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ABSTRACT

While effective therapies are available for some types of craniofacial pain, treatments for deep-tissue craniofacial pain such as temporomandibular disorders are less efficacious. Several ion channels and receptors which are prominent in craniofacial nociceptive mechanisms have been identified on trigeminal primary afferent neurons. Many of these receptors and channels exhibit unusual distributions compared with extracranial regions. For example, expression of the ATP receptor P2X₃ is strongly implicated in nociception and is more abundant on trigeminal primary afferent neurons than analogous extracranial neurons, making them potentially productive targets specifically for craniofacial pain therapies. The initial part of this review therefore focuses on P2X₃ as a potential therapeutic target to treat deep-tissue craniofacial pain. In the trigeminal ganglion, P2X₃ receptors are often co-expressed with the nociceptive neuropeptides CGRP and SP. Therefore, we discuss the role of CGRP and SP in deep-tissue craniofacial pain and suggest that neuropeptide antagonists, which have shown promise for the treatment of migraine, may have wider therapeutic potential, including the treatment of deep-tissue craniofacial pain. P2X₃, TRPV1, and ASIC3 are often co-expressed in trigeminal neurons, implying the formation of functional complexes that allow craniofacial nociceptive neurons to respond synergistically to altered ATP and pH in pain. Future therapeutics for craniofacial pain thus might be more efficacious if targeted at combinations of P2X₃, CGRP, TRPV1, and ASIC3.

KEY WORDS: trigeminal, nociception, muscle, TMD, neuropeptides.

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Emerging Peripheral Receptor Targets for Deep-tissue Craniofacial Pain Therapies

INTRODUCTION

Chronic deep-tissue pain in the craniofacial region remains a prevalent clinical problem that often responds poorly to currently available analgesics. Evidence continues to grow indicating that craniofacial pain mechanisms differ from pain mechanisms in other body regions, and that specialized craniofacial tissue types—such as tooth, periodontal ligament, jaw joint, and dura—exhibit unusual nociceptor phenotypes or distinctive nociceptive receptor distributions. For example, the ATP receptor P2X₃ is restricted to primary afferent neurons and is much more abundant on neurons relaying nociceptive feedback from deep craniofacial tissues than on analogous extracranial neurons. We propose that differences such as this may provide fruitful targets that could be exploited to direct therapies specifically at craniofacial pain. The initial part of this review therefore focuses on P2X₃ as a potential therapeutic target to treat deep-tissue craniofacial pain.

The neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) play a pivotal role in the processing of nociceptive information arising from various peripheral tissues and are co-expressed with P2X₃ receptors. Thus, recent neuropeptide antagonists which show promise in treating migraine headache may have a much wider therapeutic potential, including the treatment of craniofacial pain. Transient receptor potential channels (TRPV) and acid-sensing ion channels (ASICs) can also be concurrently activated with the P2X₃ receptor. Therefore, we review the most prominent potential peripheral therapeutic targets for deep-tissue craniofacial pain: the ATP receptor P2X₃, neuropeptides, TRPV, and ASICs.

ATP, P2X₃ RECEPTORS, AND PAIN

When ATP has been applied to the skin in human studies, painful burning sensations have been reported (Hamilton *et al.*, 2000). The injection of ATP analogues into the hindpaw of rats has also been reported to elicit nociceptive paw-lifting behavior (Hamilton *et al.*, 1999), while intrathecal administration of ATP analogues produces thermal hyperalgesia (Tsuda *et al.*, 1999). These responses indicate that ATP can evoke pain which is now known to be mediated via ATP purinoceptors (for review, see Hwang and Oh, 2007; Wirkner *et al.*, 2007). Receptors activated by ATP can be divided into P2X and P2Y families, based upon differences in receptor structure and their signal transduction mechanisms (Gever *et al.*, 2006). P2X receptors are a family of ionotropic receptors activated by ATP (for review, see Khakh and North, 2006). One member of this group, the P2X₃ receptor, has been implicated specifically in nociception (reviewed by Hwang and Oh, 2007; Wirkner *et al.*, 2007). Since these P2X₃ receptors are restricted to peripheral nociceptive sensory neurons (Vulchanova *et al.*, 1997; Bradbury *et al.*, 1998), they are promising targets for pain therapies. P2Y receptors are G-protein-coupled metabotropic receptors (Fischer and

Figure 1. Comparison of the proportion of primary afferent neurons that express the P2X₃ receptor. Note that a high percentage of neurons projecting to deep craniofacial tissues expresses P2X₃ receptors, while very few analogous extracranial neurons express P2X₃.

Krugel, 2007). Compared with the P2X receptors, the tissue distribution of P2Y receptors is quite diverse, and the role of these P2Y receptors in pain has been less-well-studied. Of the 8 cloned P2Y receptors, P2Y₁ and P2Y₂ receptors have been implicated in nociception (Okada *et al.*, 2002). Particularly, in the trigeminal ganglion and dorsal root ganglion (DRG), P2Y₁ is present in small-diameter neurons (Ruan and Burnstock, 2003) and is co-expressed with TRPV channel TRPV1 and P2X₃ receptors (Gerevich and Illes, 2004; Burnstock, 2007). P2Y₂ receptor is also expressed in small-diameter DRG neurons (Moriyama *et al.*, 2003; Stucky *et al.*, 2004) and may play a role in nociceptive transmission through TRPV1 receptors (Moriyama *et al.*, 2003; Malin *et al.*, 2008). Further studies are necessary to elucidate the contributions of P2Y receptors to craniofacial pain transmission.

Recent studies have indicated that P2X₃, P2X_{2/3} receptors are involved in a variety of pain states, including neuropathic pain (Sharp *et al.*, 2006; Shinoda *et al.*, 2007). Neuropathic pain is a complex, chronic pain state that is usually accompanied by tissue injury involving lesion(s) of the peripheral or central nervous system. P2X₃ receptor activation has also been implicated in inflammatory pain (Seino *et al.*, 2006). Inflammatory pain results from an insult to the integrity of tissues at a cellular level, which involves multiple chemical mediators. These mediators are from tissue cells and blood vessels as well as fibroblasts and mast cells. The 'chemical soup' of inflammatory mediators can directly affect peripheral nociceptive nerve terminals and cause long-term changes that sensitize them. Overt tissue damage releases a

variety of substances from the cytosol, including ATP. Since ATP directly activates P2X₃ receptors, it has been proposed that ATP released from tissue damage activates P2X₃ receptors *in vivo*. Measurements of intracellular ATP and *in vitro* simulations show that sufficient ATP is released to activate neuronal P2X₃ receptors (Cook and McCleskey, 2002). The release of ATP from damaged tissue may be particularly relevant for deep craniofacial tissues, since ATP could be released during tissue damage caused by condylar displacement, masticatory muscle myofiber damage, or dental restoration.

Neurons expressing P2X₃ receptors also interact with glial cells. For example, nerve stimulation evokes ATP release from the somata of DRG neurons, which leads to the release of TNF- α from satellite cells and an increased excitability of P2X₃ neurons (Zhang *et al.*, 2007). Processes such as these suggest that sensitization may occur not only in the CNS, but also within the ganglion (Thalakoti *et al.*, 2007).

In the trigeminal ganglion, nociceptors possess two types of desensitizing currents in response to ATP (Cook and McCleskey, 1997), suggesting that these neurons express different types of the P2X subunit. One type of ATP current rapidly desensitizes and is attributable to P2X₃, while another current is largely sustained and likely represents a P2X₃ heteromultimer (Connor *et al.*, 2005). In large neurons, P2X₃ receptors appear to be restricted to the cell body (Vulchanova *et al.*, 1997; Ramer *et al.*, 2001), implying that P2X₃ is involved in intraganglionic signaling and cross-excitation among sensory neurons (Matsuka *et al.*, 2001). Current amplitude through P2X₃ channels is altered by inflammatory acidification (Gerevich *et al.*, 2007), and inflammatory mediators such as substance P and bradykinin sensitize nociceptors through P2X₃ ion channels (Paukert *et al.*, 2001). ATP also is released concurrently with substance P from neuronal somata in the trigeminal ganglia (Matsuka *et al.*, 2001), suggesting that ATP responses could be modulated within the ganglion. Prostaglandin E₂ (PGE₂), another inflammatory mediator, has no effect on P2X_{2/3} responses but potentiates ATP currents mediated by homomeric P2X₃ receptors (Wang *et al.*, 2007).

Expression of P2X₃ in the DRG is largely restricted to non-peptidergic, unmyelinated primary afferent nociceptors (Novakovic *et al.*, 1999), while in the trigeminal ganglion about one-third of P2X₃ neurons contain a marker for myelinated axons (Staikopoulos *et al.*, 2007), indicating that many trigeminal P2X₃ neurons possess thinly myelinated axons with conduction velocities faster than those of P2X₃ DRG neurons.

The proportion of neurons expressing the P2X₃ receptor varies substantially, depending upon the type of peripheral target tissue in which the peripheral P2X₃ axon resides. For instance, while almost half of cutaneous DRG neurons express P2X₃, very few DRG joint afferent neurons express P2X₃ (Bradbury *et al.*, 1998) (Fig. 1). This differential expression implies that limb joint inflammation is not prominently mediated *via* ATP.

There are even more dramatic differences between cranial and spinal cord neurons projecting to the same type of peripheral target tissue. For example, less than 5% of DRG neurons projecting to joint tissues express P2X₃, while more than 50% of jaw joint neurons express P2X₃ (Ichikawa *et al.*, 2004).

Large differences are also present for P2X₃ muscle afferent neurons. In limb muscles, only 2% of muscle afferent neurons express P2X₃ (Bradbury *et al.*, 1998), while more than 20% of craniofacial muscle afferent neurons are immunopositive for P2X₃ (Ambalavanar *et al.*, 2005). The percentage of P2X₃ craniofacial muscle afferent neurons is reported to be even higher (55%) when electrophysiological techniques are used (Connor *et al.*, 2005). Thus, ATP is strongly implicated as a major component in craniofacial muscle and joint nociception, but not in limb muscle and joint nociception. The dramatic disparity exhibited for joint and muscle does not appear to exist for superficial tissues. Approximately 22-37% of cutaneous DRG afferent neurons express P2X₃ (Bradbury *et al.*, 1998), while a similar percentage (16-30%) of trigeminal cutaneous neurons express P2X₃ (Ichikawa and Sugimoto, 2004; Ambalavanar *et al.*, 2005). Thus, the abundance of P2X₃ expression is similar on neurons projecting from superficial tissues, while neurons expressing P2X₃ receptors are much more abundant in deep tissues of the craniofacial region than in deep tissues of the extracranial region (Fig. 2). This distribution suggests that activation of P2X₃ receptors contributes modestly to cutaneous pain throughout the body, while activation of P2X₃ receptors by ATP is a major player, specifically in deep-tissue craniofacial pain.

P2X₃ RECEPTORS ARE ABUNDANT IN DEEP CRANIOFACIAL TISSUE AND MAY PROVIDE SELECTIVE TARGETS FOR DEEP-TISSUE CRANIOFACIAL PAIN

Teeth

P2X₃ receptors are present on tooth afferent neurons (Ichikawa and Sugimoto, 2004). Within the tooth, these fibers are located in the sub-odontoblastic plexus of Raschkow, the odontoblastic layer, and advance into the predentin and dentin (Alavi *et al.*, 2001). Since about 30-40% of tooth neuronal afferents express P2X₃, and large inward currents can be evoked in tooth pulp afferent neurons by ATP (Cook and McCleskey, 1997), P2X₃ receptors are likely to be substantial contributors to nociceptive responses from the teeth. Few tooth pulp afferents co-express TRPV1 (formerly vanilloid receptor subtype I), but most co-express TRPV2 (formerly vanilloid receptor 1-like receptor, VRL-1) (Ichikawa and Sugimoto, 2004). Thus, future interventional therapies might attenuate pain by blocking neuronal responses evoked by ATP released from cell damage and inflammation within the teeth. Antagonists for P2X₃/P2X_{2/3} could even have therapeutic potential as a prophylactic intervention for dental procedures.

P2X Receptors on Jaw Joint Afferent Neurons

A large proportion of neurons (about 50%) that supply the jaw joint express P2X receptors (Ichikawa *et al.*, 2004). Injection of α,β meATP, a P2X_{1,3,2/3} receptor agonist, also produced nociceptive responses in animals with inflamed and non-inflamed jaw joints (Shinoda *et al.*, 2005). This response was likely mediated by P2X receptors, since the reduced pain pressure threshold was

Figure 2. Co-expression of P2X₃ receptor and neuropeptides in primary afferent neurons. Note the frequent co-expression of neuropeptides with P2X₃ in craniofacial primary afferent neurons supplying deep tissues. In contrast to this, very few dorsal root ganglion neurons co-express peptides with P2X₃.

blocked by a P2X_{1,3,2/3,1/5} receptor antagonist (Shinoda *et al.*, 2005). Since inflammation is frequently present in some phases of TMD involving the temporomandibular joint (Mejersjö and Wenneberg, 2008; Tanaka *et al.*, 2008), it is noteworthy that P2X₃ expression increases in jaw joint afferents following joint inflammation (Shinoda *et al.*, 2005). Within the population of P2X₃ expressing joint afferent neurons, more than 40% co-express CGRP (Ichikawa *et al.*, 2004). Since exposure of cultured trigeminal neurons to CGRP enhances ATP currents and reduces P2X₃ desensitization (Fabbretti *et al.*, 2006), this co-expression suggests analogous interactions between P2X₃ and CGRP in jaw joint afferent neurons. Thus, both CGRP and P2X₃ antagonists may prove efficacious in treating joint-based TMD.

Masticatory Muscle Pain, mTMD, and P2X₃ Receptors

Persons with TMD exhibit altered central nociceptive processing (Maixner *et al.*, 1998; Sarlani *et al.*, 2004), which is thought to be initially triggered from a peripheral source. One prime candidate for this insult is nociceptive input from muscle afferents, since it is particularly potent at generating CNS wind-up (Wall and Woolf, 1984). More specifically, oral parafunctional behavior that increases muscle tension is a good predictor of the level of jaw-muscle pain in persons with TMD (Glaros *et al.*, 2005). Since parafunctional behavior can occur for prolonged periods, and masticatory muscles have an impaired ability to

repair (Pavlati *et al.*, 1998), myofiber damage, subsequent inflammation, and increased primary afferent drive constitute a potential source to initiate or exacerbate mTMD.

Evidence of inflammation in TMDs involving myalgia (mTMD) is limited, perhaps because affected individuals are typically examined long after the initiation of the disorder, when a precipitating event involving inflammation may have subsided. However, levels of SP in the masseter muscle are reportedly reduced in persons with mTMD (Kehl *et al.*, 2006), which is consistent with decreased intramuscular levels of SP in animal studies following inflammation (Galeazza *et al.*, 1995). Some have argued that muscle inflammation is not involved in orofacial muscle pain, because it is not relieved by ibuprofen (Svensson *et al.*, 1997). In muscle pain, however, PGE₂ levels do not temporally correlate with pain intensity, indicating that PGE₂ is not the major algogenic substance involved in muscle pain (Tegeder *et al.*, 2002). Further, cyclo-oxygenase inhibitors such as ibuprofen are ineffective in relieving muscle pain (Donnelly *et al.*, 1990). Thus, the failure of ibuprofen to alleviate orofacial muscle pain should not be interpreted as evidence for a lack of inflammation in masticatory muscle pain.

Purinergic receptors in primary afferent neurons may be of particular importance for muscle pain. Intramuscular infusion of ATP, for instance, evokes pain (Mork *et al.*, 2003), and muscle nociceptors can be activated by ATP (Reinohl *et al.*, 2003). Further evidence that purinergic receptors are involved in muscle pain comes from the observation that non-specific P2X antagonists reduce nociceptive behavior in animals experiencing muscle pain (Shinoda *et al.*, 2008).

Previous studies on tissue inflammation have specifically associated P2X receptors with craniofacial nociceptive behavior (Oliveira *et al.*, 2005). It is also known that P2X₃ receptors are present on masseter muscle afferent neurons (Ambalavanar *et al.*, 2005), and that currents characteristic of P2X₃ receptors can be activated in some masseter afferents (Connor *et al.*, 2005). Following CFA-induced masseter inflammation, the number of P2X₃ muscle afferent neurons increases (Ambalavanar *et al.*, 2005), while P2X₃ muscle afferents increase 15 days following repetitive muscle contraction (Shinoda *et al.*, 2008), indicating a role for P2X₃ in muscle inflammation. One potential source of ATP to activate P2X₃ receptors is ATP released from the cytosol of damaged cells. In co-culture systems, action potentials and inward currents are evoked in nociceptors by ATP when nearby cells are mechanically damaged (Cook and McCleskey, 2002). In muscle, the concentration of ATP within myofibers is approximately 10 mM (Stewart *et al.*, 1994), a concentration which readily activates muscle primary afferent neurons *in vivo* (Reinohl *et al.*, 2003), thus demonstrating that sufficient ATP is present within myofibers to activate muscle afferent neurons. Since parafunctional muscle contraction mechanically damages myofibers (Lovering and De Deyne, 2004), it is possible that ATP released from damaged myofibers can activate P2X₃ receptors on muscle nociceptors.

Temporomandibular disorders are about 1.5-2 times more prevalent in females than in males, and 80% of persons seeking treatment for TMD are females (Dworkin *et al.*, 1990). While the origin of these gender differences is not known, TMD pain

in women is highest at times of low estrogen (LeResche *et al.*, 2003). Several studies have indicated that the central processing of sensory feedback is altered once TMD has become well-established (Sarlan *et al.*, 2004). It is not known, however, whether the different effects of sex hormones on TMD and pain emerge only after these central transformations have occurred, or whether they are present during the initial development of this disorder. Here we suggest several peripheral mechanisms that may contribute to the sex differences in TMD. [For a more encompassing review, see Warren and Fried (2001), Sarlan and Greenspan (2005), and Craft (2007).] One possibility is that primary afferent neurons are modulated by sex hormones, and, in fact, very brief exposure of DRG neurons to estradiol decreases ATP-mediated currents (Chaban *et al.*, 2003; Ma *et al.*, 2005). While this finding is consistent with the observation that greater pain is present when estrogen is low, more prolonged exposure of DRG neurons to estradiol apparently does not affect P2X currents (Xu *et al.*, 2008). Trigeminal ganglion neurons, however, do possess estrogen receptors (Bereiter *et al.*, 2005), and estradiol modulates the responses of brainstem neurons evoked by injection of ATP into the jaw joint (Tashiro *et al.*, 2007).

Levels of nerve growth factor (NGF) rise in inflamed tissue (Woolf *et al.*, 1994; Spears *et al.*, 2005). The presence of NGF also leads to enhanced up-regulation of P2X₃ receptors (Simonetti *et al.*, 2006). While NGF interacts with estrogen in a tissue-dependent manner, in some tissues estrogen increases NGF (Bjorling *et al.*, 2002). Thus, gender effects involving craniofacial pain and P2X₃ receptors could be mediated directly on P2X₃ or indirectly *via* NGF. Nerve growth factor can also stimulate CGRP expression (Lindsay and Harmar, 1989), and CGRP expression up-regulates P2X₃ receptors (Fabbretti *et al.*, 2006). Thus, sex, craniofacial pain, and P2X₃ may interact through sex hormones acting on NGF, leading to increased CGRP and subsequent P2X₃ expression (Fig. 3).

Persons with muscular TMDs report pain associated with mastication (Winocur *et al.*, 2001; Gavish *et al.*, 2002), and females with muscular TMD report more pain following chewing than do males (Karibe *et al.*, 2003). Analysis of existing data also suggests that sex hormones make muscles of females less susceptible to mechanical damage (Tiidus, 2003). Thus, estrogen may alter muscle damage and subsequent extracellular ATP concentration (for review, see Kendall and Eston, 2002).

P2X Receptors and Headache

A large percentage of trigeminal afferent neurons that innervate the dura express P2X receptors. About half of dura afferents express P2X₃, P2X₂, or both (Staikopoulos *et al.*, 2007). It has recently been shown that exposure of trigeminal ganglion neurons to CGRP up-regulates the expression of P2X₃ receptors (Fabbretti *et al.*, 2006). The level of nerve growth factor (NGF) is also increased in the cerebrospinal fluid of persons with some types of headache (Sarchielli *et al.*, 2001), and neutralization of NGF decreases P2X₃ receptor activity on trigeminal ganglion neurons (D'Arco *et al.*, 2007). Thus, CGRP antagonists and anti-NGF agents may prove to be useful targets to modulate P2X₃ receptor function.

Peripheral Trigeminal Nerve Damage

The trigeminal nerve is routinely encountered during dental procedures, during which it can be damaged and produce nerve dysfunction (Sandstedt and Sorensen, 1995; Robert *et al.*, 2005). Injury to the inferior alveolar nerve, for instance, produces enhanced nociceptive behavior and cutaneous mechanical allodynia (Saito *et al.*, 2008). Following inferior alveolar nerve injury, P2X₃ receptors are also up-regulated in the trigeminal ganglion (Eriksson *et al.*, 1998). Some of the responses exhibited following damage to branches of the trigeminal nerve appear to differ from those of other nerves, leading to the suggestion that new therapeutic approaches need to be developed to treat injury-induced trigeminal pain (Robinson *et al.*, 2004). These differences include differences in the time-course of ectopic discharge after injury, involvement of sympathetic fibers, and changes in the expression of some neuropeptides, including galanin and vasoactive intestinal peptide. Whether P2X receptor antagonists or agonists have therapeutic potential for pain and nerve recovery remains to be determined.

Deep-tissue Craniofacial Peptidergic Afferent Neurons Co-express P2X₃

While P2X₃ neurons in the DRG rarely express neuropeptides, there is extensive co-expression of P2X₃ and neuropeptides in trigeminal neurons, suggesting a far greater interaction between neuropeptides and P2X₃ in deep craniofacial neurons. Out of the population of DRG neurons that express CGRP, only 14-26% express P2X₃ (Bradbury *et al.*, 1998; Ruan *et al.*, 2005). In trigeminal neurons expressing CGRP, about 80% co-express P2X₃ (Fabbretti *et al.*, 2006). We found that 75% of the CGRP and about 50% of the SP muscle afferent population in the trigeminal ganglion express P2X₃ (Ambalavanar *et al.*, 2005). About half of jaw joint afferents express both P2X₃ and CGRP (Ichikawa *et al.*, 2004). Thus, a striking difference exists between the percentage of peptidergic neurons that co-express P2X₃ in the trigeminal ganglion and DRG (Fig. 2). This extensive co-localization of P2X₃ with neuropeptides likely reflects interactions between neuropeptides and P2X₃ in trigeminal neurons. For instance, exposure of trigeminal neurons to CGRP increases trafficking of P2X₃ receptors to the cell membrane (Fabbretti *et al.*, 2006).

ENCOURAGING PROGRESS TREATING DEEP-TISSUE PAIN WITH NEUROPEPTIDE ANTAGONISTS

Peripheral receptor activation through neuropeptide release is an important effector mechanism coupled with nociceptor stimulation. In particular, calcitonin gene-related peptide (CGRP) and substance P (SP) are specifically implicated in nociceptive mechanisms involving deep tissues (Schaible, 2004), including muscle (Reinert *et al.*, 1998). The widespread involvement of these neuropeptides in deep-tissue pain is exciting, because the recent development of neuropeptide antagonists to treat migraine headache may have broader therapeutic applications, including deep-tissue craniofacial pain.

Figure 3. Schematic representation of the inter-relationships between the P2X₃ receptor and tissue injury, nerve growth factor, and CGRP. Note that sex hormones may directly or indirectly modulate the expression of the P2X₃ receptor in primary afferent neurons.

Calcitonin Gene-related Peptide

Calcitonin gene-related peptide is associated with orofacial inflammation (Hutchins *et al.*, 2000; Carleson *et al.*, 2004; Ambalavanar *et al.*, 2006). For example, levels of CGRP are elevated in the trigeminal ganglia following craniofacial inflammation, including the jaw joint (Devor, 1991) and masseter muscle (Carleson *et al.*, 2004; Ambalavanar *et al.*, 2006). In persons with temporomandibular disorders, a positive correlation has also been reported between CGRP levels in the temporomandibular joint and the severity of joint pain (Amano *et al.*, 1986). The up-regulation of CGRP production, therefore, is a characteristic feature of craniofacial deep-tissue pain.

The significance of CGRP in craniofacial pain has been further substantiated by studies where blockade of CGRP transmission resulted in anti-nociceptive effects (Olesen *et al.*, 2004; Ambalavanar *et al.*, 2006; Ho *et al.*, 2008). In particular, the clinical effectiveness of a potent CGRP antagonist for acute treatment of migraine headache (Olesen *et al.*, 2004; Ho *et al.*, 2007) represents a potential therapeutic venue for other chronic pain disorders, such as TMD. Intravenous injection of the CGRP antagonist BIBN4096 and an orally active non-peptide antagonist, MK-0974 (Ho *et al.*, 2008), has shown tremendous promise in the treatment of migraine headache. These studies indicate that neuropeptide antagonists can be safe for human use and may prove useful in treating TMD in which levels of CGRP are elevated.

Substance P

Substance P is present in about 20% of trigeminal ganglion neurons (Hokfelt *et al.*, 1975) and is associated with orofacial inflammation and pain. For instance, tooth pulp is supplied by

SP-containing neurons, and SP mediates inflammation and pain arising from the tooth pulp (Sabino *et al.*, 2002). Levels of SP are also increased in arthritic temporomandibular joints (Carleson *et al.*, 1997) and trigeminal ganglia following jaw joint inflammation (Hutchins *et al.*, 2000). Masseter muscle afferent neurons also express SP, and this SP is up-regulated following muscle inflammation (Ambalavanar *et al.*, 2006). Additional evidence supporting a role for NK1 in craniofacial vasoregulation and nociception is the fact that activation of NK1 increases blood flow in the human temporalis muscle (Tuxen *et al.*, 1989). Although many studies show the anti-nociceptive effects of NK1 receptor antagonists, several clinical trials have failed to demonstrate the analgesic efficacy of these compounds in humans (reviewed by Hill and Oliver, 2007), though they have been successful in the treatment of other diverse conditions, including depression, chemotherapy-induced emesis, and inflammatory bowel disease (Duffy, 2004). Future studies are necessary to evaluate the usefulness of NK1 receptor antagonists to treat craniofacial deep-tissue pain.

Neuropeptides Interact with Growth Factors and P2X₃

Nociception involves multiple interactions between and among growth factors, neuropeptides, and nociceptive receptors. For instance, NGF not only induces increased levels of CGRP and SP (Skoff and Adler, 2006), but also increases P2X₃ expression (Simonetti *et al.*, 2006; D'Arco *et al.*, 2007). Because inflammation and partial nerve-injury-induced hyperalgesia are partly dependent upon NGF production (Boucher *et al.*, 2000), increased NGF levels following injury or inflammation in craniofacial tissue may increase neuropeptide synthesis, subsequent release, and P2X₃-mediated ATP currents (Fig. 3). P2X₃ receptor synthesis and trafficking can also be up-regulated by CGRP independent of NGF (Fabbretti *et al.*, 2006).

TRANSIENT RECEPTOR POTENTIAL CHANNELS: TRPV1

The mammalian transient receptor potential channels represent a family of ion channels subdivided into 6 subfamilies (for review, see Vennekens *et al.*, 2008). Of these receptors, the vanilloid receptors TRPV1, TRPV2, TRPV3, and TRPV4 are involved in nociception and thermo-sensation. TRPV2 (formerly vanilloid receptor-like receptor VRL1) is a non-selective ligand-gated cation channel that responds to noxious heat (> 52°C) (Caterina *et al.*, 1999) and membrane stretch, as well as osmolarity changes (Muraki *et al.*, 2003). Compared with TRPV1, however, TRPV2 may serve diverse physiological functions, and its therapeutic potential in hyperalgesia remains unclear (Szallasi *et al.*, 2007). Similarly, studies on the role of TRPV3 and TRPV4 in pain are inconsistent (for review, see Szallasi *et al.*, 2007; Vennekens *et al.*, 2008).

TRPV1 (formerly vanilloid receptor VR1) (reviewed by Szallasi *et al.*, 2007), a voltage-dependent cation channel, is highly expressed in nociceptive primary afferent neurons and

is gated by capsaicin, noxious heat (> 45°C), acidic pH (< 5.3), components of endogenous inflammatory mediators (bradykinin, PGE₂), and anandamide (Vellani *et al.*, 2001). Recently, several TRPV1 antagonists have been reported to alleviate or reverse mechanical and thermal hyperalgesia associated with inflammatory pain (Gavva *et al.*, 2005; Jia *et al.*, 2005; Szallasi *et al.*, 2007). In some persons, the oral TRPV1 antagonist SB705498 (Chizh *et al.*, 2007) is effective in alleviating capsaicin- and UV-induced hyperalgesia. These encouraging pre-clinical and clinical findings indicate that TRPV1 can be a potential target for the treatment of human pain conditions.

Evidence for the involvement of TRPV1 receptors in craniofacial pain comes from studies on tooth pulp (Rodd *et al.*, 2007; Kim *et al.*, 2008) as well as meningeal afferents (Dux *et al.*, 2003; Geppetti *et al.*, 2008). Tooth pulp and jaw joint afferents express TRPV1 (see Table). In addition, TRPV1 plays a role in thermal hyperalgesia and cold pain following inflammation of the face (Pei *et al.*, 2007). Persons with burning mouth syndrome also show a positive correlation between TRPV1 expression and pain score (Yilmaz *et al.*, 2007).

Recent studies demonstrated that TRPV1 may play a role specifically in muscle pain (Hoheisel *et al.*, 2004; Rau *et al.*, 2007). Injection of capsaicin into the masseter muscle causes pain in humans (Sohn *et al.*, 2000), and masseter muscle afferent neurons in the trigeminal ganglion express TRPV1 receptors (Connor *et al.*, 2005), suggesting that TRPV1 may be involved in craniofacial muscle pain.

The P2X₃ receptor is sometimes co-localized with TRPV1 (Table). In the trigeminal ganglion, about 35% of P2X₃ neurons express VR1 (Ichikawa *et al.*, 2004; Ichikawa and Sugimoto, 2004). In contrast, approximately 75% of P2X₃ DRG neurons express TRPV1 (Guo *et al.*, 1999). The differences in overlap of these receptors may indicate a differential interaction or co-activation of different receptors.

ACID-SENSING ION CHANNELS (ASICs)

Activation of acid-sensing ion channels (ASICs) on primary afferent neuron terminals may be at least partly responsible for the sensitization of neurons during hyperalgesia, since low tissue pH occurs in muscle during exhaustive exercise, ischemic muscle contraction, and inflammation (Issberner *et al.*, 1996; Reeh and Steen, 1996). Acid-sensing ion channel 3 (ASIC3) receptors are present in trigeminal ganglion neurons that innervate the masseter muscle, tooth pulp, and facial skin (Ichikawa and Sugimoto, 2002) (see Table). Nearly all trigeminal ganglion P2X₃ muscle afferent neurons also express ASIC3 (Connor *et al.*, 2005), implying that tissue pH is a relevant stimulus for craniofacial neurons.

Acidic saline injected into limb muscles activates a proportion of muscle afferents (Hoheisel *et al.*, 2004) and produces hyperalgesia (Sluka *et al.*, 2001). Even though ASIC3 channels are present on craniofacial muscle afferents (Connor *et al.*, 2005; Ambalavanar *et al.*, 2007), injection of acidic saline into the masseter muscle does not produce hyperalgesia or alter CGRP or SP expression (Ambalavanar

Table. Expression of TRPV and ASICs in Trigeminal Ganglion Neurons Innervating Different Craniofacial Tissues and Their Co-expression with CGRP and P2X₃

Receptor/Channel	TG Neurons (%)	Cutaneous (%)	Tooth Pulp (%)	Jaw Joint (%)	Muscle* (%)
TRPV1 (VR-1)	20 ¹	26 ¹	8 ¹	25 ⁴	66 ⁶
TRPV2 (VRL-1)	14 ²	9 ²	36 ²	41 ⁴	—
ASIC3	23	13	33	—	64 ⁶
TRPV1 & P2X ₃	35% of P2X ₃ ³	16 ³	6 ³	—	50 ⁶
TRPV1 & CGRP	57 ¹	18 ¹	100 ¹	38% of CGRP ⁴	—
TRPV2 & P2X ₃	9% of P2X ₃ ³	8 ³	23 ³	—	—
TRPV2 & CGRP	—	25 ²	45 ²	30% of CGRP ⁴	—
ASIC3 & CGRP	26 ⁵	37% of CGRP ⁵	36% of CGRP ⁵	—	—
TRPV1, ASIC3 & P2X ₃	—	—	—	—	5 ⁶

1 Ichikawa and Sugimoto, 2001.

2 Ichikawa and Sugimoto, 2000.

3 Ichikawa and Sugimoto, 2004.

4 Ichikawa *et al.*, 2004.

5 Ichikawa and Sugimoto, 2002.

6 Connor *et al.*, 2005.

* Electrophysiological study.

et al., 2007). While *in vitro* studies indicate that trigeminal ganglion (Connor *et al.*, 2005) and DRG neurons (Hoheisel *et al.*, 2004; Molliver *et al.*, 2005) respond to very small changes in pH, injection of acidic saline into the masseter muscle *in vivo* failed to evoke nociceptive responses (Ambalavanar *et al.*, 2007).

The co-expression of P2X₃ and ASIC3 also appears to vary considerably between the cranial and extracranial regions. In the trigeminal ganglion, nearly every P2X₃ primary muscle afferent neuron also expresses ASIC3 (Connor *et al.*, 2005). In contrast, less than 5% of DRG neurons express both P2X₃ and ASIC3 (Molliver *et al.*, 2005).

INTERACTIONS BETWEEN CRANIOFACIAL NOCICEPTIVE RECEPTORS AND CHANNELS

Many primary afferent neurons co-express more than 2 nociceptive receptors or channels. For instance, more than 50% of masseter muscle afferent neurons in the trigeminal ganglion express not only TRPV1, but also P2X₃ as well as ASIC3 receptors in the same neuron (Connor *et al.*, 2005). CGRP is also highly co-expressed with TRPV1 in the trigeminal ganglion (Dux *et al.*, 2003). Previous studies demonstrated that the release of CGRP is induced by low pH in trigeminal ganglion neurons (Goodis *et al.*, 2006), and that ASIC3 receptors are co-localized with CGRP (Ichikawa and Sugimoto, 2002). Thus, the role of ASIC receptors in pain processing may be influenced by their subunit composition and the contributions of TRPV1 receptors in proton sensitivity (Ugawa *et al.*, 2002; Poirrot *et al.*, 2006). Thus, it seems likely that concurrent activation of different molecular receptors in trigeminal ganglion neurons occurs during normal and pathological states. This type of interaction has recently been shown in DRG neurons, which respond primarily to a combination of muscle metabolites (Light *et al.*, 2008). Further studies with physiologically relevant stimuli are necessary to determine whether the co-expression of

nociceptive receptors and channels in trigeminal neurons conveys a synergistic action of 2 or more receptors and represents functional complexes.

THERAPEUTIC POSSIBILITIES

The therapeutic potential of providing pain therapies by blocking P2X₃ receptors may initially appear limited, since P2X₃ receptors exhibit rather long-lasting desensitization (Cook *et al.*, 1998; Cook and McCleskey, 2002). However, heteromultimers containing P2X₃ exhibit sustained ATP currents (Connor *et al.*, 2005), and thus may be productive therapeutic targets. The development of the selective P2X₃ receptor antagonist, 5-[[[(3-Phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-*n*-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate, A-317491, represents a milestone in the development of purinergic-based pain therapies (Jarvis *et al.*, 2002). This antagonist reduces thermal and mechanical hyperalgesia in CFA pain models (Jarvis *et al.*, 2002; Wu *et al.*, 2004). An important characteristic of A-317491 is that it exhibits limited penetration into the CNS (Wu *et al.*, 2004), and thus may be suited for the selective targeting of peripheral pain. Development of additional P2X₃ receptor antagonists—such as the endogenous peptide spinorphin, which shows potent antagonism at the human P2X₃ receptor (Jung *et al.*, 2007)—is clearly warranted. Slow ATP responses in P2X_{2/3} DRG and nodose ganglion neurons can also be inhibited by cannabinoids (Krishtal *et al.*, 2006), which may also have potential for modulating deep-tissue craniofacial pain. Since P2X₃ receptors are much more abundant on neurons supplying deep craniofacial tissue, and P2X₃ receptors have prominent interactions with CGRP, TRPV1, and ASIC, they currently appear to be very promising therapeutic targets for deep craniofacial pain.

Sustained exposure of trigeminal neurons to CGRP not only enhances ATP currents through P2X₃ receptors, but also accelerates the recovery of P2X₃ receptors from desensitization (Fabbretti *et al.*, 2006). Thus, CGRP antagonists have therapeutic potential,

not only because of their ability to reduce plasma extravasation significantly, but also because they can block the facilitatory effects of CGRP on P2X₃ receptors. CGRP and P2X₃ antagonists, perhaps in combination, are therefore strong candidate targets for craniofacial pain, while antagonists for SP currently appear less promising.

Unfortunately, little information is available on the use of TRPV1 antagonists to alleviate deep-tissue craniofacial pain. With the recent advances in the understanding of TRPV1 physiology and pharmacology, together with the availability of potent, selective TRPV1 antagonists, future studies should lead to further understanding of the role of TRPV1 receptors and of the TRPV1 receptor as a potential therapeutic target for the treatment of craniofacial pain, including TMD. Complete, selective antagonists for ASIC3 are not currently available (Hayes *et al.*, 2007). Studies are needed to develop selective ASIC3 antagonists and to determine whether ASIC3 currents can be modulated indirectly *via* other receptors.

CONCLUDING REMARKS

Chronic craniofacial pain often leads to long-term alterations in central nociceptive processing (*i.e.*, central sensitization). While these transformations can result in a state in which pain becomes independent of peripheral input, the initiation of these central transformations is likely to involve a peripheral stimulating event or trigger. In the craniofacial region, P2X₃ receptors are restricted to primary afferent neurons and are particularly abundant on neurons relaying nociceptive feedback from deep craniofacial tissues. Thus, transmission through P2X₃ neurons represents one prominent pathway by which nociceptive signaling from deep craniofacial tissues could be conveyed to the central nervous system. The recent development of specific P2X₃ antagonists which do not readily cross the blood-brain barrier thus might be particularly effective in reducing nociceptive feedback from deep craniofacial tissues and attenuating peripheral triggers that might evoke central sensitization. Therapeutics directed at NGF and CGRP may also be promising therapeutic targets for deep-tissue craniofacial pain, since not only are they involved in peripheral nociceptive mechanisms, but they also up-regulate P2X₃ receptors. The potential for TRPV1 antagonists to treat craniofacial pain needs further study, while the role of ASIC3 in craniofacial pain remains problematic. Thus, P2X₃ and CGRP antagonists currently appear to be the most promising potential targets to treat deep-tissue craniofacial pain.

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