampus, and in cortical areas [12,76]. 5-HT\textsubscript{1A} receptor activation inhibits neuronal firing by the opening of G-protein-coupled inwardly rectifying K\textsuperscript{+} channels, causing hyperpolarization [76]. Agonists for the 5-HT\textsubscript{1A} receptor include the full agonist 8-OH-DPAT, and partial agonists such as the anxiolytics buspirone and
<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Receptor-effector mechanisms</th>
<th>Selective agonists</th>
<th>Selective antagonists</th>
<th>Tissue location</th>
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</thead>
<tbody>
<tr>
<td>5-HT_{1A}</td>
<td>Inhibition of AC</td>
<td>8-OH-DPAT</td>
<td>WAY100635</td>
<td>Mesencephalic raphe nuclei, hippocampus, cortical areas</td>
</tr>
<tr>
<td></td>
<td>Opening of G-protein-coupled K⁺ channels</td>
<td>DP-5-CT</td>
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<tr>
<td>5-HT_{1B}</td>
<td>Inhibition of AC</td>
<td>Naratriptan</td>
<td>N/A</td>
<td>Dorsal and median raphe nuclei, hippocampus, cerebral arteries, trigeminal and dorsal root ganglia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sumatriptan</td>
<td></td>
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<td></td>
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<td>Zolmitriptan</td>
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<tr>
<td>5-HT_{1D}</td>
<td>Inhibition of AC</td>
<td>Naratriptan</td>
<td>N/A</td>
<td>Trigeminal ganglia</td>
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<tr>
<td></td>
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<td>Sumatriptan</td>
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<td>Zolmitriptan</td>
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</tr>
<tr>
<td>5-HT_{2A}</td>
<td>Stimulation of PLC and PLA2</td>
<td>α-methyl-5-HT</td>
<td>Ketanserin</td>
<td>Cortex, caudate nucleus, hippocampus, basal ganglia, skeletal muscle, smooth muscle, platelets, kidneys, trigeminal and dorsal root ganglia</td>
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<td></td>
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<td>Sarpogrelate</td>
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<tr>
<td>5-HT_{3}</td>
<td>Ligand-gated to an ion channel</td>
<td>2-methyl-5-HT</td>
<td>Granisetron</td>
<td>Cerebral area postrema, hippocampus, spinal cord, trigeminal and dorsal root ganglia</td>
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<td></td>
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<td>Ondansetron</td>
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<td>Tropisetron</td>
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Table 1.1. The table summarizes the receptor-effector mechanisms, selective agonists and antagonists, and tissue locations of the 5-HT_{1A,1B,1D,2A,3} receptor subtypes. AC: Adenylyl cyclase, PLC: Phospholipase C, PLA2: Phospholipase A2.
gepirone [76]. A number of antagonists have been developed for this receptor, but nearly all have shown poor selectivity with the exception of a few more recent ligands, most notably WAY 100635, which is the most potent to date [12,76]. It is worth mentioning that among the non-selective antagonists are the beta adrenergic receptor blockers such as pindolol and propranolol, which bind to the 5-HT\textsubscript{1A} receptor due to its similarity to the beta adrenergic receptor [12].

The 5-HT\textsubscript{1B} receptor is highly expressed in the CNS, and mRNA encoding this receptor has been detected in neurons within the dorsal and median raphe nuclei using in situ hybridization histochemistry [20,96]. 5-HT\textsubscript{1B} receptor mRNA has also been identified centrally in cells of the hippocampus, the frontal cortex, the primary olfactory cortex, and in the Purkinje cells of the cerebellum, among numerous other sites [20,96]. In the periphery, 5-HT\textsubscript{1B} receptors are most notably present on vascular tissues such as the cerebral arteries. 5-HT\textsubscript{1B} receptor mRNA has been labeled in rat trigeminal [108,169,174] and dorsal root ganglia [19,20,131,174]. In human trigeminal ganglia, immunocytochemistry revealed the expression of 5-HT\textsubscript{1B} receptors in 40% of the neuronal cells that were studied [75]. Interestingly, it was also shown in rats that 5-HT\textsubscript{1B} receptor mRNA-containing cells were immunoreactive for the excitatory amino acid glutamate, the neuropeptide CGRP, but did not express substance P [108,169]. Indeed, in rats the majority (approximately 70%) of 5-HT\textsubscript{1B} receptor-positive trigeminal ganglion cells were shown to express CGRP [109]. In guinea pig, double labeling in situ hybridization revealed the colocalization of 5-HT\textsubscript{1B} receptor mRNA with mRNA for substance P and CGRP in trigeminal ganglion cells [18]. However, it has not yet been
determined in rats or humans whether 5-HT$_{1B}$ receptors are expressed by trigeminal ganglion cells that specifically innervate masticatory muscle.

Like the 5-HT$_{1A}$ receptor, the 5-HT$_{1B}$ receptor has been linked to G/G$_0$ proteins that inhibit the actions of AC [96,135]. It has also been reported to stimulate cAMP formation and PLC activity, thereby increasing intracellular Ca$^{2+}$ levels [135]. Some studies have reported that the 5-HT$_{1B}$ receptor stimulates endothelial nitric oxide (NO) production [135]. In the CNS, the 5-HT$_{1B}$ receptor is believed to function as a terminal autoreceptor by inhibiting presynaptic 5-HT release from serotonergic cells, as well as terminal heteroreceptors by inhibiting or modulating the release of other neurotransmitters, some of which may be glutamate, noradrenaline, acetylcholine, dopamine, and GABA [76,96].

The 5-HT$_{1B}$ receptor is very similar to the 5-HT$_{1D}$ receptor in terms of its pharmacological properties; the main pharmacological difference is seen in the fact that the 5-HT$_{1B}$ receptor has a uniquely high affinity for beta-adrenergic receptor blockers, whereas the 5-HT$_{1D}$ receptor does not [135]. A number of clinically effective 5-HT$_{1B/1D}$ receptor agonists, collectively called the “triptans”, have been developed for the treatment of migraine headache, and include sumatriptan, zolmitriptan, naratriptan, and rizatriptan [76]. The ergot alkaloids ergotamine and dihydroergotamine are also used to treat migraine by their non-selective binding of the 5-HT$_{1B/1D}$ receptors [96].

The 5-HT$_{1D}$ receptor shares 63 % of its structure with the 5-HT$_{1B}$ receptor [76]. However, it is sparsely expressed compared to the 5-HT$_{1B}$ receptor, particularly in brain tissue [76,96]. Like the 5-HT$_{1B}$ receptor, it couples to G/G$_0$ proteins to inhibit the activity of AC [96]. It also regulates ion channels, as it has been shown to inhibit N-type Ca$^{2+}$
channels and stimulate Ca\(^{2+}\)-dependent K\(^+\) channels [135]. The 5-HT\(_{1D}\) receptor, like the 5-HT\(_{1B}\), is believed to be involved in the pharmacological actions of triptan drugs [96]. In fact, PNU 109291, a selective 5-HT\(_{1D}\) receptor agonist, has been shown to decrease plasma protein extravasation (or neurogenic inflammation) that is believed to underlie, at least in part, the development of migraine headache [76,96]. In rat trigeminal ganglia, the presence of 5-HT\(_{1D}\) receptor-encoding mRNA has been demonstrated by means of in situ hybridization histochemistry, and in the ganglia, mRNA molecules encoding this receptor subtype were identified on large cell bodies, many of which were also immunopositive for the excitatory neurotransmitter glutamate [19,20,108,174].

The 5-HT\(_{1E}\) and 5-HT\(_{1F}\) receptors have been less studied and so their respective distributions and physiological roles are not as well known [76]. It has been suggested that both receptors couple negatively to adenylyl cyclase, as has been demonstrated in studies using transfected cells [96]. Although the function of these receptors is poorly understood, due to the affinity of sumatriptan and naratriptan for the 5-HT\(_{1F}\) receptor, it has been proposed that agonism of the 5-HT\(_{1F}\) receptor may decrease neurogenic inflammation associated with the development of migraine, and subsequent studies have indeed shown that selective 5-HT\(_{1F}\) receptor agonists decrease this inflammation [96]. 5-HT\(_{1F}\) receptors are also expressed by trigeminal ganglion neurons that also appear to contain glutamate [108]. It is clear that the 5-HT\(_{1B}\), the 5-HT\(_{1D}\), and possibly the 5-HT\(_{1F}\) receptors are involved in the mediation of pain and inflammation, particularly associated with migraine. However, the respective roles of these receptors in the mediation of muscle pain associated with TMDs and FM require further investigation.
5-HT₂ Receptors

The 5-HT₂ receptor family consists of the 5-HT₂A, 5-HT₂B, and 5-HT₂C receptor subtypes [76]. It is currently understood that all 5-HT₂ receptors couple to G₉/₁₁ proteins to stimulate PLC, which catalyzes the formation of the second messenger inositol trisphosphate (IP₃), thereby increasing intracellular Ca²⁺ levels through intracellular stores and extracellular influx [76,102]. In addition, all three 5-HT₂ receptors couple to phosphlipase A₂ (PLA₂), activation of which results in the release of arachidonic acid (AA), a precursor to prostaglandins [102,135].

The 5-HT₂A receptor couples mainly to the PLC pathway to increase intracellular Ca²⁺ levels [135]. 5-HT₂A receptor activation has been shown to mediate contractile responses in vascular smooth muscle cells [135]. The involvement of 5-HT₂A receptors in vascular smooth muscle contraction has in fact been well documented in bronchial, intestinal, uterine and urinary smooth muscle preparations [76]. The 5-HT₂A receptor is involved in the release of Ca²⁺ from intracellular stores and the activation of voltage-gated and voltage-independent Ca²⁺ channels, all of which work to increase intracellular Ca²⁺ [135]. The most selective 5-HT₂A receptor antagonists to date are ketanserin and MDL 100907 [76].

The 5-HT₂A receptor is expressed extensively throughout the CNS and in the periphery [76,102]. In the brain, it has been identified in the cortex, caudate nucleus, hippocampus, and basal ganglia [76,135]. Peripherally, the 5-HT₂A receptor has been localized in a variety of tissues including skeletal muscle, smooth muscle, platelets, and the kidneys [135]. Polymerase chain reaction (PCR) and in situ hybridization techniques revealed mRNA for 5-HT₂A receptors in rat trigeminal and dorsal root ganglia.
[123,131,174], as well as human dorsal root ganglia [132]. Furthermore, all 5-HT$_{2A}$ receptor-positive dorsal root ganglion neurons were CGRP-immunopositive in rats [123]. Immunohistochemical techniques have been used to identify 5-HT$_{2A}$ receptors on unmyelinated sensory axons in rat glabrous skin [32], however, their expression by afferent fibres in deeper tissue, particularly muscle, is not known.

In contrast to the contractile responses associated with 5-HT$_{2A}$ receptor activation, 5-HT$_{2B}$ receptor activation has been shown to result in vasorelaxation, which has been demonstrated in pig pulmonary artery endothelial cells and in the rat jugular vein [76]. The vasodilatatory effects of 5-HT$_{2B}$ receptor activation have been attributed to an increased production of nitric oxide (NO) [135]. The 5-HT$_{2C}$ receptor is present in the choroid plexus and other CNS sites [76]. Activation of this receptor is believed to inhibit dopaminergic and adrenergic transmission, and has been associated with hypophagia, hypoactivity, and anxiogenesis [76]. However, a broader understanding of the physiological role of the 5-HT$_{2C}$ receptor has yet to be elucidated, and to date has not been successful due to the poor selectivity of ligands for this receptor [76]. In particular, its role in nociceptive transmission remains elusive since studies are contradictory as to whether activation of this receptor subtype increases or decreases nociceptive transmission [45].

5-HT$_3$ Receptors

The 5-HT$_3$ receptor belongs to the superfamily of ligand-gated ion channels, and mediates rapid neuronal depolarization characterized by fast desensitization and resensitization [66,76]. 5-HT$_3$ receptor-mediated depolarization occurs as a result of the
opening of cation-selective ion channels that facilitate the influx of Ca\(^{2+}\) and Na\(^{+}\) and the efflux of K\(^{+}\) ions [76].

The 5-HT\(_3\) receptor is known for its role in emesis and inflammatory pain [66]. Selective antagonists for this receptor, which include ondansetron, granisetron, and tropisetron, have a clinical application in the treatment of chemotherapy-induced and postoperative nausea and vomiting and offer an advantage over conventional antiemetics by causing little or no sedative effects [66]. The 5-HT\(_3\) receptor has also been implicated in the mediation of nociception, and this will be discussed in greater detail later (Chapter I, 5-HT involvement in peripheral pain). In the CNS, the 5-HT\(_3\) receptor is distributed in the cerebral area postrema, the hippocampus and the spinal cord [65,76]. In the periphery, the receptor is present on autonomic and rat trigeminal and dorsal root ganglion neurons [131,174] and in the heart [76].

**Tropisetron, a selective 5-HT\(_3\) receptor antagonist**

Tropisetron, an indole compound also known as ICS 205-930, is a highly potent and selective antagonist directed at the 5-HT\(_3\) receptor [98]. It has virtually no affinity for the 5-HT\(_1\) and 5-HT\(_2\) receptors and relatively weak affinity for the 5HT\(_4\) receptor [98]. Further, tropisetron has negligible affinity for \(\alpha_1\), \(\alpha_2\), \(\beta_1\), and \(\beta_2\) adrenoceptors, histamine H\(_1\) and H\(_2\) receptors, dopamine D\(_1\) and D\(_2\) receptors, muscarinic receptors, and benzodiazepine receptors [36,98,136]. Tropisetron has been reported to concentration-dependently decrease isolated inwardly rectifying K\(^{+}\) currents (IC\(_{50}\): 1.95 x 10\(^{-5}\) M) at a test voltage of -40 mV, and Na\(^{+}\) and Ca\(^{2+}\) currents at 10\(^{-4}\) M in whole-cell patch clamped ventricular myocytes, with the greatest block on K\(^{+}\) and the smallest block on Ca\(^{2+}\).
currents [150]. A recent study identified tropisetron as a potent and selective partial agonist at the α7 nicotinic receptor, which, like the 5-HT3 receptor, is a ligand-gated cation channel [110]. A radioligand binding study in rat brain tissue demonstrated that tropisetron competes for 5-HT3 receptor binding with the selective antagonist 3H-GR65630 [90]. Bolus 5-HT injections into the rat jugular vein caused dose-dependent reflex bradycardia (i.e. von Bezold-Jarisch reflex). Pretreatment with systemic tropisetron (1 µg/kg) resulted in a right shift of the 5-HT dose-response curve that was restored by increasing the 5-HT concentration fourfold [136]. Collectively, these findings provide evidence that tropisetron is a reasonably selective, competitive 5-HT3 receptor antagonist, and is therefore a good candidate for pharmacological studies.

Tropisetron is clinically used as an antiemetic to prevent and control nausea and vomiting associated with chemotherapy (e.g. cisplatin) [98]. It is currently understood that chemotherapy and radiation therapy damage the intestinal mucosa, thereby causing the release of 5-HT from enterochromaffin cells in the GI tract [98]. The mechanism of action of tropisetron is believed to occur via antagonism of 5-HT3 receptors in the periphery (on vagal afferents in the GI tract) and centrally (in the chemoreceptor trigger zone where vagal afferents terminate) to block the actions of 5-HT, thereby offsetting the vomiting reflex [98].

Tropisetron is administered orally and intravenously and so it follows that its pharmacokinetic properties have been characterized based on these routes of administration [88]. Following oral administration, tropisetron absorption is rapid and nearly complete, but it undergoes hepatic first pass metabolism, albeit non-extensively, in a dose-dependent manner [36,88]. Due to its lipophilic properties, tropisetron has a
large volume of distribution ($V_D$, 678 L during steady state and 554 L during the terminal elimination phase) [36,88]. The pharmacokinetic parameters of IV and oral tropisetron have been described in humans [57,88], however, there appear to be no studies conducted on IM tropisetron, making it difficult to speculate on the pharmacokinetic parameters of tropisetron in muscle.

2-methyl-5-HT, a selective 5-HT$_3$ receptor agonist

2-methyl-5-HT is a selective 5-HT$_3$ receptor agonist [78]. Whole-cell patch clamp studies in rat trigeminal ganglion neurons revealed that application of 2-methyl-5-HT at $10^{-4}$ M mimicked currents activated by $10^{-4}$ M 5-HT, (and that this current was therefore mediated by the 5-HT$_3$ receptor), although the amplitude of the 2-methyl-5-HT-induced current was slightly smaller than that induced by 5-HT [78]. The binding affinities of 2-methyl-5-HT and 5-HT for the cloned human 5-HT$_{3A}$ receptor are also very similar, with $pK_i$ values of 6.18 ± 0.09 and 6.47 ± 0.17, respectively [83]. 2-methyl-5-HT and 5-HT also display comparable EC$_{50}$ values in contracting isolated guinea pig colon preparations (3.1 µM for 2-methyl 5-HT and 4.8 µM for 5-HT) and in producing positive chronotropic effects in guinea pig right atria (3.9 µM and 11 µM respectively) [83]. These data, collectively, suggest that 2-methyl-5-HT and 5-HT are relatively equipotent.

5-HT$_{4,7}$ Receptors

The remaining 5-HT receptors 5-HT$_{4,5,6,7}$ have been identified only recently, and therefore less is known about them in comparison to the 5-HT$_{1,2,3}$ receptors, especially with respect to pain transduction. The 5-HT$_4$ receptor is believed to be involved in the
pathogenesis of irritable bowel syndrome (IBS) [29]. The novel selective 5-HT₄ agonist tegaserod was shown to be effective in reducing the symptoms of IBS, including abdominal pain and discomfort [13,29]. The 5-HT₅ receptor has not been sufficiently characterized pharmacologically, and although the 5-HT₆ (limited largely to the brain [35]) and 5-HT₇ receptor subtypes have been linked to disorders such as epilepsy and affective states such as depression [133,168], there is still much that needs to be studied and understood about these receptors. Due to the lack of information about these receptors in peripheral pain transduction, their respective roles in pain of masticatory origin have yet to be elucidated. PCR techniques have identified mRNA for the 5-HT₄ and 5-HT₇ receptors in rat trigeminal and dorsal root ganglia [174]. 5-HT₇ receptors were also identified in human dorsal root ganglia using PCR [132].

5-HT Involvement in Peripheral Pain

Following tissue injury or inflammation, platelets, mast cells, or basophils infiltrate the site of injury and release 5-HT [154,173], an inflammatory mediator in the periphery during the initial phase of the inflammatory response [73]. Although the exact mechanism(s) by which 5-HT contributes to pain and inflammation is unclear, the 5-HT₁, 5-HT₂, and 5-HT₃ receptor subtypes have been implicated in the inflammatory pain process [156].

In a study by Sufka et al (1992), methysergide (a 5-HT₁ receptor antagonist) was more potent than ketanserin (5-HT₂ receptor antagonist) and ondansetron at blocking 5-HT-evoked edema and algesia of the rat hindpaw [156], suggesting that the 5-HT₁ receptor contributes the most to peripheral nociception. Taiwo and Levine (1992)
demonstrated that injection of the selective 5-HT$_{1A}$ agonists 8-OH-DPAT and DP-5-CT into the rat hindpaw mimicked the dose-dependent mechanical hyperalgesia (as determined by paw withdrawal thresholds) produced by 5-HT, whereas selective agonists for the 5-HT$_{1B}$, 5-HT$_{2A/C}$ ($\alpha$-methyl-5-HT), and 5-HT$_{3}$ (2-methyl-5-HT) receptors did not produce hyperalgesia [158]. However, other rat behavioral studies have reported that the 5-HT$_{2A}$ rather than the 5-HT$_{1A}$ receptor produces hyperalgesia, thus contradicting the results of the aforementioned studies and questioning the extent of 5-HT$_{1A}$ receptor involvement in the development of hyperalgesia [1,164].

The involvement of the 5-HT$_{2A}$ receptor in pain and hyperalgesia has been demonstrated in a number of pain models in rats. In a study by Obata et al (2000), subcutaneous injection of formalin into the rat hindpaw caused flinches that were dose-dependently suppressed by pre- and post-treatment with local and intraperitoneal sarpogrelate, a selective 5-HT$_{2A}$ receptor antagonist [122]. The lack of an antinociceptive effect with intrathecal sarpogrelate pretreatment, coupled with the drug's poor penetration of the blood brain barrier, the authors claim, suggest that sarpogrelate exerts an antinociceptive effect via a peripheral 5-HT$_{2A}$ receptor mechanism [122]. Okamoto et al (2002) demonstrated that oral administration of sarpogrelate reduced thermal hyperalgesia in the CFA-inflamed rat hindpaw, and made the suggestion that the 5-HT$_{2A}$ receptor may potentiate peripheral inflammatory nociception [123]. In a study employing the thermal injury model in the rat hindpaw heel, pre-treatment with local and intraperitoneal sarpogrelate was shown to significantly attenuate primary thermal hyperalgesia and secondary mechanical allodynia in a dose-dependent manner [145]. Subcutaneous microdialysis of the primary and secondary areas revealed that 5-
HT concentrations increased 1997 ± 441% and 814 ± 235 %, respectively, immediately following thermal injury [145]. In the carrageenan-inflammed rat hindpaw model, pre-treatment with intraplantar ketanserin, a selective 5-HT$_{2A}$ receptor antagonist, reversed carrageenan-induced thermal hyperalgesia in a dose dependent manner, and reduced paw edema and c-fos-like immunoreactivity in the ipsilateral lumbar dorsal horn [164]. Tokunaga et al (1998) demonstrated that intradermal injection of the selective 5-HT$_{2A}$ receptor agonist α-methyl-5-HT but not the 5-HT$_{3}$ receptor agonist 2-methyl-5-HT into the rat hindpaw caused hyperalgesia, as determined by latency to paw withdrawal to heat [160]. In addition, pre-treatment with the selective 5-HT$_{2A}$ receptor antagonist ketanserin decreased 5-HT-induced hyperalgesia, whereas the selective 5-HT$_{3}$ receptor antagonist tropisetron did not [160]. Collectively, these studies support the involvement of the 5-HT$_{2A}$ receptor in nociception and most notably sensitization in cutaneous tissue.

The involvement of the 5-HT$_{2A}$ receptor in muscle nociception has not been studied previously with the exception of a recent study by Okamoto et al (2005). In rats with persistent CFA-induced temporomandibular joint (TMJ) inflammation, orofacial nocifensive behaviours evoked by injection of formalin into the masseter muscle were significantly decreased by pretreatment with local and systemic ketanserin, a selective 5-HT$_{2A}$ receptor antagonist [124].

Perhaps the strongest evidence for 5-HT receptor involvement in nociception rests with the 5-HT$_{3}$ receptor. In a study in mice, Zeitz et al (2002) reported that tissue injury-induced pain depends on the excitation of nociceptive afferents mediated by 5-HT$_{3}$ receptor activation, since genetic disruption of this receptor subtype significantly reduced nociception [173]. Based on behavioural responses, it was concluded that both
central and peripheral 5-HT$_3$ receptors are involved in the mediation of nociception [173]. The involvement of 5-HT$_3$ receptors is supported by other behavioural studies in rats. Peripheral edema and algesia induced by intraplantar injection of 5-HT were significantly attenuated by pretreatment with ondansetron, a selective 5-HT$_3$ receptor antagonist [156]. Local treatment with the selective 5-HT$_3$ receptor antagonists tropisetron and MDL 72222 significantly attenuated paw edema and behavioural responses to acute (formalin-evoked) and chronic (CFA-evoked) inflammatory pain in the rat hindpaw [62]. Intraplantar injection of tropisetron into the rat hindpaw significantly attenuated carrageenan-evoked mechanical sensitization in the ipsilateral but not contralateral paw, indicating that tropisetron decreases carrageenan-evoked mechanical sensitization via a peripheral 5-HT$_3$ receptor mechanism [52]. In a human study by Voog et al (2000), the selective 5-HT$_3$ receptor antagonist granisetron was shown to reduce jaw joint pain in patients with inflammatory arthritis, suggesting that the 5-HT$_3$ receptor is involved in the mediation of such pain [162]. Eide and Hole (1992), after reviewing the results of a number of studies, concluded that peripheral 5HT$_3$ receptor activation increases nociceptive transmission [45].

The speculation of a possible indirect effect of 5-HT in peripheral nociceptive transduction has been previously made by Hong and Abbott [74]. In their study, it was demonstrated that intraplantar co-injection of 5-HT and either BK, histamine, prostaglandin E$_2$ (PGE$_2$), or substance P produced synergistic behavioural pain responses (i.e. increased paw favouring, licking or lifting) in rats, while co-injection of any combination without 5-HT produced only additive pain responses [74]. These results indicated that 5-HT may exert an indirect effect by enhancing the algesic effects
of other pain/inflammatory mediators [74]. Pre-injection of the 5-HT_{2A/2C} receptor antagonist ketanserin nearly completely abolished licking and lifting behaviour evoked by a combination of 5-HT and PGE_{2}, whereas antagonists for the 5-HT_{1} (BMY 7378) and 5-HT_{3} (tropisetron) receptors had no effect [1]. Furthermore, spiperone (a selective 5-HT_{2A} receptor antagonist) dose-dependently suppressed paw lifting and licking evoked by both formalin and a combination of α-methyl-5-HT and PGE_{2}, thereby supporting the authors' claim that the potentiation of the nociceptive actions of other inflammatory mediators is mediated by 5-HT_{2A} receptor activation [1]. Studies in animal [115] and human muscle [10, 85] provide further evidence that 5-HT may have an indirect action on nociceptive afferents to produce pain and/or hyperalgesia, and will be discussed in the sections that follow.

Regardless of whether 5-HT has a direct or indirect effect on nociceptive afferents, the exact roles of the 5-HT_{1}, 5-HT_{2A}, and 5-HT_{3} receptors in peripheral nociceptive transduction require further delineation. Most studies on 5-HT in nociceptive transduction have been done in cutaneous tissue, as illustrated in this section. Further, the great majority of these studies are behavioural pain studies that fail to distinguish between peripheral and central mechanisms. In light of the fact that the most clinically relevant pain syndromes involve muscle rather than cutaneous tissue, and that peripheral mechanisms in muscle pain are poorly understood, particularly with respect to 5-HT involvement, there is a need to explore the transduction of muscle pain in relation to peripheral 5-HT receptor mechanisms.
Animal 5-HT Studies in Muscle

In contrast to the study of 5-HT in cutaneous nociception, very few studies have addressed the role of 5-HT in muscle nociception. In a study by Fock and Mense (1976), it was shown that 5-HT effectively stimulated muscular group IV afferent units (or C fibres) when injected into the sural artery supplying the excised gastrocnemius-soleus muscle of the cat [58]. When compared to the other pain-producing substances histamine and K$^+$ ions, 5-HT was a more effective stimulant by far; however, it was considerably less potent than BK [58]. In addition, 5-HT (as well as PGE$_2$) was shown to sensitize group IV muscle receptors to BK, indicating that 5-HT-induced chemical sensitization of group IV muscle receptors to BK may be important in the production of pain in inflamed or injured muscle [115].

Okamoto et al (2004) suggested the involvement of peripheral 5-HT$_3$ receptors in TMD-type masseter muscle pain [125] on the grounds that both systemic (intraperitoneal) and local (intramuscular) injection of tropisetron significantly suppressed late-phase orofacial nocifensive behaviours evoked by formalin injection into the masseter muscle. However, the antinociceptive effects of tropisetron were only seen in rats with CFA-evoked inflammation of the TMJ, whereas tropisetron had no effect in rats without CFA inflammation. Given that the majority of myofascial TMD patients lack overt signs of gross inflammation in the temporomandibular region, the method of masseter muscle and TMJ inflammation used in this study poorly models TMDs. The authors' speculation of a 5-HT$_3$ receptor involvement in the mediation TMD pain however is quite plausible, since other studies in humans have also supported this theory [49].
Temporomandibular Disorders (TMDs)

TMDs are a group of musculoskeletal conditions marked by chronic pain in the TMJ and/or masticatory (jaw-closing) muscles [44,100]. TMDs affect anywhere from 5 to 15% of North American adults [44,63,100,103,106] and are more prevalent in adults than in the elderly and children [100,105]. Among adults, TMDs are on average twice as common in women than in men (affecting anywhere from 8 to 15% women and 3 to 10% of men) [99,100]. The diagnosis of TMDs is based on criteria that categorize TMDs into three groups: (I) muscle disorders, (II) disc displacements, and (III) arthralgia, arthritis, and arthrosis [43].

Muscle or myofascial TMDs are the most common of the three groups, reported by approximately 60 to 70% of TMD patients [134,149] and are characterized by dull, aching pain and tenderness in the masticatory muscles that worsen during function [42,113]. Pain is often confined to the masticatory muscles; however, it can also be referred to other regions, for example, the face, jaw, inner ear, temples, and front teeth [113]. Many patients complain of localized tender points within masticatory muscle, which some clinicians refer to as myofascial trigger points [113]. The diagnosis of myofascial TMDs is especially difficult since it is rarely associated with gross pathological changes such as inflammation and relies heavily upon subjective pain reports by patients [42].

Disc displacement (Group II) disorders occur when the disc moves anterolaterally or anteromedially from its original position between the articular eminence and condyle and can be diagnosed with limited jaw opening and reduction, a process in which the disc returns to its normal position upon full opening, causing a clicking noise [42].
Arthralgias, the least severe of Group III disorders, are characterized by pain and tenderness in the joint region, often originating from the capsule and synovial lining of the TMJ [42]. More severe Group III disorders such as osteoarthritis and osteoarthrosis are marked by erosion or sclerosis of joint components [43].

Pathophysiology

The pathophysiology underlying myofascial TMDs is poorly understood. One controversial theory is that mechanical overloading of the muscle and the ensuing ischemia and trauma lead to myofascial pain [116]. This concept is illustrated in human experimental pain studies which have shown that mechanical overloading of the masticatory muscles by controlled repetitive bruxing movements in healthy subjects causes pain and prolonged soreness [8]. Mechanical overloading (e.g. via repetitive muscle movements for instance associated with bruxism) is associated with the development of myofascial trigger points (located in taut bands or regions of thickened skeletal muscle), which when active become locally painful and tender [152]. In vivo microdialysis carried out to examine the “biochemical milieu” of myofascial trigger points within the human trapezius muscle demonstrated significantly elevated 5-HT levels as well as other neurochemicals such as glutamate, BK, substance P, CGRP, norepinephrine, tumor necrosis factor-α, and IL-1β in subjects with active trigger points compared to healthy subjects [152]. It is possible that the development of myofascial trigger points in masticatory muscle can lead to similar elevations in neurochemicals, in particular 5-HT.
Indeed, a role for elevated skeletal muscle levels of the endogenous biogenic amine 5-HT in myofascial TMDs and FM has been suggested by Ernberg et al (1999) [47]. 5-HT levels were found to be elevated in the masseter muscle of patients with TMD-related masseter muscle pain, and these levels were associated with pain intensity [47]. Injection of 5-HT into the masseter muscle of healthy female subjects evoked short-lived pain and mechanical allodynia (an increased sensitivity to normally non-noxious stimuli) in the region of injection [50]. Co-injection of 5-HT with the selective 5-HT\textsubscript{3} receptor antagonist granisetron blocked both 5-HT-evoked pain and mechanical sensitization [49]. Taken together, these results suggest the involvement of 5-HT in the transduction of masticatory muscle pain via a peripheral mechanism involving the 5-HT\textsubscript{3} receptor.

There is also evidence that the excitatory amino acid glutamate may play a role in the development and maintenance of masticatory muscle pain. Human studies have demonstrated that local injection of glutamate into the masseter muscle evokes pain and mechanical allodynia in humans, and that glutamate-evoked pain is greater in females than in males [24,157]. In parallel, electrophysiological studies in rats have shown that injection of glutamate into the masseter muscle evokes afferent discharge and decreases the MT of putative nociceptive afferent fibres. Also, glutamate-evoked afferent discharge, but not mechanical sensitization, is greater in female rats than in male rats [23]. Glutamate involvement in masticatory muscle nociception is clearly evident based on human and animal studies, and the revelation of sex-related differences makes stronger the case for glutamate, as myofascial pain conditions such as TMDs exhibit sex-related differences.
A decrease in muscle pH, a phenomenon observed in the human trapezius muscle [152], may also contribute to the development of myofascial pain associated with TMDs. This theory is somewhat supported by a study in which experimental ischemia in the working human forearm muscles (by the “submaximal effort tourniquet technique”) resulted in a decrease in intradermal pH in the skin directly overlying the muscles [81]. Although intradermal pH is not representative of intramuscular (IM) pH (the investigators decided against inserting the pH probe directly into the muscle out of concern of developing clots around the probe during muscle contractions), it may reflect to some degree what is happening in the underlying muscle as well. Furthermore, when acid phosphate buffer was continuously infused into the flexor muscles of the human forearm, subjective pain scores increased considerably and were correlated with the infusion rate [81]. In the human masseter muscle, magnetic resonance spectroscopy (MRS) revealed significantly decreased pH during isometric clenching compared to resting state [95]. Although a decrease in masticatory muscle pH has not been demonstrated in TMDs per se, evidence of pH drops in skeletal muscle suggests that a similar trend is possible in myofascial TMDs associated with muscle overactivity.

It remains to be determined to what extent biological factors play a role in sex-related differences in the prevalence of TMDs. Clinical studies have shown that menstrual cycle stage influences masseter muscle pain report [100] and mechanical sensitivity measured by pressure pain thresholds (PPTs) [40,82]. Recent animal studies indicate that the mechanical sensitivity of putative masseter muscle nociceptive afferent fibres is positively correlated with estrogen concentration, such that MT is lowest when estrogen levels are at their lowest level (perimenstrually) [112]. Masseter
muscle afferent discharge in response to certain chemical stimuli, such as glutamate, is not only greater in females than in males but also appears to vary across the estrus (menstrual) cycle [22]. While these studies suggest that reproductive hormones, particularly estrogen, may influence pain in chronic myofascial pain conditions, the underlying mechanisms are likely complex and multi-faceted, and require further investigation [30].

**Fibromyalgia (FM)**

Initially thought to be a painful inflammatory condition affecting fibrous tissue, FM was previously referred to as fibrositis, but the demonstration of the absence of any inflammatory or other histopathological involvement led to the revision of this misnomer to the currently accepted term fibromyalgia [129]. FM is a syndrome of chronic (at least three months in duration) widespread musculoskeletal pain and tenderness [4,114,167]. FM affects approximately 2 to 2.4% of North American adults, and like TMDs, it is more prevalent in women (3.4%) than in men (0.5%) [101,114]. In the Canadian population, the prevalence in women and men were reported at 5% and 1.6%, respectively, clearly indicating a higher prevalence in women [140].

FM pain is often characterized as having a continuous component described as a dull ache and an intermittent component of a shooting quality, and usually affects the neck, shoulder and back muscles [14,41]. The majority (73-85%) of FM patients also report symptoms of fatigue, stiffness, and sleep disturbances, and a smaller yet substantial (45-69%) proportion of patients report headache, paresthesias, and psychological distress such as anxiety [4,167]. Recently developed diagnostic criteria
for FM (American College of Rheumatology, 1990) include the presence of widespread pain and concomitant tenderness to palpation in at least eleven out of eighteen possible tender point sites [167]. Under this classification scheme, widespread pain refers to pain in the left and right side of the body, above and below the waist, and in the axial skeleton [167]. The presence of tender points is determined by digital palpation of eighteen predetermined tender point sites, with pain upon palpation of any one site counting it as one of the eleven required for a positive diagnosis [167]. Patients with FM often exhibit signs of masticatory muscle pain and tenderness, which are classical signs of TMDs.

Pathophysiology

The pathophysiology underlying FM is poorly understood. More recently, a myriad of etiological postulates have been tested in a drawn-out attempt to explain the mechanisms underlying this condition, including psychopathogenic mechanisms [2,33,41,80,129,165,170] and sympathetic overactivity [143,170].

Of particular interest over the years was the role of the biogenic amine 5-HT and its essential amino acid precursor tryptophan. In 1978, Modolfsky and Warsh reported a significant inverse relationship between plasma free tryptophan levels and pain severity in eight FM patients, which they related to a CNS 5-HT deficiency [120]. A parallel inverse relationship was reported between 5-HT and pain response in animal studies, in which 5-HT depletion, for example by medial forebrain bundle lesion in rats, produced an enhanced behavioral response to noxious stimuli as measured by electrical shock and hot-plate methods [69]. In another study, dietary tryptophan
deprivation in rats seemed to enhance their jump response to electric shock [117]. There are, however, severe limitations to the application of these animal results to chronic muscle pain conditions such as FM and TMDs since they were obtained from cutaneous rather than muscle models of pain.

Although numerous studies seem to support the theory of a CNS 5-HT deficiency, other findings contradict this relationship between 5-HT and FM symptoms. For example, Modolfsky et al (1976) showed a lack of improvement in pain and mood disturbances in FM patients given high dose oral tryptophan [119]. Another human study by Wolfe et al (1997) reported a positive rather than an inverse correlation between serum 5-HT and tender point count as well as depression and anxiety [166].

The relationship between plasma free tryptophan and FM symptoms led to the investigation of other serum amino acids in FM. Russell et al (1989) measured the serum levels of 29 amino acids in FM patients, and found that in addition to tryptophan, levels of alanine, citrulline, histidine, lysine, proline, phosphoserine, serine and threonine were significantly lower in FM patients (n = 20) in comparison to matched healthy controls [142]. However, there was no apparent correlation between tryptophan concentration and severity of FM symptoms [142]. Yunus et al (1992) expounded on these findings by measuring the plasma levels of 22 amino acids, and as an indirect measure of brain 5-HT activity, the transport ratios of tryptophan and other large neutral amino acids across the blood/brain barrier in FM [171]. Plasma histidine and serine concentrations were significantly lower in FM patients than in healthy controls; however, when comparing the transport ratios of large neutral amino acids between the two groups, only the transport ratio of tryptophan was found to be significantly lower in FM
patients compared to controls [171]. Negative correlations were found between plasma tryptophan levels and FM symptoms such as pain and stress, but interestingly, the tryptophan transport ratio correlated with FM symptoms only when the FM and control groups were combined [171]. These findings lend further support to the theory of a central 5-HT deficiency in FM. A peripheral 5-HT deficiency, either alone or in combination with a central deficiency, is possible since most claims of a central 5-HT deficiency were made on the observation of serum (peripheral) 5-HT deficiencies in FM patients. A peripheral defect is further supported by the report of increased 5-HT reuptake receptor density on peripheral platelets in FM patients [141].

In direct contrast, however, recent studies suggest that rather than a peripheral deficiency, a peripheral elevation of 5-HT occurs in FM [47]. FM patients suffering from localized masseter muscle pain had significantly increased muscle 5-HT levels within the masseter muscle compared to healthy controls [47]. As already mentioned in the TMD section, it was demonstrated in healthy female subjects that injection of 1 mM 5-HT into the masseter muscle evoked significant pain and allodynia [50] that were attenuated by co-injection with granisetron [49]. In FM patients, however, 5-HT injection did not cause masseter muscle pain and allodynia/hyperalgesia [50]. In addition, injection of granisetron into the painful masseter muscle of FM patients did not influence local pain and allodynia/hyperalgesia [51].

Human experimental pain studies in the tibialis anterior muscle of the leg demonstrated a significant increase in subjective pain scores to IM injection of 5-HT compared to isotonic saline, although the mean peak pain scores (as determined by visual analog scale peaks, abbreviated VAŚ-peaks) to 5-HT did not exceed two on a
scale of ten (where “0” denotes no pain and “10” denotes worst pain imaginable) [10,11]. Injection of either BK or 5-HT alone did not cause any mechanical sensitization as determined by PPT measurements [11]; however, a combination of 5-HT + BK resulted in significant mechanical sensitization [10]. Similarly, a combination of 5-HT + BK evoked significantly greater pain than did a combination of 5-HT + isotonic saline and isotonic saline + BK. The demonstration that pre-injection of 5-HT augmented BK-evoked pain and mechanical hyperalgesia suggests that 5-HT may enhance the algesic and hyperalgesic effects of other substances such as BK, signifying an indirect role of 5-HT in myofascial pain mechanisms [9,10]. Essentially, this idea assumes the involvement of more than one endogenous substance, which from a physiological standpoint may be more realistic, and exemplifies just how complex and intricate the etiological picture may become.

Another theory on the pathophysiology of FM, although controversial and largely speculative, involves the presence of microcirculatory disturbances in the muscle that lead to hypoxic conditions [72]. Fassbender and Wegner (1973) observed swollen muscle capillary endothelial cells upon morphological examination of trapezius muscle biopsies in FM likely associated with local hypoxic regions [55,107]. Bengtsson et al (1986) described “ragged red” and “moth-eaten” fibres in muscle biopsies from FM patients [15], both of which have been observed following experimentally-induced ischemia/hypoxia [70]. Lund et al (1986), however, failed to demonstrate that FM patients have lower muscle oxygenation (as measured by muscle surface oxygen pressures, or pO2, in the trapezius muscle) than healthy subjects, although FM patients exhibited abnormal muscle oxygenation in comparison to healthy controls [107]. A
recent study by Jeschonnek et al (2000) demonstrated disturbed blood flow via laser
doppler flowmetry in the skin overlying multiple myofascial tender points in FM patients
[86]. To date, it is still not clear what causes these disturbances in microcirculation in
FM, but it has been theorized that fatigue and poor aerobic fitness may be contributing
factors [72]. Poor sleep causes fatigue and therefore inactivity, leading to unfit muscle
and subsequent microtrauma of the muscle [72]. Microtrauma may then result in an
increased excitability of muscle nociceptive afferent fibres perhaps through the release
of a number of neurochemicals, in particular, 5-HT [72]. A lack of obvious aberrations in
capillary morphology and blood flow was demonstrated in the nail-fold of FM patients
[59]. The examination of cutaneous rather than muscle tissue to study a predominantly
myofascial pain syndrome, however, makes problematic the application of these
findings to FM. Findings of a reduction in the energy substrate ATP in the trapezius
muscle of humans suffering from chronic work-related muscle pain may also indicate an
“energy crisis” that arises from hypoxic conditions in the muscle due to microcirculation
impairment [97]. This energy imbalance can hypothetically cause damage to muscle
membranes [72], which can in turn lead to the leakage of K\(^+\) ions and release of other
excitatory neurochemicals including 5-HT into the interstitial fluid, where they could
excite and or sensitize muscle nociceptive afferent fibres [72].

**Pharmacotherapy of TMDs and FM**

Treatment of TMDs and FM is similar to that of other musculoskeletal disorders,
and consists mainly of non-steroidal anti-inflammatory drugs (NSAIDs), muscle
relaxants, corticosteroids, opioids and antidepressants [67,104]. However, these drugs
are not always effective and pose the risk of side effects. For example, prolonged NSAID use can cause gastro-intestinal ulceration, exacerbation of hypertension, and renal dysfunction. Newer treatments based on the experimental findings discussed in the previous section are being tested and include the 5-HT receptor antagonist granisetron [51].

Clinical studies using short term treatment with the selective 5-HT$_3$ receptor antagonists tropisetron (5 mg orally once daily for ten days) and ondansetron (8 mg orally twice daily for five days) demonstrated significant reductions in pain intensity and tender points compared to control in FM patients [54,77]. A pilot study evaluating tropisetron (5 mg daily) therapy for four weeks in ten female FM patients (three of whom dropped out due to side effects) showed similar improvements in FM symptoms, although the lack of a placebo control may compromise the integrity of the results [127,155]. Inconsistent with orally administered 5-HT$_3$ receptor antagonists, IM injection of granisetron (1 mg/ml) into the masseter muscle of female FM patients did not improve symptoms of muscle pain and allodynia within thirty minutes of injection [51].

The use of highly selective 5-HT receptor antagonists such as those targeted at the 5-HT$_3$ receptor offers an effective means of studying the role of the different receptor subtypes in FM while minimally interfering with other receptor systems. However, it is difficult to interpret the results of short-term studies on chronic pain syndromes such as FM. In a six-month clinical study comparing the effects of amitriptyline and cyclobenzaprine to placebo in FM patients, significant clinical improvements were observed after one month in amitriptyline patients compared to placebo [31]. There is good reason to believe that serotonergic mechanisms and in
particular the 5-HT$_3$ receptor is involved in FM since most drugs used to manage FM act on these mechanisms and are clinically effective.

It appears that in humans injection of 5-HT into masticatory muscle causes brief, low intensity pain and more prolonged mechanical sensitization [11, 48, 50, 85]. Since in vivo electrophysiology at the trigeminal afferent fibre level is not feasible in humans, experiments of this nature are largely limited to reduced animal species such as the rat. In particular, the Sprague Dawley rat strain exhibits consistent sex-related differences in the response of putative nociceptive afferent fibres to masseter muscle injection of noxious substances (e.g. glutamate) that mimic differences displayed in humans [24]. Therefore, the purpose of our study was to better understand peripheral serotonergic mechanisms in human myofascial pain by extrapolating results we obtained from a more detailed study in rats. We anticipate that the results of our experiments will shed some light on the peripheral mechanisms underlying chronic myofascial pain conditions associated with TMDs and FM.

**Experimental Hypothesis**

IM injection of 5-HT into the rat masticatory (masseter/temporalis) muscle will cause an increase in muscle afferent fibre excitability via 5-HT$_3$ receptor (ligand-gated to an ion channel) activation and a reduction in MT via 5-HT$_1$ and/or 5-HT$_2$ receptor (G-protein linked) activation in a sex-dependent manner.
Research Objectives

The objectives of this study are the following:

(i) To determine the frequency of expression of the 5-HT$_{1B}$, 5-HT$_{2A}$, and 5-HT$_3$ receptor subtypes by masseter muscle trigeminal ganglion cells in male and female rats.

(ii) To determine the effects of repeated IM 5-HT injection on the excitability and MTs of masticatory muscle afferent fibres in male and female rats.

(iii) To examine the possibility of sex-related differences in the response of masticatory muscle afferent fibres to IM injection of 5-HT.

(iv) To determine which 5-HT receptor subtype(s) contribute(s) to the change(s) in excitability and MT evoked by IM injection of 5-HT.
II METHODS

Immunohistochemistry

Immunohistochemistry experiments were carried out to determine the frequency of 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>3</sub> receptor subtype expression in masseter muscle ganglion cells. Bilateral injection of Fast Blue, (FB 2%, 10 μl, purchased from Polysciences<sup>®</sup>, Inc., Warrington, PA) a fluorescent tracer taken up by axons, was made into the right and left masseter muscles of four adult Sprague Dawley (two male, two female) rats anesthetized with isoflurane. Care was taken to avoid injection of FB into the skin overlying the muscles. Following a survival period of seven days, rats were sacrificed with an overdose of IV pentobarbital (100 mg/kg). Rats were perfused with 120 ml cold heparinized saline (5%) followed by 120 ml of the fixative paraformaldehyde (4%) using an infusion pump (KD Scientific Model 200 Series, Holliston, MA). After perfusion, rats were decapitated and intact heads were placed in a paraformaldehyde (4%) bath for several days. The left and right trigeminal ganglia were removed and cut on a vibratome into 40 μm sections. Sections were then sorted into three groups, each to be incubated with one of three primary antibodies. Each group contained tissue sections from both male and female ganglia so that sex-related differences in the expression of each receptor subtype could be assessed. Consecutive sections from one ganglion were assigned to different groups in order to minimize the possibility of double counting cells.

Sections were incubated in 5% normal goat serum (NGS) in phosphate buffered saline for 1 hour at room temperature followed by overnight incubation in a humidity chamber with one of three commercially-available primary antibodies in 1 % NGS:
affinity purified rabbit anti-serotonin 5-HT_{1B} receptor polyclonal antibody (1:800 dilution, purchased from Chemicon\textsuperscript{®}) with reactivity in human and mouse species; purified mouse anti-serotonin 5-HT_{2A} receptor monoclonal antibody (1:100 dilution, purchased from BD PharMingen\textsuperscript{®}) with reactivity in human, monkey, and rat species; and rabbit anti-serotonin 5-HT_{3} receptor monoclonal antibody (1:33 dilution, purchased from Sigma\textsuperscript{®}, St. Louis, MO) reactive against mice and rats.

Following incubation with primary antibodies, sections were washed several times with phosphate buffered saline and subsequently incubated for 60 min (in the dark, at room temperature) with a secondary antibody conjugated with a fluorescent compound. We used Cy\textsuperscript{TM} 3-conjugated AffiniPure Goat Anti-Rabbit IgG (1:200 dilution, purchased from Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for the 5-HT_{1B} and 5-HT_{3} receptors and Cy\textsuperscript{TM} 3-conjugated AffiniPure Goat Anti-Mouse IgG (1:200 dilution) for the 5-HT_{2A} receptor. After washing several times with PBS, sections were mounted onto slides, coverslipped with Immunofluor\textsuperscript{®} mounting medium, and visualized under a Leica\textsuperscript{®} DML fluorescent microscope. Images for analysis were acquired with a Cool Snap\textsuperscript{®} CCD camera attached to the microscope. Sections that were not incubated with the primary antibody served as a control [38,91].

**Sample Size**

A sample size of greater than one hundred masseter ganglion cells was used for the immunohistochemical identification of each receptor subtype. This ensured that the frequencies of expression we reported were relatively accurate and reliable.
Data Analysis

Trigeminal ganglion sections were examined for FB-positive neuronal cells under a Leica DML fluorescent microscope and photographs were captured using a Cool Snap CCD camera attached to the microscope. The cell body diameters of all FB-positive cells were estimated by calculating the distance from the left edge to the right edge of each cell using Adobe® Photoshop®. The frequency and distribution of cell diameters of all FB-positive cells were determined. In addition, FB-positive cells were examined for 5-HT$_{1B}$-, 5-HT$_{2A}$-, and 5-HT$_{3}$-like immunoreactivity.

Statistical Analysis

Differences in the frequency of 5-HT$_{1B}$, 5-HT$_{2A}$, and 5-HT$_{3}$ receptor expression between male and female masseter ganglion cells were assessed using Chi-square analysis, with p < 0.05 required for significance.

Electrophysiology

Surgical Preparation

Adult Sprague-Dawley rats of both sexes (225 – 450g) were prepared for acute, in vivo extracellular recording of single trigeminal muscle (masseter and/or temporalis) afferent fibre activity. Anywhere from one to a maximum of three afferent fibre(s) were studied per rat, with a total recording time of 90 minutes per fibre. Single unit recording was performed for using an AC amplifier (A-M systems model 1800) routed into a computer equipped with Spike 2 (Cambridge Electronic Design, Cambridge, U.K.) for online data collection. The surgical preparation and recording techniques used are
identical to those previously described by Cairns et al [23-28]. After weighing rats, anesthesia was induced with isoflurane delivered in O<sub>2</sub> via a facemask. In all female rats, a vaginal lavage was performed to determine estrus stage by microscopic examination of vaginal epidermal cells. A tracheotomy was performed, allowing for the initiation of mechanical ventilation. Isoflurane and oxygen flow rates were maintained at 1.5 to 3%, and 0.3-0.4 L/min, respectively [23]. Core body temperature was monitored with a rectal temperature probe and the rat was placed on a homeothermic heating pad to maintain body temperature within the normal physiologic range. Electrocardiogram (ECG) leads were affixed to monitor heart rate throughout the experiment. Blood pressure was measured through a pressure transducer connected to the femoral artery via polyethylene tubing and continuously monitored throughout the experiment.

The rat's head was placed in a Kopf® stereotaxic frame and the skull exposed by incising the skin over it. A trephination was made on the right side of the skull to allow the lowering of a recording microelectrode into the trigeminal ganglion located beneath the brain. In addition, the dura overlying the caudal brainstem was removed, allowing the placement of a stimulating electrode directly against the caudal brainstem. The isoflurane level was adjusted throughout the experiment to maintain an adequate level of anesthesia. Parameters such as heart rate, mean arterial blood pressure, expired CO<sub>2</sub>, and core body temperature were monitored and maintained within the range of 300-350/min, 60-80 mmHg, 20-50 mmHg, and 36.8-37.2 °C, respectively [23]. All protocols were performed in accordance with the Guidelines issued by the University of British Columbia Animal Care Committee.
Recording Techniques

Single masticatory muscle afferent units were found by applying mechanical search stimuli to the masseter and temporalis muscles with a blunt-tipped applicator while a parylene-coated tungsten recording microelectrode was lowered through the brain into the trigeminal ganglion (Figure 2.1).

When a unit was found, its receptive field was carefully assessed to ensure that it responded to deep (muscle) rather than superficial (cutaneous) stimulation. This assessment was made by gently pulling the skin away from the muscle and applying brush, pinch, and pressure stimuli solely to the skin. A lack of response to cutaneous stimulation confirmed the location of the fibre's mechanoreceptive field within the muscle.

Considering that the majority of masticatory muscle nociceptors project centrally to the caudal brainstem [151], only fibres that projected to this region were studied. A description of the methods we used to identify these fibres follows.

Orthodromic-Antidromic Collision Techniques

To confirm the projection of fibres to the caudal brainstem, orthodromic-antidromic collision techniques were applied whereby antidromic action potentials generated by electrical stimulation of the caudal portion of the brainstem were collided with orthodromic actions potentials generated by mechanical stimulation of the fibre's muscle receptive field (Figure 2.2) [23].

The CV of each fibre was calculated by dividing the distance between the two electrodes by the antidromic latency. Slow Aδ and C fibres were used in this
Figure 2.1. A. The diagram illustrates the methodology employed to identify masticatory muscle afferent fibres with caudal brainstem projections. B. The traces outline masseter muscle afferent fibre action potential discharge (upper trace) in response to increasing force applied to the muscle with an electronic Von Frey hair (lower trace).
Figure 2.2. The top trace illustrates the generation of an orthodromic spike by mechanical stimulation of the temporalis muscle and an antidromic spike by electrical stimulation of the caudal brainstem. By shortening the delay between the oppositely-going spikes (middle trace), collision was achieved, causing the antidromic spike to disappear (*). Lengthening the delay between the two spikes resulted in recovery of the antidromic spike, as shown in the lower trace.
experiment, since these are the fibres that respond the greatest to algesic substances like glutamate and hypertonic saline, and are considered to be nociceptors.

**Experimental Design**

**Experiment 1:** The first series of experiments were carried out to determine if repeated IM injection of 5-HT excites or sensitizes masseter/temporalis afferent fibres *in vivo*. Groups of afferent fibres received either control (phosphate buffered isotonic saline) or 5-HT (0.1 mM, 1 mM, or 10 mM). These data were used to construct a concentration-response curve.

In all rat experiments, baseline MT was recorded for 10 min prior to injection of any substance into the muscle using an electronic Von Frey hair (model 1601C IITC) (Figure 2.1A and 2.3). The baseline MT was determined by averaging the thresholds for ten consecutive mechanical stimuli applied at 1-min intervals.

IM injections were made with a 27-gauge needle connected by polyethylene tubing to a 50 µl Hamilton syringe. The needle tip was inserted into the receptive field of the muscle afferent fibre and baseline activity was recorded for 10 min prior to injection in order to assess any spontaneous discharge (Figure 2.3). Following baseline, 10 µl of control or 5-HT solution was slowly injected (over a 5 to 10 s period), and any evoked activity was recorded for a period of 10 min. A second identical injection was made 30 min after the first one to assess the reproducibility of the response to 5-HT, and again evoked activity was recorded for a period of 10 min. 41 min after the first injection, 30 consecutive MT measurements were recorded at 1-min intervals to assess any sensitization that may have occurred in response to 5-HT injection.
Figure 2.3. The experimental protocol outlined above was applied to each masticatory muscle trigeminal ganglion neuron identified. The horizontal axis represents time in minutes, and the double-headed arrows represent the time span for the recording of evoked activity (following injections 1 and 2, ▲) and MTs.
Experiment 2: The second series of experiments were undertaken to determine whether the 5-HT\textsubscript{3} receptor subtype mediated any significant increase in evoked activity or decrease in MTs. These experiments followed the same timeline as in Experiment 1 (refer to Figure 2.3). Initially, 5-HT was injected at a fixed concentration of 10 mM. This concentration was chosen because it evoked the greatest response and elicited a significant change in activity, as determined from Experiment 1. Thirty min after the initial injection, a second injection was made of 10 mM 5-HT alone (control), or in combination with the selective 5-HT\textsubscript{3} receptor antagonist tropisetron at a concentration of 1 mM. This concentration ensured a 10:1 (agonist:antagonist) ratio, which, in past studies, was sufficient to block 5-HT-mediated inward current in trigeminal ganglion neurons [78,79]. As in Experiment 1, baseline and post-second injection MTs were assessed. As a final confirmation that the effects of 5-HT injection were mediated, at least in part, by the 5-HT\textsubscript{3} receptor, selective agonist experiments were carried out with the selective 5-HT\textsubscript{3} receptor agonist 2-methyl-5-hydroxytryptamine maleate (2-methyl-5-HT). These experiments, designed to replicate the effects of 10 mM 5-HT, involved the repeated IM injection of 10 mM 2-methyl-5-HT [78,79]. All solutions were phosphate-buffered to achieve a pH of approximately 7.4. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO. USA).

Terminal Procedures

At the end of each experiment, rats were euthanized with an over-dose of pentobarbital (100mg/kg IV).
Sample Size

In the electrophysiology experiments, data from at least 20 afferent fibres (10 from males, 10 from females) were collected for each 5-HT concentration and for each selective agonist and antagonist used. These numbers are based on a sample size estimate using a one-way ANOVA with a power of 0.8 and an expected difference of at least 25% in evoked activity between control and treatment. Therefore, a grand total of at least 120 afferent fibres were studied. Whenever possible, data were collected from one masseter and one temporalis muscle afferent fibre per rat. If a third fibre was found in either muscle, it was examined only if its receptive field was at least one centimeter away from any other fibre already studied in the same muscle.

Data Analysis

Evoked Discharge: The response to injection of buffered isotonic saline and 5-HT was analyzed in terms of magnitude as well as frequency. Peri-stimulus time histograms were generated from each fibre's raw discharge data using 1-s bins. From these histograms, peak afferent fibre discharge rate, or peak discharge (Pk), was determined to be the highest rate of discharge (in Hz) recorded during 10 min following injection. Because the responses to injection of 5-HT were very rapid and short-lived, a response was defined as a peak discharge of greater than 1 Hz. The relative peak was calculated by dividing the peak afferent fibre discharge from the second injection by that from the first, and this served as a measure of the reproducibility of the response to 5-HT injection.
The frequency of response to injection of buffered isotonic saline and each concentration of 5-HT was determined by dividing the number of cells that responded to injection (i.e. had a Pk of greater than 1 Hz) by the total number of cells within that particular treatment group, and expressing this ratio as a percentage. Concentration-response plots were constructed from both the magnitude (peak afferent discharge) and frequency of response data.

**Mechanical Threshold:** Changes in the mechanical sensitivity of afferent fibres were assessed by recording MTs every minute for 10 minutes before the first injection and for 30 minutes post-second injection with an electronic von Frey hair. MT measurements were averaged over 10 min epochs (time periods) and normalized to the baseline MT [23,28].

**Statistical Analysis**

**Evoked Discharge:** Peak discharges (Pk) evoked by buffered isotonic saline and 5-HT (0.1, 1, 10 mM) were compared by a Kruskal-Wallis one-way ANOVA on Ranks with treatment as a single factor, followed by post-hoc analysis with Dunn's Method. An increase in peak discharge following 5-HT injection compared to buffered isotonic saline was interpreted as an effect. In addition to assessing the evoked discharge data from a magnitudinal perspective, these data were also analyzed in terms of the frequency of response using Chi-square analysis.

A paired t-test was used to assess the reproducibility of the response to repeated injection of 5-HT. The same test was used to assess the attenuation of 5-HT-evoked
discharge by a second co-injection of 5-HT and tropisetron. Relative peak discharge was compared between 10 mM 5-HT and 10 mM 2-methyl-5-HT groups with a t-test (since data for relative peak discharge passed tests for normality and equal variance).

Mechanical Threshold: For MT data, a GLM repeated measures ANOVA was used to compare saline control to each 5-HT concentration, with time as a repeated factor and concentration and sex as non-repeated factors. For data that was normally distributed, the mean ± SE is given. For data that was non-normal, the median and interquartile range (IQR) is given. A p-value of less than 0.05 was required for significance in all statistical tests.
III RESULTS

Immunohistochemical Identification of 5-HT Receptors on Masseter Ganglion Cells

A total of 839 FB-positive (masseter ganglion) cells with a median cell body diameter of 74.5 μm (IQR 54.2 – 89.7 μm, range 11.6 – 151.1 μm) were identified from ganglion sections in this series of experiments. The pattern of distribution of cell diameters was similar to those observed in previous histological studies in rat and human trigeminal ganglia [6,75], and there was no obvious difference in size distribution between male and female masseter ganglion cells. Although there was a wide range of cell sizes, most cells were relatively large in diameter and fell in the 60 to 100 μm range (Figure 3.1.A).

The frequency of 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>3</sub> receptor expression by masseter ganglion cells was 28.0 (n = 354), 11.1 (n = 207), and 52.5 (n = 278) %, respectively. In male and female masseter ganglion cells, the respective frequencies were 24.8 and 30.0 % for the 5-HT<sub>1B</sub> receptor, 14.2 and 7.4 % for the 5-HT<sub>2A</sub> receptor, and 38.5 and 68.5 % for the 5-HT<sub>3</sub> receptor. There were no obvious sex-related differences in receptor expression with the exception of the 5-HT<sub>3</sub> receptor, which was more frequently expressed in female than in male masseter ganglion cells. The greater frequency of 5-HT<sub>3</sub> receptor expression by female masseter ganglion cells could be seen at all cell diameters (Figure 3.1.C).
Figure 3.1. A. The bar graph illustrates the frequency of FB-positive cells (%) versus cell body diameter (μm). B. The paired images show examples of three FB-positive cells (blue color) that are immunoreactive for the 5-HT$_{1B}$ (purple color), 5-HT$_{2A}$ (green color), and 5-HT$_{3}$ (red color) receptor subtypes. C. The bar graphs display the frequency of 5-HT$_{1B}$ (left), 5-HT$_{2A}$ (middle), and 5-HT$_{3}$ (right) receptor expression versus estimated cell diameter in male (black bars) and female (white bars) FB-positive (masseter ganglion) cells.
5-HT-Evoked Afferent Discharge

Baseline Properties

A total of 152 (80 male and 72 female) masticatory muscle afferent fibres having caudal brainstem projections were examined. The median CV, MT and other properties were calculated for each sex and for both sexes combined, and are summarized in Table 3.1. Male weights were significantly higher than female weights (p<0.05, Mann-Whitney Rank Sum Test). There were no significant sex-related differences in CV and baseline MT. The influence of estrus cycle stage on the CV and MT of female masticatory afferent fibres was examined to determine whether there were any cyclic changes in fibre properties. However, no estrus-stage related differences with respect to CV and MT were found (Kruskal-Wallis one-way ANOVA on ranks).

One-hundred-and-fourteen (or 75% of) fibres had CVs less than 10 m/s. One-hundred-and-ten of these fibres were slow A\(^\delta\) fibres (CV between 2 to 10 m/s), and the remaining four were C fibres (CV less than 2 m/s).

During the 10 min pre-injection baseline, 52% (69/132) of masticatory muscle afferent fibres exhibited spontaneous discharge. The median discharge rate was 0.013 Hz (range: 0.002 to 2.375 Hz).

Response to 5-HT Injection

Injection of 5-HT into masticatory muscle resulted in rapid, short-lived action potential discharge typically lasting several seconds. Because the responses to 5-HT were rapid and quickly desensitizing, we defined a response as a peak discharge (Pk)
<table>
<thead>
<tr>
<th>Sex (n)</th>
<th>Body Weight (g)</th>
<th>CV (m/s)</th>
<th>MT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (80)</td>
<td>337 (297-391)</td>
<td>5.8 (4.7-8.7)</td>
<td>17.2 (7.5-40.0)</td>
</tr>
<tr>
<td>Female (72)</td>
<td>265 (253-275)</td>
<td>7.4 (5.0-11.3)</td>
<td>12.4 (8.2-22.8)</td>
</tr>
<tr>
<td>Combined (152)</td>
<td>285 (263-340)</td>
<td>6.8 (4.8-9.9)</td>
<td>15.6 (7.5-29.0)</td>
</tr>
</tbody>
</table>

**Table 3.1.** The baseline properties of all masticatory (masseter and temporalis) muscle afferent fibres projecting to the caudal brainstem are summarized. Median values are shown with their respective interquartile ranges in brackets. *p < 0.05, Mann-Whitney Rank Sum Test.*
of greater than 1 Hz following the initial injection. In analyzing the magnitude of evoked response, we initially attempted a three-way ANOVA to detect any significant treatment, sex, and muscle effects on evoked responses and any interactions between factors; however, because the evoked data failed normality, non-parametric tests (on ranks), which can accommodate at most one factor, were carried out instead.

IM injection of 5-HT evoked significant concentration-related increases in peak discharge (p < 0.05, Kruskal-Wallis one-way ANOVA on Ranks) (Figure 3.2.A). Post-hoc analysis using Dunn's Method revealed that injection of 1 mM and 10 mM 5-HT evoked significantly greater peak discharge than saline control; however, the responses to 5-HT showed a lot of variability in terms of magnitude. There were no sex-related differences in the magnitude of evoked response to IM injection of 5-HT (0.1, 1, and 10 mM) and saline control (p > 0.05, Mann-Whitney Rank Sum Test).

In addition, injection of 5-HT evoked significant concentration-related increases in the frequency of response (p < 0.05, Chi square). Injection of 0.1, 1, and 10 mM 5-HT evoked responses in 33.3% (7/21), 61.9% (13/21), and 78.3% (18/23) of masticatory afferent fibres, respectively, while saline control evoked responses in only 18.2% of fibres (4/22) (Figure 3.2.B). Among fibres that responded to an initial injection of 10 mM 5-HT (n = 18), the mean peak discharge evoked by the first and second injections were $8.3 \pm 3.1$ Hz, ± SEM) and $10.0 \pm 4.2$ Hz, and did not differ significantly (p > 0.05, paired t-test). This indicated that repeated injection of 10 mM 5-HT evoked relatively reproducible responses (in terms of magnitude). The mean relative peak discharge, calculated as an additional means of assessing the reproducibility of response, was $1.0 \pm 0.2$ (± SEM, n = 18), indicating again that the response to repeated injection of
Figure 3.2. A. A line and scatter plot illustrating peak discharge (Pk, Hz) versus 5-HT concentration (mM) (*: p < 0.05 Kruskal-Wallis one-way ANOVA on Ranks). B. A bar graph showing frequency of response (Pk > 1) versus 5-HT concentration indicated a significant difference in the frequency of response dependent on concentration (p < 0.05, Chi square analysis).
10 mM 5-HT was reproducible (Figure 3.3). The mean relative peak discharge in the masseter and temporalis muscles was $0.86 \pm 0.22$ (± SEM, $n = 6$) and $1.1 \pm 0.3$ (± SEM, $n = 12$) respectively, indicating that the response to repeated injection of 10 mM 5-HT was reproducible in both muscles and did not differ between muscles ($p > 0.05$, t-test).

It was necessary to demonstrate reproducibility of 5-HT-evoked afferent discharge in order to continue on with the selective antagonist experiments involving an initial injection of 10 mM 5-HT followed by a second co-injection of 10 mM 5-HT and 1 mM tropisetron. In knowing that the responses to repeated injection of 5-HT were reproducible, we were able to determine whether tropisetron was effective in attenuating an initial response to 5-HT.

Data from all fibres that received an initial injection of 10 mM 5-HT ($n = 68$, taken from the 10 mM 5-HT experiments and selective antagonist experiments employing tropisetron) were combined to create a larger data pool from which any relationships between response to 5-HT injection and baseline properties such as baseline MT and CV could be identified. After combining evoked data from all fibres that received an initial injection of 10 mM 5-HT, the frequency of response was calculated to be 57.4 %. Although there was a trend toward an inverse correlation between baseline MT and CV, it was not significant ($p > 0.05$, Spearman Rank Order Correlation) (Figure 3.4). Furthermore, there was a complete lack of a relationship between CV/baseline MT and the magnitude of response to 10 mM 5-HT in each muscle separately and in both muscles combined ($p > 0.05$, Spearman Rank Order Correlation analysis),
A. The voltage trace illustrates an example of the rapid discharge evoked by injection of 10 mM 5-HT into the mechanoreceptive field of a slow Aδ masseter afferent fibre (CV 6.0 m/s, baseline MT 6.4 g). B. Collision of the orthodromic and antidromic action potentials (*) in the same fibre indicated caudal brainstem projection. C. The peri-stimulus time histogram illustrates the rapid and reproducible response of a masseter afferent fibre to repeated injection (▲) of 10 mM 5-HT into its muscle receptive field.
Figure 3.4. The above scatter plot illustrates the association between baseline MT and CV of 68 masticatory muscle afferent fibres, 39 of which responded (●) and 29 of which did not respond (○) to an initial injection of 10 mM 5-HT.
indicating that the excitation of masticatory muscle afferent fibres by 5-HT is not very selective.

Effect of Tropisetron on 5-HT-Evoked Response

Although injections of 1 mM and 10 mM 5-HT evoked reproducible responses, injection of 10 mM 5-HT evoked the greatest magnitude of response and activated the highest frequency of fibres. Therefore, we decided to employ this concentration of 5-HT in the selective 5HT₃ receptor antagonist experiments. All fibres that were included in this series of experiments had to respond to the initial injection of 10 mM 5-HT. A total of 45 fibres were studied of which only 21 responded to the initial injection and were therefore included for analysis.

In these experiments, an initial injection of 10 mM 5-HT evoked a mean peak discharge of 7.4 ± 1.0 Hz (± SEM, n = 21). A subsequent co-injection of 10 mM 5-HT and 1 mM tropisetron evoked a mean peak discharge of 4.2 ± 0.86 Hz (a 43 % reduction in peak discharge), which indicated that tropisetron significantly decreased the magnitude of 5-HT-evoked peak discharge (p < 0.05, paired t-test) (Figure 3.5). The percentage decrease in 5-HT-evoked discharge upon co-injection with tropisetron was similar in male and females fibres (44.4 % in male vs. 42.1 % in female fibres).

Effect of 2-methyl-5-HT on Evoked Response

An initial injection of 10 mM 2-methyl-5-HT evoked a response (Pk > 1) in 40 % of masticatory afferent fibres (n = 20). This frequency of response was significantly lower than that evoked by 10 mM 5-HT (p < 0.05, Chi-square), but similar to that
Figure 3.5. A. The peri-stimulus time histogram illustrates the attenuation of 5-HT-evoked discharge by co-injection of 10 mM 5-HT + 1 mM tropisetron (△) following an initial injection of 10 mM 5-HT (∆) into the mechanoreceptive field of a male temporalis afferent fibre (CV 5.4 m/s, baseline MT 21.1 g). B. The bar graph illustrates the mean peak discharges evoked by an initial injection of 10 mM 5-HT and a second co-injection of 10 mM 5-HT + 1 mM tropisetron. Tropisetron significantly attenuated the response to the initial injection of 10 mM 5-HT (p < 0.05, paired t-test, error bars: SEM).
evoked by 0.1 mM and 1 mM 5-HT (p > 0.05, Chi-square). It was also greater than the frequency of response evoked by buffered isotonic saline, but not significantly (p > 0.05, Chi-square). There was no sex-related difference in the magnitude (p < 0.05, Mann-Whitney Rank Sum Test) and the frequency of response of masticatory muscle afferent fibres to 2-methyl-5-HT (p > 0.05, Fisher Exact Test).

Among fibres that responded to an initial injection of 10 mM 2-methyl-5-HT (n = 8), the mean peak discharge evoked by the first and second injections was 9.8 ± 3.1 and 5.5 ± 2.3 Hz, respectively. The magnitude of these responses did not differ significantly (p > 0.05, paired t-test) (Figure 3.6.C). The mean relative peak discharge was 0.78 ± 0.29 (± SEM), again indicating that the response to repeated injection of 10 mM 2-methyl-5-HT was relatively reproducible, and that like 10 mM 5-HT, 10 mM 2-methyl-5-HT evoked responses of comparable magnitude upon repeated injection (p > 0.05, t-test).

Effect of 5-HT on MT

The post-injection MTs decreased during the first 10 min (T1) epoch regardless of whether buffered isotonic saline or 5-HT (0.1, 1, or 10 mM) was injected into the muscle (Figure 3.7). However, the MTs following injection of saline control returned to baseline (C) levels by the 20 to 30 min (or third, T3) epoch whereas the MTs following injection of 5-HT remained decreased throughout the entire 30 min recording interval. Statistically, there was no significant difference between relative MTs following injection of 5-HT and buffered isotonic saline (GLM repeated measures ANOVA with treatment and sex as non-repeated and time as a repeated factor). Because the greatest
Figure 3.6. A. The voltage trace illustrates an example of the rapid and brief discharge evoked by injection of 10 mM 2-methyl-5-HT into the mechanoreceptive field of a slow Aδ temporalis afferent fibre (CV 5.6 m/s, baseline MT 15.4 g). B. Collision of the orthodromic and antidromic action potentials (*) in the same fibre indicated caudal brainstem projection. C. The peri-stimulus time histogram illustrates the reproducibility of response to repeated injection of 10 mM 2-methyl-5-HT into the temporalis muscle.
Figure 3.7. The grouped bar graph illustrates the effects of 5-HT (0.1, 1, and 10 mM) and saline control on the MTs (relative to baseline, C) of masticatory muscle afferent fibres 1 to 10 min (T1), 11 to 20 min (T2), and 21 to 30 min (T3) following repeated injection (▲). The bars indicate median relative MTs and the error bars represent the interquartile ranges. Injection of buffered isotonic saline resulted in a brief period of mechanical sensitization, whereas injection of 5-HT at all three concentrations resulted in a prolonged sensitization of muscle afferent fibres.
difference in relative MTs between the saline control and 10 mM 5-HT groups was seen during the T3 epoch, this epoch was re-analyzed in terms of the frequency of sensitization. The relative MT during the T3 epoch was neither correlated with baseline MT or CV (p > 0.05, Spearman Rank Order Correlation).

The frequency of mechanical sensitization, no change, and desensitization was assessed during the 21-30 min epoch following injection of saline control and 5-HT. Mechanical sensitization was defined as a MT of at least two standard deviations below the mean baseline threshold, and desensitization was defined as a MT of at least two standard deviations above the mean baseline threshold. Having defined these terms, the frequency of sensitization following injection of 0.1, 1 and 10 mM 5-HT was 28.6% (6/21), 28.6% (6/21), and 21.7 % (5/23), respectively (Figure 3.8). Injection of buffered isotonic saline mechanically sensitized only 5.0 % (1/ 20) of fibres. Despite a trend toward mechanical sensitization following injection of 5-HT, there was no significant difference in the frequency of sensitization between the buffered isotonic saline and 5-HT groups (p > 0.05, Chi-square).

**Effect of Tropisetron on MT**

Following co-injection of 10 mM 5-HT and 1 mM tropisetron, there was no significant change in MT during the 1 to 10, 11 to 20, and 21 to 30 minute epochs as compared to the mean baseline MT (p > 0.05, Friedman Repeated Measures ANOVA on Ranks). The relative post-injection MTs did not differ between fibres that received repeated injections of 10 mM 5-HT and fibres that received a second co-injection of 10 mM 5-HT and 1 mM tropisetron (p > 0.05, GLM three-way repeated measures ANOVA
Figure 3.8. The grouped bar graph illustrates the frequency (%) of sensitization, no change, and desensitization of masticatory muscle afferent fibres during the T3 (21 to 30 min) epoch following IM injection of buffered isotonic saline (n = 20) and 0.1 mM (n = 21), 1 mM (n = 21), and 10 mM (n = 23) 5-HT. There were no significant differences in the frequency of sensitization, no change, and desensitization between 5-HT and saline control groups (p > 0.05, Chi-square).
with treatment and sex as non-repeated factors and time as a repeated factor). Also, there was no significant time or sex-related differences in MTs, and no significant interactions between the three factors tested (p > 0.05, same test as mentioned above).

In experiments in which an initial injection of 10 mM 5-HT was followed by a second co-injection of 10 mM 5-HT and 1 mM tropisetron, 14.3% of fibres (3/21) were mechanically sensitized (i.e. the MT was at least two standard deviations below the mean baseline threshold) during the 21-30 min epoch. This did not differ significantly from the frequency of sensitization in fibres that received repeated injection of 10 mM 5-HT alone (p > 0.05, Fisher Exact Test).

The frequency of desensitization (i.e. a MT of at least two standard deviations above the mean baseline threshold) during the 21-30 min epoch in fibres that received the second co-injection of 10 mM 5-HT and 1 mM tropisetron was 33.3% (7/21), which was significantly greater than the frequency in fibres that received repeated injection of 10 mM 5-HT alone (p < 0.05, Fisher Exact Test). There was no correlation between the relative MT during the 21-30 min epoch and baseline MT or CV (p > 0.05, Spearman Rank Order Correlation).

**Effect of 2-methyl-5-HT on MT**

Following repeated injection of 10 mM 2-methyl-5-HT, there was no significant change in MT during the 1 to 10, 11 to 20, and 21 to 30 minute epochs compared to the mean baseline MT (p > 0.05, Friedman Repeated Measures ANOVA on Ranks). Although there appeared to be a smaller decrease in MT following repeated injection of 10 mM 2-methyl-5-HT compared to 10 mM 5-HT, the difference was not significant (p >
0.05, GLM three-way repeated measures ANOVA with treatment and sex as non-repeated factors and time as a repeated factor). Injection of 10 mM 2-methyl-5-HT resulted in mechanical sensitization of 31.6 % of fibres (6/19), desensitization in 10.5 % of fibres, but no change in the majority (57.9 %) of masticatory muscle afferent fibres during the 21 to 30 min epoch. The frequency of sensitization following repeated injection of 10 mM 2-methyl-5-HT did not differ significantly from that following repeated injection of 5-HT at all three concentrations (p > 0.05, Chi-square). Neither baseline MT nor CV was correlated with the relative MT during the 21 to 30 min epoch (p > 0.05, Spearman Rank Order Correlation).

**Effect of 5-HT on Blood Pressure**

The greatest and most unexpected response to 5-HT was seen in the blood pressure. Injection of 5-HT into masticatory muscle resulted in a rapid and prolonged decrease in median blood pressure with slow recovery (Figure 3.9).

The blood pressure and the duration of decreased pressure, decreased and increased, respectively, in a concentration related manner (Figure 3.10). There was a small decrease in blood pressure following injection of saline. However, injection of 10 mM 5-HT resulted in a significantly greater median drop in blood pressure (-12.4 mmHg, IQR: -16.2 to -9.5 mmHg) than saline controls (-0.6 mmHg, IQR: -2.2 to 1.3 mmHg). In addition, the duration of decreased blood pressure after injection of 10 mM 5-HT into masticatory muscle (554.5 s, IQR: 394.5 to 569.0 s) was significantly longer than saline controls (40.0 s, IQR: 0 to 89.0 s) (p < 0.05, Kruskal-Wallis one-way ANOVA on Ranks, Dunn's Method as post hoc).
Figure 3.9. The line trace (above) and peri-stimulus time histogram (below) illustrate blood pressure (BP) and afferent discharge, respectively, after injection of 5-HT (▲:10 mM) into the mechanoreceptive field of a temporalis afferent fibre (CV 7.8 m/s, baseline MT 14.8 g) in a female rat. The inset illustrates a collision (*) between an orthodromic and antidromic spike recorded from the same fibre.
Figure 3.10. A. The line and scatter plot illustrates the duration of decreased blood pressure following IM injection of saline control and 5-HT. B. The change in blood pressure versus 5-HT concentration. (*p < 0.05, Kruskal-Wallis one-way ANOVA on Ranks, post hoc analysis with Dunn's Method).
Effect of Tropisetron on Blood Pressure

The initial injection of 10 mM 5-HT resulted in a median blood pressure drop of -16.3 mmHg (IQR: -22.6 to -13.0 mmHg). The median duration of decreased blood pressure was 565.0 s (IQR: 507.5 to 576 s). The second injection of 10 mM 5-HT combined with tropisetron 1 mM resulted in a similar drop in median blood pressure (-18.4 mmHg (IQR: -21.3 to -13.1 mmHg) for a similar duration of time (562.0 s, IQR: 431.5 to 580 s) (Figure 3.11). There was no significant difference in the median blood pressure drops between the first (10 mM 5-HT) and second (10 mM 5-HT + 1 mM tropisetron) injections (p > 0.05, Paired t-test); nor was there a significant difference in the duration of decreased blood pressure between the two injections (p > 0.05, Wilcoxon Signed Rank Test). This confirmed that tropisetron did not have an effect on decreased blood pressure.

Effect of 2-methyl-5-HT on Blood Pressure

Injection of 10 mM 2-methyl-5-HT into masticatory muscle resulted in a blood pressure decrease for a median duration of 62.0 s (IQR: 31.8 to 180.5 s), which did not differ significantly from saline controls (p > 0.05, Kruskal-Wallis one-way ANOVA on Ranks, Dunn's Method as post hoc). The median drop in blood pressure (-1.4 mmHg, IQR: -2.5 to -0.2 mmHg) also did not differ significantly from saline controls (p > 0.05, Kruskal-Wallis one-way ANOVA on Ranks, Dunn's Method as post hoc).
Figure 3.11. The line traces (above) illustrate the heart rate and blood pressure, and the peri-stimulus time histogram (below) the afferent discharge in response to an initial injection of 10 mM 5-HT (△) and a second co-injection of 10 mM 5-HT + 1 mM tropisetron (Δ) into the mechanoreceptive field of a temporalis afferent fibre (CV 7.8 m/s, baseline MT 14.8 g) in a female rat.
IV DISCUSSION

The main objective of this study was to determine whether injection of 5-HT into masticatory muscle increased afferent fibre excitability and decreased MTs via 5-HT\textsubscript{3} and 5-HT\textsubscript{1B/2A} receptor activation, respectively, so as to provide insight into the mechanisms underlying 5-HT-evoked masticatory muscle pain and allodynia evidenced in past human experimental pain studies [50], and on a broader scale to gain a better understanding of the contribution of peripheral serotonergic mechanisms to the pathophysiology of myofascial pain conditions such as TMDs and FM. Immunohistochemistry experiments were carried out to determine the frequency of expression of the 5-HT\textsubscript{1B}, 5-HT\textsubscript{2A} and 5-HT\textsubscript{3} receptors in rat masseter ganglion cells. Our detection of low frequencies of 5-HT\textsubscript{1B} and 5-HT\textsubscript{2A} but a relatively high frequency of 5-HT\textsubscript{3} receptors indicated that our electrophysiology experiments would be focused on characterization of 5-HT\textsubscript{3} receptor activation. Our electrophysiology experiments demonstrated that repeated injection of 5-HT into rat masticatory muscle evoked concentration-related increases in afferent fibre excitability and resulted in a prolonged mechanical sensitization. However, the decrease in MTs did not differ significantly from that following injection of saline control. Further, co-injection of 10 mM 5-HT and 1 mM tropisetron significantly attenuated the evoked response to 5-HT, suggesting the involvement of 5-HT\textsubscript{3} receptors in the mediation of 5-HT-evoked discharge. There were no sex-related differences in the baseline or response properties of afferent fibres. There was, however, a significant sex-related difference in the frequency of 5-HT\textsubscript{3} receptor expression.
Immunohistochemistry

This study presents novel data on the expression of 5-HT receptor subtypes by masseter ganglion cells. FB-positive (masseter ganglion) cells that were identified through immunohistochemistry had a pattern of size distribution similar to that of rat masseter ganglion cells as well as human trigeminal ganglion cells reported in previous histological studies [6,7,75].

All three receptor subtypes (5-HT\textsubscript{1B}, 5-HT\textsubscript{2A}, and 5-HT\textsubscript{3}) were expressed by medium to large diameter masseter ganglion cells. However, there was a complete lack of expression of all three receptor subtypes in small diameter cells (< 20 \textmu m). Our observation of relatively low frequencies of 5-HT\textsubscript{1B} and 5-HT\textsubscript{2A} receptor expression (28.0 and 11.1 \%, respectively) suggests that the respective roles of these receptors in peripheral nociceptive transduction in the masseter muscle may be minor compared to that of the 5-HT\textsubscript{3} receptor, which was expressed by a considerably greater proportion of masseter ganglion neurons. In fact, our report of so few 5-HT\textsubscript{2A} receptors contradicts the numerous findings in support of a 5-HT\textsubscript{2A} receptor involvement in peripheral pain transduction [1,122,124,145,160,164], since one would expect a much higher frequency of expression if this were the case. However, one could argue that the vast majority of studies supporting a 5-HT\textsubscript{2A} receptor involvement in peripheral pain transduction have been performed in cutaneous rather than muscle tissue and that often, models of inflammatory pain were used. Perhaps these differences may explain at least in part the lack of concordance between our study and previous findings. Nevertheless, on the basis of such low frequencies of expression for the 5-HT\textsubscript{1B} and 5-HT\textsubscript{2A} receptors, it was
concluded that pursuing selective agonist/antagonist experiments for these receptors would be impractical and time-consuming.

In human trigeminal ganglia, it was determined that 40 % of ganglion neurons expressed the 5-HT$_{1B}$ receptor [75]. Our frequency of 5-HT$_{1B}$ receptor expression (28.0 %) was not so different, but this slightly lower frequency may be attributable to interspecies differences in expression or due to our exclusive examination of ganglion cells that project to the masseter muscle. To the best of my knowledge, masticatory muscle ganglion cells have not been previously labeled for specific 5-HT receptor subtypes, and therefore no direct comparisons can be made with other studies.

Baseline Properties

Collectively, the baseline properties of all 152 masticatory muscle afferent fibres recorded were similar to those of masticatory afferent fibres characterized in past studies [28,39]. In accordance with previous reports, the majority (72 %) of masticatory muscle afferent fibres with caudal brainstem projections were slowly-conducting A$\delta$ fibres, while only a small percentage (~3 %) were C fibres [28,39]. During baseline, 52 % of masticatory muscle afferent fibres exhibited spontaneous discharge, a percentage slightly higher than but still within a reasonable range of the percentages reported in masseter (31 %) [24] and temporalis (41 %) fibres in previous work [39]. The median baseline MT of fibres studied in males did not differ significantly from that studied in females, although fibres from females exhibited a trend toward lower thresholds (Table 3.1). The lack of a significant sex-related difference in baseline MT is in agreement with what was previously reported in both the masseter and temporalis
muscles [23,39]. In addition, CV did not differ significantly between fibres recorded from male versus female rats, and this coincides with previous reports in both masseter and temporalis fibres [23,39]. In our study, the only significant sex-related difference was that male rats weighed significantly more than female rats.

There was no significant inverse relationship between baseline MT and CV in masticatory afferent fibres, which does not agree with a previous report in masseter afferent fibres [23]. Among fibres that received an initial injection of 10 mM 5-HT, there was no significant inverse correlation between baseline MT and CV (see Figure 3.4). Among the 29 fibres that actually responded to an initial injection of 10 mM 5-HT, the lack of a relationship between response and CV or MT suggests that 5-HT is not completely selective for nociceptive fibres. This is in agreement with our immunohistochemistry results - our observation that a wide range of cell diameter fibres were positive for the 5-HT$_3$ receptor supports the idea that 5-HT is nonselective in the types of fibres it excites, granted 5-HT evokes afferent discharge via 5-HT$_3$ receptor activation.

There was no significant influence of estrus cycle stage on afferent fibre CV and baseline MT in female rats. Indeed, it is difficult to detect significant estrus cycle-related differences in the response properties of masticatory afferent fibres. In a past study, masseter afferent fibre data collected from female rats had to be pooled over a six year period and re-analyzed in order to detect a small difference in the baseline MTs of putative masseter nociceptors between diestrus and proestrus [22]. Over this six-year period, a total of 10 fibres were studied during proestrus, which illustrates the rarity of finding a fibre in this stage. In our study, only one fibre was studied from a female rat in
proestrus, which is too few to detect a statistically significant estrus stage-related difference.

**Evoked Activity**

The afferent discharge data demonstrate that IM injection of 5-HT into masticatory muscle evokes rapid and short-lived discharge in a relatively reproducible manner. The rapid response to 5-HT (within several seconds following injection), suggests the activation of ionotropic 5-HT$_3$ receptors, since these are the only 5-HT receptors capable of mediating fast cellular responses. The short-lived discharge correlates well with reports in human experimental pain studies, wherein IM injection of 5-HT into the masseter muscle evoked a rapid increase in pain intensity lasting at most several minutes [50].

The magnitude of the response to 5-HT was significant yet small, especially when compared to the responses to other endogenous substances like glutamate [23] and hypertonic saline [118]. Again, this parallels human experimental pain findings, in which IM injection of 1 mM 5-HT into the masseter muscle resulted in a small (albeit statistically significant) increase (less than 20 %) in subjective pain scores [50]. In contrast, injection of 10 µM 5-HT into the human temporalis muscle resulted in a non-significant increase in pain [85]. One could speculate that the temporalis is less sensitive than the masseter to the effects of 5-HT, however the more likely explanation is that the concentration injected was not sufficiently high to excite nociceptive afferents. In the human tibialis anterior muscle of the leg, injection of 40 µM 5-HT was reported to cause a significant increase in pain compared to isotonic saline; however, the mean
VAS-peak was no greater than two on a scale of ten, indicating that the pain was not clinically significant. In comparing our results to those of previous clinical studies however, caution must be exercised in interpreting any parallel findings, since human reports of pain are subjective and involve central processes in addition to the peripheral ones that we examined in our study.

In cats, injection of 135 μg 5-HT into the sural artery supplying the gastrocnemius-soleus muscle excited group IV (C fibre) muscle afferents [10]. However, because the muscle was excised, it is possible that the resulting inflammation had an effect on the excitability of muscle afferents. The results from human clinical and animal behavioural studies suggest that 5-HT in muscle evokes modest pain in humans [11,48,50,85] and relatively mild nocifensive responses (e.g. paw favouring, licking and lifting) in rats [74]. Similarly, our electrophysiological findings in rat masticatory muscle indicate that the effects of 5-HT on the excitability of slowly conducting putative nociceptive afferents are modest.

The magnitude of the evoked response to 5-HT increased in a concentration-dependent manner. In our study, a minimum 5-HT concentration of 1 mM was required to evoke a response that was significantly greater than that evoked by saline control. The same 5-HT concentration was required to evoke significant masseter muscle pain in healthy female subjects [48,50], indicating that IM injection of 5-HT in rats can model pain evoked by IM injection of 5-HT in humans. A similar correlation between evoked response in rats and pain perception in humans was shown with glutamate injection [24]. When the response to 5-HT was analyzed in terms of frequency as opposed to magnitude, a clear concentration-dependent increase in the frequency of response was
observed. In other words, the higher the concentration of 5-HT injected, the greater the percentage of fibres that responded. Responses were evoked in approximately 57% of masticatory afferent fibres that received an initial injection of the highest concentration (10 mM) of 5-HT. Based on our supposition that the 5-HT$_3$ receptor mediates 5-HT-evoked discharge, we could then speculate that approximately 57% of masticatory afferent fibres express the 5-HT$_3$ receptor. Our immunohistochemistry results demonstrated that approximately 53% of masseter ganglion cells express the 5-HT$_3$ receptor, and this number closely agrees with the percentage we deduced from the frequency of response to injection of 10 mM 5-HT.

Indeed, our pharmacological experiments suggest that the 5-HT$_3$ receptor mediates 5-HT-evoked discharge. Co-injection of 10 mM 5-HT and 1 mM tropisetron significantly decreased the magnitude of 5-HT-evoked discharge, signifying an important role for the 5-HT$_3$ receptor in the immediate effects of 5-HT in masticatory muscle. Analogously, in human experimental pain studies, co-injection of granisetron (another selective 5-HT$_3$ receptor antagonist) and 5-HT significantly attenuated 5-HT-evoked masseter muscle pain [49]. Okamoto et al (2004) reported that in the formalin-inflamed rat masseter muscle, locally and systemically administered tropisetron significantly decreased orofacial nocifensive behaviours [125]. It is noteworthy to mention, however, that the antinociceptive effect of tropisetron (local and systemic) was only seen in rats with CFA-induced inflammation of the TMJ and not in rats without inflammation [125]. These findings suggest that the 5-HT$_3$ receptor plays a significant role in the transduction of pain during inflammatory injury affecting both the masseter muscle and the TMJ. Unfortunately, the clinical relevance of these findings is
questionable, since the majority of TMD cases are not associated with TMJ inflammation, let alone myositis of the masseter muscle [42].

Thus far, we have discussed a direct excitatory effect of 5-HT on masticatory muscle afferents through 5-HT₃ receptor activation. However, it is possible that 5-HT may also have an indirect effect on masticatory muscle afferents, primarily by enhancing the algesic properties of other chemical mediators, particularly BK. This phenomenon was demonstrated in animal [115] and human [10] muscle studies. Infusion of 5-HT followed by BK into the human tibialis anterior muscle significantly increased the mean peak pain to BK, as reported by subjects [9]. In the cat gastrocnemius-soleus muscle, when 5-HT was administered prior to BK, the excitability of muscular group IV (C) fibres was increased. These studies collectively show that 5-HT may sensitize or "prime" muscle afferent fibres to the effects of BK, thereby playing an indirect role in nociceptive transduction.

**Mechanical Threshold**

The results of our study indicate that although injection of 5-HT resulted in mechanical sensitization of masticatory afferent fibres, the post-injection decrease in MTs was not significantly different from that following injection of saline control. Behavioural studies in rats have demonstrated (mechanical) hyperalgesia following subcutaneous injection (usually into the hindpaw) of either 5-HT or selective 5-HT receptor agonists [158]. One must take into consideration that in measuring behavioural responses to peripherally-applied mechanical or thermal stimuli, inherent in the measurements are the influences of central processes (for example the integration
of nociceptive signals in higher brain centres) in addition to peripheral ones. In our study, only the peripheral component of pain transduction was examined without consideration of the central effects. Mechanical sensitization following IM injection of 5-HT has not been previously studied in animals. However, in the healthy human masseter muscle, injection of 0.01 and 1 mM 5-HT significantly decreased PPTs (by a mean of approximately 11%) during the 30 min recording interval compared to saline control [50]. Interestingly, injection of saline control into the masseter muscle also significantly lowered PPTs compared to $10^{-7}$ M 5-HT [50]. In the human temporalis muscle, injection of 10 μM 5-HT did not significantly decrease PPTs [85]. Neither did injection of 4, 8 or 40 μM 5-HT into the human tibialis anterior muscle significantly decrease PPTs [11]. The majority of human studies show that IM injection of 5-HT does not cause mechanical sensitization. The only animal study that demonstrated mechanical sensitization to 5-HT was in the cutaneous tissue of the goat palate [68]. Single unit recordings within the trigeminal ganglion showed that injection of 12 mM 5-HT into the goat palate mechanically sensitized palatal nociceptors [68]. It is possible that the muscle is different from the skin in terms of the 5-HT receptor subtypes that are expressed and their ratios of expression. In rat glabrous skin of the toe for instance, 32% of unmyelinated primary afferent fibres expressed the 5-HT$_{2A}$ receptor [32], while in our study only 11% of muscle primary afferents fibres expressed the 5HT$_{2A}$ receptor. Thus, a tissue-related difference in mechanical sensitization may be explained, at least in part, by the differential expression of 5-HT$_{2A}$ receptors on afferent fibres that innervate skin versus muscle tissue. Further, it is possible that some 5-HT receptor subtypes may be inhibitory, while others are excitatory. If the ratio of inhibitory to
excitatory receptors is higher in the muscle than in skin, this may explain why less sensitization has been observed in muscle as compared to skin.

Co-injection of 10 mM 5-HT and 1 mM tropisetron did not cause a significant change in MT compared to baseline and compared to repeated injection of 10 mM 5-HT. The frequency of mechanical sensitization did not differ between fibres that received repeated injection of 10 mM 5-HT and fibres that received a second co-injection of 10 mM 5-HT and 1 mM tropisetron. However, the frequency of mechanical desensitization was significantly greater in fibres that received a second co-injection of 10 mM 5-HT and 1 mM tropisetron compared to fibres that received repeated injection of 10 mM 5-HT alone. Repeated injection of the selective 5-HT\textsubscript{3} receptor agonist 2-methyl-5-HT (10 mM) did not cause a significant change in MT. These results, taken together, suggest that peripheral 5-HT\textsubscript{3} receptors do not play a significant role in the mediation of 5-HT-induced mechanical sensitization.

**Blood Pressure**

One of our most unexpected findings was the pronounced change in blood pressure in response to 5-HT. Injection of 5-HT into masticatory muscle caused a significant drop in blood pressure (latency to onset ~ 20 to 30 seconds, duration ~ 555 seconds), the magnitude and duration of which were concentration-dependent. Although the effect observed on the cardiovascular system was more than apparent, the mechanism(s) underlying this phenomenon is/are far from distinguishable due to the complexity of the cardiovascular effects of 5-HT, and as such, the ideas presented herein are largely speculative.
It is believed that 5-HT exerts a direct contractile effect on peripheral blood vessels (via 5-HT\textsubscript{2} receptor activation) [66]. Unfortunately, very few studies, if any, have been done in which 5-HT was injected into skeletal muscle and blood pressure monitored. In the rabbit masseter muscle, local injection of 1 nM 5-HT significantly decreased blood flow as measured by laser-Doppler flowmetry, but 5-HT at higher concentrations had no effect on the microcirculation [93]. Further, the microcirculatory decrease in rabbit masseter blood flow was inhibited by ritanserin, a selective 5-HT\textsubscript{2} receptor antagonist, suggesting that the effect was 5-HT\textsubscript{2} receptor-mediated [92]. In the human masseter muscle, laser-Doppler flowmetry did not detect any significant change in blood flow following 5-HT injection compared to saline [48]. In anesthetized cats, intra-arterial injection of 5-HT into the hindlimb muscles did not affect blood pressure or heart rate significantly [146].

As demonstrated in this study, injection of noxious substances into masticatory muscle activates potentially nociceptive fibres. Noxious sensory activation, for example by injection of formalin into the rat hindpaw, has been shown to initiate reflex sympathetic responses (i.e. increases in blood pressure and heart rate) that correlate with nocifensive behaviours (i.e. flinching) [159]. Such a reflex response has previously been demonstrated with injection of NMDA [38]. However, following injection of 10 mM 5-HT, the opposite occurred, which suggests that a reflex sympathetic response is a highly unlikely explanation for our observations. We can only assume that the blood pressure drop is due to a peripheral effect of 5-HT, since 5-HT does not readily cross the blood-brain-barrier [163]. A possible mechanism for the drop in blood pressure is a direct action of 5-HT on the heart. For 5-HT to act directly on the heart, it must
physically reach the heart by leaving the muscle and entering the systemic circulation. Unfortunately, the clearance of 5-HT from muscle tissue has not been studied; therefore, we cannot comment directly on the ease with which 5-HT leaves skeletal muscle following IM injection. However, the clearance of the excitatory amino acid glutamate has been studied in the masseter muscle using MRS [61]. It was determined that the half-life of glutamate is approximately 108 seconds and its clearance from the muscle is approximately 10 minutes following injection [61]. Although 5-HT is not an amino acid per se, it is derived from an amino acid, so we can estimate its pharmacokinetic parameters from glutamate's (i.e. a half-life of approximately 100 seconds and clearance from the muscle of approximately 10 minutes following injection). The latency to onset of the fall in blood pressure following injection of 10 mM 5-HT was approximately 20 to 30 seconds, which is conceivably enough time for a fraction of the injectate to clear the muscle and reach the heart as well as other peripheral tissues.

Recent evidence demonstrated a lack of sensitivity of isolated rat atrial muscle strips and ventricular papillary muscles to application of 5-HT [172]. However, in isolated human atrial muscle preparations, application of 5-HT resulted in concentration-dependent increases in the force of contraction, as well as an increase in Ca^{2+} currents in atrial myocytes [84]. In isolated electrically-stimulated rat atria, 5-HT produced a positive inotropic effect that was concentration-dependently diminished by the selective 5-HT_{2A} receptor antagonist ketanserin [94]. Administration of ketanserin in anesthetized normotensive rats resulted in hypotension, an effect that was attributed to 5-HT_{2A} receptor blockade [126]. As these studies suggest, it is believed that 5-HT has a
primarily positive chronotropic and inotropic effect in mammalian heart, mainly mediated by the 5-HT$_{2A}$ receptor in rats and the 5-HT$_4$ receptor in humans and pigs [71,163].

The majority of 5-HT's cardiovascular effects are believed to be mediated mainly by 5-HT$_1$, 5-HT$_2$, and 5-HT$_3$ receptors (although recent evidence suggests the involvement of the 5-HT$_4$ receptor) [148]. The involvement of the three main receptors can be illustrated in the triphasic response of arterial blood pressure to intravenously administered 5-HT [87,148]. An initial short-lived depressor phase characterized by bradycardia (the Bezold-Jarisch reflex) is followed by a pressor (second) phase, which is succeeded by a third phase of prolonged hypotension. The initial depressor phase is believed to be mediated by 5-HT$_3$ receptors (located on cardiac vagal afferent nerve endings [87,148]. The pressor phase is 5-HT$_2$ receptor-mediated (it was antagonized by ketanserin, a selective 5-HT$_2$ receptor antagonist), and the third depressor phase is apparently 5-HT$_1$ receptor-mediated [87]. It is possible that this triphasic response would also occur to intramuscularly administered 5-HT since it is likely that some 5-HT will enter the systemic circulation following IM administration.

In our study, tropisetron did not block and 2-methyl-5-HT failed to mimic the 5-HT-induced decrease in blood pressure, indicating that the drop in blood pressure was not mediated by the 5-HT$_3$ receptor. It seems highly unlikely that the 5-HT$_{2A}$ receptor would mediate a prolonged hypotension since it has been reported in the rat heart that 5-HT$_{2A}$ receptors mediate positive inotropy [94]. Moreover, 5-HT$_{2A}$ receptors are believed to mediate blood vessel contraction [148]. The 5-HT$_4$ receptor mediates positive inotropic effects in failing rat heart papillary muscles and no effect on Sham papillary muscles, so it seems unlikely that this receptor would mediate hypotension.
This leaves us with the 5-HT₁ receptor, which is the most likely culprit since it supposedly mediates prolonged hypotension in the late depressor phase of the triphasic blood pressure response [148]. Several 5-HT₁ receptor-mediated mechanisms have been proposed to explain this prolonged hypotension, and include inhibition of noradrenergic release from postganglionic sympathetic neurons, relaxation of vascular smooth muscle, and induction of endothelium-derived relaxant factor (EDRF) release [148]. It is possible that one or more of the proposed 5-HT₁ receptor-mediated mechanisms contributed to the prolonged hypotension we observed.

Finally, it is possible that 5-HT causes a decrease in blood pressure secondary to the release of other chemical mediators. One may speculate that once 5-HT reaches the blood, it can bind to receptors on mast cells to cause degranulation and the subsequent release of chemical mediators, some of which may enter the systemic circulation to exert direct negative inotropic effects on the heart or cause vasodilation. However, this theory seems unlikely since it has been shown that 5-HT does not induce the release of mediators such as histamine from intestinal mast cells [17]. Another possible mechanism stems from what is known about the pathophysiology of migraine headache. It is theorized that migraine may result from vasodilation of cranial blood vessels in response to the release of CGRP, a highly potent vasodilator [34, 109]. The antimigraine properties of triptan drugs are attributable to the inhibition of CGRP release by 5-HT₁B/₁D receptor activation [109]. When 5-HT, a nonselective agonist at 5-HT₁B/₁D receptors, is injected into the muscle, it may cause a similar release of CGRP (as well as other vasoactive neuropeptides substance P or neurokinin A) from the peripheral endings of primary afferent fibres [34]. Considering that the co-expression of 5-HT₁B/₁D
receptors and CGRP have already been demonstrated in rat trigeminal ganglion neurons [109], the speculation of a CGRP-induced hypotension is feasible. However, in order to determine the exact mechanism(s) by which IM 5-HT decreases blood pressure, further studies will have to be done.

Limitations in the design of the experiment

Our study did not take into consideration the influence of the inhalational anesthetic isoflurane. Because afferent fibre electrophysiological recordings were carried out in rats under isoflurane anesthesia, it is possible that the anesthetic could have influenced our results, for instance through a decrease in systemic arterial blood pressure [53]. The mechanism by which isoflurane decreases systemic blood pressure is by decreasing vascular resistance, particularly in the skin and muscle [53]. A decrease in vascular resistance is associated with an increase in blood flow, and this phenomenon in the masseter muscle may result in the acceleration of 5-HT clearance from the muscle following injection. Responses to 5-HT may therefore be smaller than expected. Isoflurane has been shown to potentiate 5-HT₃ receptor currents at alveolar concentrations below that needed for anesthesia [128]. If this occurred in our experiments, it is possible that the discharge evoked by 5-HT may have been potentiated by isoflurane through ionotropic 5-HT₃ receptors.

As already discussed, 5-HT did not cause significant mechanical sensitization because saline also caused an initial drop in MT, which was unexpected. However, the drop in MT following saline injection recovered to baseline within the 30 min recording period. In past studies, injection of saline into the rat masseter muscle did not
mechanically sensitize muscle afferents within a 30 min period [23] or even during a longer (six hour) recording period [112], which makes the interpretation of our results difficult. The only obvious difference between our study and previous studies showing no mechanical sensitization of muscle afferents to saline control lies in the design of the experiment. In previous studies, a single injection of a substance was made prior to assessment of MTs, whereas in our study, repeated injection of a substance was made prior to MT measurements. The decrease in MTs we observed following repeated injection of isotonic saline may therefore be a consequence of having made a second injection into the same region of tissue. The combined volume of the two injections in itself may have been adequate in producing an edema that was conducive to mechanical sensitization of nociceptive afferents. Ideally, we would have assessed changes in MT after only a single injection as was done in past studies. However, for us to do this, we would have had to divide the study into two separate components: one to measure evoked discharge, and the other to measure changes in mechanical sensitization following injection. Although this design was considered, for the purpose of efficiency in both time and in reducing the number of rats sacrificed, we decided to combine these two experiments into a single one. Another important reason why we decided to make repeated injections was the high inter-fibre variability of responses to 5-HT injection. The use of repeated injections allowed us to assess the reproducibility of response within the same fibre and therefore reduce our n values.
Future Directions

Immunohistochemistry of the Masticatory Muscles: In our study, we immunohistochemically identified the presence of the 5-HT1, 2 and 3 receptors in the cell bodies of masseter ganglion cells. Our results therefore indicate that these cells contain the machinery necessary to translate these receptors from their respective mRNA molecules. However, what our results do not tell us is whether these receptors are also present on the peripheral terminal endings of these cells. 5-HT receptors can be identified on the terminal endings by injecting FB into the ganglion and then excising the muscles for subsequent incubation with the various antibodies. In addition, other 5-HT receptor subtypes can be examined, including the 5-HT4, 5, 6, and 7 receptors. However, there may be considerable difficulty in finding selective antibodies for some receptor subtypes, since they are newer and are not as well characterized pharmacologically.

Longer Assessment of MTs: Following 5-HT injection, we observed a decrease in MT that persisted throughout the entire 30 min recording period, and may have persisted for even longer. In order to establish the duration of decreased MTs, measurements can be taken every minute for a longer period of time (perhaps one hour). A significant difference between the MTs following 5-HT and saline control injection may develop after 30 minutes. In order to detect this difference, prolonged MT measurements can be made.

Colocalization of 5-HT and BK: Studies in animals and humans have reported that 5-HT enhances the effects of BK [9,74,115]. However, it has never been determined
whether 5-HT receptors and BK are colocalized on muscle afferent ganglion cells. It is possible that 5-HT has an indirect rather than a direct effect on masticatory muscle afferent fibres, particularly through the potentiation of BK’s effects. Therefore, it may be worthwhile to determine whether 5-HT receptors and BK are co-expressed in masticatory muscle ganglion cells.

**Blood Pressure Studies:** The mechanism(s) underlying the 5-HT-evoked decrease in blood pressure can be investigated using pharmacological studies. To determine whether CGRP is mediating the blood pressure drop, a CGRP antagonist can be given IV to an anesthetized rat prior to injection of 5-HT into the masticatory muscles. If pre-administration of a CGRP antagonist attenuates the 5-HT-evoked decrease in blood pressure, then the blood pressure effects can be attributed to the well-documented effects of CGRP on vasculature tissue. This experiment can also be carried out using other neuropeptides (such as substance P and neurokinin A) in place of CGRP to determine the potential involvement of other vasoactive mediators in 5-HT-evoked hypotension.
V SUMMARY OF CONCLUSIONS

Our immunohistochemistry results show that the frequency of expression of 5-HT_{1B} and especially 5-HT_{2A} receptors by masseter ganglion cells is low compared to that of 5-HT_{3} receptors, which is relatively high. In addition, the frequency of expression of 5-HT_{3} receptors is significantly greater in female than in male masseter ganglion cells.

Our electrophysiology results show that injection of 5-HT into masticatory muscle evokes rapid and short-lived discharge in putative muscle nociceptors in a relatively reproducible and concentration-related manner. In agreement with our hypothesis, the results from our pharmacology experiments employing selective agonists and antagonists indicate that the increase in excitability upon IM injection of 5-HT is mediated by a peripheral receptor mechanism involving the 5-HT_{3} receptor. However, we found that IM injection of 5-HT did not significantly sensitize putative masticatory muscle nociceptors to mechanical stimuli. Perhaps the most surprising finding in our study was the marked decrease in blood pressure to IM injection of 5-HT, the mechanism of which can only be explained through further study.
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