

The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword

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ABSTRACT

Bee venom injection as a therapy, like many other complementary and alternative medicine approaches, has been used for thousands of years to attempt to alleviate a range of diseases including arthritis. More recently, additional therapeutic goals have been added to the list of diseases making this a critical time to evaluate the evidence for the beneficial and adverse effects of bee venom injection. Although reports of pain reduction (analgesic and antinociceptive) and anti-inflammatory effects of bee venom injection are accumulating in the literature, it is common knowledge that bee venom stings are painful and produce inflammation. In addition, a significant number of studies have been performed in the past decade highlighting that injection of bee venom and components of bee venom produce significant signs of pain or nociception, inflammation and many effects at multiple levels of immediate, acute and prolonged pain processes. This report reviews the extensive new data regarding the deleterious effects of bee venom injection in people and animals, our current understanding of the responsible underlying mechanisms and critical venom components, and provides a critical evaluation of reports of the beneficial effects of bee venom injection in people and animals and the proposed underlying mechanisms. Although further studies are required to make firm conclusions, therapeutic bee venom injection may be beneficial for some patients, but may also be harmful. This report highlights key patterns of results, critical shortcomings, and essential areas requiring further study.

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 12-HETE, 12-hydroxyeicosatetraenoic acids; AA, arachidonic acid; AC, adenylate cyclase; AIDA, 1-aminoindan-1,5-dicarboxylic acid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid; AP5, D,L-2-amino-5-phosphonopentanoic acid; ASIC, acid-sensing ionic channel; ATP, adenosine triphosphate; BK1/2, bradykinin receptors 1/2; BVT, bee venom therapy; Ca^{2+} - K^{+} , calcium-dependent potassium channel; cAMP, cyclic adenosine monophosphate; CFA, complete Freund's adjuvant; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin-gene related peptide; CH, chelerythrine chloride; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CNS, central nervous system; COX, cyclooxygenase; CPZ, capsaizine; cRF, cutaneous receptive field; CRPN, chemically relevant persistent nociception; DAG, diacylglycerol; DRG, dorsal root ganglion; EAAs, excitatory amino acids; EGLU, (S)- α -ethylglutamic acid; ERK, extracellular signal-regulated kinase; GABA, γ -aminobutyric acid; Glu, glutamate; H1, histamine receptor type 1; IAAs, inhibitory amino acids; iGluRs, ionotropic glutamate receptors; IL, interleukin; JNK, c-Jun N-terminal kinase; KA, kainite acid; Kir, inward-rectifier potassium channel; LOX, lipoxygenase; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MCD peptide, mast cell degranulating peptide; MCL peptide, mastocytolytic peptide; mGluRs, metabotropic glutamate receptors; MRPH, mechanically relevant primary hyperalgesia; MSOP, (RS)- α -methylserine-O-phosphate; NKA, neurokinin A; NKB, neurokinin B; NMDA, N-methyl-D-aspartic acid; NO, nitric oxide; NOS, nitric oxide synthase; NS, nociceptive specific; NSAID, non-steroidal anti-inflammatory drug; PAF, platelet-activated factor; PGs, prostaglandins; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PKs, protein kinases; PLA₂, phospholipase A2; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; RAST, radioallergosorbent test; RMM, rostral medial medulla; RT-PCR, reverse transcription-polymerase chain reaction; sGC, soluble guanylate cyclase; SMU, single motor unit; SP, substance P; TCM, traditional Chinese medicine; TNF α , tumor-necrosis factor α ; TRMIH, thermally relevant mirror-image hyperalgesia; TRPH, thermally relevant primary hyperalgesia; TRPV1, transient receptor potential vanilloid receptor 1; TRSH, thermally relevant secondary hyperalgesia; TTX, tetrodotoxin; VAS, visual analog scale; VDCC, voltage-dependent calcium channel; VDPC, voltage-dependent potassium channel; VIT, bee venom immunotherapy; WDR, wide dynamic range.

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1. Introduction

Bee venom therapy (BVT) is the application of live honeybee stings to patients for therapeutic purposes. BVT is one of the most traditional complementary and alternative therapeutic methods that have long been believed to be effective in the treatment of many diseases, including rheumatic arthritis, bursitis, tendinitis, shingles (herpes zoster), multiple sclerosis, wounds, gout, burns and infections. The distinguishing feature for the application of this therapeutic approach to those disease sufferers is relief of pain and inflammation and restoration of normal body functions (Son et al., 2007). More recently, BVT is also being considered as a potential cancer treatment (Orsolich et al., 2003; Yin et al., 2005; Putz et al., 2006; Hu et al., 2006a,b,c; Liu et al., 2008b).

The use of BVT in patients suffering from rheumatism or arthritis has a long history that can be traced back to ancient Egypt, Greece, and China. However, it had not been well recognized until 1888 when the book "Report about a Peculiar Connection Between the Bee Stings and Rheumatism" was published by the Austrian, Philip Terc, believed to be the first physician introducing the application of BVT to treatment of rheumatic patients (see website of American Apitherapy Society: <http://www.apitherapy.org/>). In 1935, Dr. Bodog F. Beck (1871–1942), a follower of Philip Terc, published another book on the use of BVT in the treatment of rheumatic arthritis (Beck, 1935) and brought the therapeutic method to the United States of America and educated many BVT users (see <http://www.apitherapy.org/> for An Introduction to Bee Venom Therapy, a foreword to reprint version of the book in 1981

by Charles Mraz). Charles Mraz (1905–1999), a beekeeper and follower of Dr. Beck, was recognized in the United States as the pioneer of the use of BVT to treat various disorders, particularly autoimmune diseases. In addition to initiating clinical research with scientists, he established the standard for purity for dried whole venom for the U.S. Food and Drug Administration and was the supplier of venom to pharmaceutical companies throughout the world (see <http://www.apitherapy.org/>). Although BVT is more likely to be promising as a therapeutic alternative for treatment of chronic pain and other diseases (Son et al., 2007), so far its efficacy and safety have not been approved by food and drug authorities worldwide. Thus, it still remains a challenge for both physicians and patients to accept BVT as a mainstream therapy.

One justified impediment to acceptance of BVT as a mainstream therapy is that stings by bees and other insects of Hymenoptera may cause allergic reactions in sensitized individuals, including systemic or anaphylactic reactions (Golden, 1989, 2006; Kay and Lessof, 1992; Annala, 2000; Bernstein et al., 2008; Simons et al., 2008). Allergy to bee venom is dangerous and life-threatening. In Australia, the mortality incidence was shown to be 0.086/1 million population per year during 1960–1981 and is likely to differ from region to region (Harvey et al., 1984). An epidemiological investigation showed that in developed countries up to 3% of adults have had a systemic reaction to sting, while about 25% might have an allergic risk as indicated by a positive skin test or RAST (radioallergosorbent test) for specific antibodies (Golden, 1989). Moreover, our ability to predict systemic reactions to bee venom is limited as it was reported that among 51 patients with negative

skin test responses at their first sting challenge 22% (11 out of 51) had systemic reactions to subsequent sting challenge including hives, respiratory depression and hypotension; RAST-determined antibody levels also do not reliably predict systemic reaction history or response to challenge (Golden et al., 2001). Until now, intensive demographic epidemiological investigations of the population allergic to bee stings are still lacking.

A second justified impediment to acceptance of BVT is the common knowledge that bee stings cause pain and inflammation and a suspected, but poorly studied, relationship between repeated bee stings and the development of signs and symptoms of arthritis in beekeepers (Cuende et al., 1999). In the past decade, intensive experimental studies on the nociceptive and anti-nociceptive effects of bee venom and its polypeptides have been conducted mainly by two separate groups of investigators. In one series of experimental studies, the nociceptive and inflammatory responses of mice, rats, cats and humans to subcutaneous injection of bee venom and melittin have been well observed by examining behavioral responses, electrophysiological properties of pain-signaling neurons, neurochemical substrates of pain pathways, and pharmacological blocking of the effects of nociceptive signal mediation (Chen, 2003, 2007, 2008). The results have provided lines of solid evidence demonstrating acute pharmacological and toxicological effects of bee venom and its major polypeptide (melittin) on the pain-producing and -conducting system, revealing the natural biological significance of bee stings as a harmful invasion of the human body. In contrast, another group has published a series of experimental studies demonstrating anti-nociceptive and anti-inflammatory effects of acute and repeated BVT in rats and patients by local subcutaneous injection of bee venom into an acupoint based on traditional Chinese medicine (TCM) (Son et al., 2007). The results have revealed effectiveness of this 'acupuncture' procedure on various kinds of inflammatory pain models including the complete Freund's adjuvant (CFA)-induced arthritis model (Kwon et al., 2001d, 2002; Kang et al., 2002; Lee et al., 2004, 2005a,b; Suh et al., 2006), the formalin test (Kwon et al., 2001b; Kim et al., 2005; Roh et al., 2006), carrageenan-induced inflammation (Chen et al., 1993), the intraperitoneal acetic acid writhing test (Kwon et al., 2001a,b), and on neuropathic pain modeled by chronic constriction injury (CCI) of a peripheral nerve (Roh et al., 2004). Here, we review these two opposing aspects of the biological actions of bee venom on the somatosensory system to determine the empirical rationale underlying the undesirable and desirable aspects of live bee stings and BVT on the human somatosensory system.

2. Biological constituents of bee venom

Envenomation by a honeybee has been carefully studied and the process is known to be initiated by the insertion of the sting apparatus into the victim's skin that is followed by autotomy or separation of the sting apparatus and venom sac from the abdomen when the bee pulls away from the embedded stinger (Schumacher et al., 1992, 1994). After separation, the embedded stinger continues to inject venom from the venom sac due to autonomous repetitive contraction of muscles in the sting apparatus. Quantitative analysis shows a time-dependent increase in the amount of bee venom delivered at the site of embedding of the sting apparatus; Following 2–7 s, 8–16 s and more than 16 s, the amount of bee venom found to be injected was $71.5 \pm 36.7 \mu\text{g}$, $114.8 \pm 57.6 \mu\text{g}$ and $141.9 \pm 89.2 \mu\text{g}$, respectively (Schumacher et al., 1994). Generally, given sufficient time, one sting can deliver approximately $140 \mu\text{g}$ of bee venom. Interestingly, the dose-response studies in animals suggest that 100–200 μg of bee venom per injection is an optimal dose to induce the most intense pain-related behaviors (Lariviere and Melzack, 1996).

(Honey) bee venom (of *Apis mellifera*) is a complex composition of polypeptides, enzymes, amines, lipids and amino acids (Habermann, 1972; Lariviere and Melzack, 1996; Son et al., 2007) (see Table 1). Some of the components of bee venom have been shown to have anti-inflammatory and antinociceptive effects, others to have toxic or detrimental effects, and some to have both beneficial and adverse effects in different conditions. In general, bee venom polypeptides are likely to act on the nervous system in both the periphery and the CNS as modulators of ion channels and other molecular targets, leading to various biological, pharmacological and toxicological activities (Table 1). Some key effects of the constituents found in greatest abundance are described below.

2.1. Peptide constituents

Among the chemical constituents of bee venom are unique biologically active substances including the polypeptides melittin (Habermann, 1954; Neumann and Habermann, 1954; Habermann and Reiz, 1965), apamin (Habermann and Reiz, 1965; Spoerri et al., 1973, 1975), mast-cell degranulating (MCD) peptide (Fredholm, 1966; Breithaupt and Habermann, 1968), mastocytolytic (MCL) peptide (Habermann and Breithaupt, 1968), minimine (Lowy et al., 1971), secapin (Gauldie et al., 1978; Hider and Ragnarsson, 1981), tertiapin (Hider and Ragnarsson, 1981; Xu and Nelson, 1993), melittin F (Gauldie et al., 1978), cadiopep (Vick et al., 1974) and adolapin (Shkenderov and Koburova, 1982; Koburova et al., 1984, 1985).

2.1.1. Melittin

Although some of the polypeptides of bee venom are toxic, few are demonstrated to be allergenic. Thus, studies of biological, pharmacological and toxicological activities of these bee venom polypeptides on mammals, particularly humans, are of great importance. For example, melittin (Fig. 1, Table 1), a strongly basic 26 amino-acid polypeptide which constitutes 40–60% of the whole dry honeybee venom, has various biological, pharmacological and toxicological actions including strong surface activity on cell lipid membranes, hemolyzing activity, antibacterial and antifungal activities (Habermann, 1972, 1974; Gauldie et al., 1976; Lariviere and Melzack, 1996) and antitumor properties (Orsolich et al., 2003; Liu et al., 2008a,b). Recently, melittin has also been demonstrated to cause neural plastic changes along pain-signaling pathways by activation and sensitization of nociceptor cells via phosphorylation of mitogen-activated protein kinases (MAPK) (Hao et al., 2008; Yu et al., 2009), activations of thermal nociceptive channels (transient

Melittin (26 amino acid residues)

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-GlnNH₂

Apamin (18 amino acid residues)

Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-HisNH₂

Mast cell degranulating peptide (22 amino acid residues)

Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-AsnNH₂

Secapin (24 amino acid residues)

Tyr-Ile-Ile-Asp-Val-Pro-Pro-Arg-Cys-Pro-Pro-Gly-Ser-Lys-Phe-Ile-Lys-Asn-Arg-Cys-Arg-Val-Pro-Val

Fig. 1. Amino acid sequences of bee venom polypeptides: melittin, apamin, mast cell degranulating peptide and secapin.

Table 1
Ingredients of venom of *Apis mellifera* and their biological effects (partially adapted from Lariviere and Melzack, 1996, with permission).

Ingredients	Biological effects
Acetylcholine ¹	Agonist at muscarinic and nicotinic acetylcholine receptors ⁷
Adolapin (1%) ⁵	Anti-nociceptive and anti-inflammatory through inhibition of cyclooxygenase activity ⁵ , antipyretic ⁸
Amino acids ¹	Unspecified
Apamin (2–3%) ^{1–4}	Selective blocker of the calcium-dependent potassium channel ⁹
Carbohydrates (2%) ¹	Unspecified
Cardiopep (0.7%) ¹	β-Adrenergic and anti-arrhythmic effects ¹⁰
Dopamine ^{1,3}	Agonist at dopamine receptors ¹¹
Esterase ¹	Unspecified
Histamine (1.5%) ^{1–4}	Agonist at histamine receptors ¹²
Hyaluronidase (<3%) ^{1–4}	Hyaluronic acid hydrolyzing enzyme ¹³ Allergenicity ¹⁴ , Histamine release ¹⁵
Lipids (5%) ¹	Unspecified
Mast-cell degranulating peptide (2–3%) ^{1,2,4}	Histamine release ^{1,2,4} Induction of LTP in hippocampus ^{16,17} , inhibition of epilepsy ¹⁸ inhibiting a voltage-dependent K(+)–channel in brain membranes ¹⁸ Activation of pertussis toxin-sensitive G-protein ¹⁹ A selectively mastocytolytic factor ⁶ Strong surface activity on cell lipid membrane ^{1–4} Hemolyzing activity ^{1–4} Activation of PLA2 ^{1–4} Antibacterial and antifungal activity ^{20–22} Antitumor activities ^{23–25} Activation and sensitization of nociceptors ^{26–32} Anti-nociceptive and anti-inflammatory ³³
MCL-peptide ⁶	Unspecified
Melittin (40–60%) ^{1–4}	Unspecified
Melittin F (<1%) ^{1,34}	Agonist at adrenoceptors
Minimine ^{1,35}	Antigenicity and allergenicity ^{1–4}
Noradrenaline ^{1,3}	Inflammatory and nociceptive ^{36,37}
Phospholipase A (12%) ^{1–4}	Interaction with melittin ³⁸ Activation of neurons and glial cells ³⁹ Nerve regeneration ⁴⁰ Facilitation of neurotransmitter release ⁴¹
Phospholipase B ³	Unspecified
Procamine ¹	Unspecified
Promelittin ¹	Unspecified
Protease inhibitor ¹	Unspecified
Secapin (<1%) ¹	High-affinity binding sites in rat brain ⁴²
Tertiapin (<1%) ¹	High-affinity binding sites in rat brain ⁴² Inhibition of the enzyme-activating capacity of calmodulin ⁴³ Inhibition of activity of soluble phosphodiesterase ⁴⁴

Note: values in parentheses for ingredients (left) indicate percent by weight of dry bee venom. LTP, long-term potentiation.

¹Tu (1977); ²Habermann (1971); ³Minton (1974); ⁴Habermehl (1981); ⁵Shkenderov and Koburova (1982); ⁶Habermann and Breithaupt (1968); ⁷Brown (2006); ⁸Koburova et al. (1984); ⁹Hugues et al. (1982); ¹⁰Vick et al. (1974); ¹¹Marsden (2006); ¹²Parsons and Ganellin (2006); ¹³Soka et al. (2001); ¹⁴Aukrust et al. (1982); ¹⁵Clinton et al. (1989); ¹⁶Cherubini et al. (1987); ¹⁷Kondo et al. (1992); ¹⁸Gandolfo et al. (1989); ¹⁹Fujimoto et al. (1991); ^{20,21}Habermann (1972, 1974); ²²Gaudie et al. (1976); ²³Orsolic et al. (2003); ^{24–25}Liu et al. (2008a, b); ²⁶Hao et al. (2008); ²⁷Yu et al. (2009); ²⁸Li and Chen (2004); ²⁹Shin and Kim (2004); ^{30–31}Chen et al. (2006a,b); ³²Lu et al. (2008); ³³Son et al. (2007); ³⁴Gaudie et al. (1978); ³⁵Lowy et al. (1971); ³⁶Hartman et al. (1991); ³⁷Landucci et al. (2000); ³⁸Koumanov et al. (2003); ³⁹Sun et al. (2004a); ⁴⁰Edstrom et al. (1996); ⁴¹Yue et al. (2005); ⁴²Taylor et al. (1984); ⁴³Miroshnikov et al. (1983); ⁴⁴Dudkin et al. (1983).

receptor potential vanilloid receptor 1, TRPV1) (Li and Chen, 2004; Shin and Kim, 2004; Chen et al., 2006a,b) and ATP P2X and P2Y receptors (Lu et al., 2008). Contrarily, melittin has also been believed to be the major biologically active substance of bee venom to play a role in production of anti-nociceptive and anti-inflammatory effects when applied to the acupoint of a subject ('apipuncture') (Son et al., 2007). The molecular and cellular mechanisms underlying the nociceptive and anti-nociceptive effects of melittin are not entirely clear and remain to be further clarified by intensive experimental studies.

2.1.2. Apamin

Apamin (Fig. 1, Table 1), another important bee venom neurotoxic polypeptide of 18 amino acids comprising 2–3% of dry bee venom, possesses a selective inhibitory action on calcium-dependent potassium channels that are involved in regulation of the after-hyperpolarization period and frequency of action potential generation in the central nervous system (CNS) (Hugues et al., 1982). Injections of 0.3 mg/kg (but not 0.1 mg/kg) in the rat can cause behavioral side effects of grooming at the injection site, excitation, wet dog shakes and lying on the belly; a higher dose of

1.0 mg/kg produces the same side effects in addition to Straub symptoms and clonic seizures (van der Staay et al., 1999).

2.1.3. Mast cell degranulating (MCD) peptide

MCD peptide, also known as peptide 401 (Fig. 1, Table 1), a bee venom polypeptide with 22 amino acids and constituting 2–3% of dry bee venom, was originally named due to its biological action of causing release of histamine from mast cells (Jasani et al., 1979; Banks et al., 1990). However, studies of its biological and pharmacological actions in the CNS have been emerging gradually. MCD peptide has specific binding sites in the hippocampus and application of this peptide onto hippocampal slices was shown to result in the production of long-term potentiation (LTP) in the CA1 area that is distinct from the LTP evoked by conditioning electrical stimulation (Taylor et al., 1984; Cherubini et al., 1987; Bidard et al., 1987a; Mourre et al., 1988; Ben et al., 1989; Kondo et al., 1990; Sequier and Lazdunski, 1990; Neuman et al., 1991; Kondo et al., 1992). MCD peptide may also be involved in the pathogenesis of epilepsy (Bidard et al., 1989; Ide et al., 1989). The molecular and cellular mechanisms underlying MCD peptide-evoked LTP in the hippocampus are not clear, but some reports showed that it has

selective binding sites and actions on voltage-dependent potassium channels (Bidard et al., 1987b; Schmidt et al., 1988; Rehm and Lazdunski, 1988; Rehm et al., 1988; Gandolfo et al., 1989; Kondo et al., 1992).

2.1.4. Adolapin

Adolapin (Table 1), a basic polypeptide with 103 amino acids residues and comprising 1% of dry bee venom, is the only one that has been shown to have anti-nociceptive, anti-inflammatory and antipyretic effects primarily (Shkenderov and Koburova, 1982; Koburova et al., 1984, 1985). Adolapin can inhibit prostaglandin synthesis via inhibition of cyclooxygenase activity (Shkenderov and Koburova, 1982).

2.1.5. Other peptides

Similar to MCD peptide, tertiapin and secapin (Fig. 1, Table 1) are both demonstrated to be neurotoxins (Taylor et al., 1984), but their biological, pharmacological and toxicological activities in the nervous system are poorly studied. Both of the two bee venom polypeptides comprise less than 1% of dry bee venom and are likely to be modulators of voltage-dependent potassium channels due to their similar distribution of binding properties in the CNS as MCD peptide (Taylor et al., 1984; Mourre et al., 1988; Kondo et al., 1992). This presumption has been strongly supported by a series of studies showing that tertiapin is a high-affinity inhibitor for inward-rectifier K⁺ channels (Jin and Lu, 1999; Kanjhan et al., 2005; Felix et al., 2006; Ramu et al., 2008).

2.2. Enzymes

2.2.1. Phospholipase A₂ (PLA₂)

PLA₂ (Table 1), which constitutes 10–12% of dry bee venom, has inflammatory and nociceptive effects (Hartman et al., 1991; Landucci et al., 2000). PLA₂ is a membrane-associated phospholipid converting enzyme that is important in the production of arachidonic acid, which is further metabolized to prostaglandins by cyclooxygenase and to leukotrienes by lipoxygenase. PLA₂ exhibits complex interactions with melittin that can result in potentiation of secretory PLA₂ effects or in inhibition depending on the peptide/phospholipid ratio (Koumanov et al., 2003). PLA₂ has effects in a range of cells related to nociception including astrocytes and neurons and possibly microglial cells (Sun et al., 2004a). PLA₂ is involved in nerve regeneration (Edstrom et al., 1996), but also in pronociceptive glutamergic neurotransmission in the substantia gelatinosa of the dorsal horn of the spinal cord (Yue et al., 2005), and delayed neurotoxic effects *in vitro* and *in vivo* (Clapp et al., 1995).

2.2.2. Hyaluronidase

Hyaluronidase (Table 1) constitutes 1.5–2% of dry bee venom (Lariviere and Melzack, 1996). Hyaluronidases break down hyaluronic acid in tissues such as in synovial bursa of rheumatoid arthritis patients (Barker et al., 1964). Hyaluronidase in bee venom shares this property with endogenous hyaluronidase (Barker et al., 1963).

3. Nociceptive and inflammatory effects of subcutaneous bee venom injection

Bee venom contains many components capable of producing pro-inflammatory and pronociceptive adverse events. These have been studied extensively in the past decade and a half.

3.1. Experimental human studies

Due to the risk of anaphylaxis and systemic reactions to bee venom allergens, so far there has been no report of injection of

whole honeybee venom in humans for the primary purpose of studying pain or nociception. Only rare case reports exist carefully describing the quantity and quality of pain in patients receiving BVT or bee venom immunotherapy (VIT). Thus, the spatial and temporal features of pain induced by bee stings have been greatly neglected by the scientific research field even though pain sensation serves as the immediate alarm response for an individual who is being stung by a bee. However, due to increased interest in animal studies on bee venom-induced nociception or pain, the first experimental study on pain and inflammatory responses of seven healthy adults (2 women and 5 men) to intradermal (i.d.) injection of melittin was carefully conducted and published in 2000 (Koyama et al., 2000). The human subjects reported the pain intensity experienced using a 0–10 visual analog scale (VAS) in which scores of 0, 5 and 10 indicated “no pain”, “moderate pain” and “intolerable pain”, respectively. A sharp pain sensation (score > 8.0) was reported in all 7 subjects immediately after intradermal injection of melittin (5 µg in 50 µl saline) into the volar aspect of one forearm. The pain sensation declined gradually and totally disappeared at 3 min after melittin injection. It was clearly described that there was no itch or burning sensation following melittin stimulation, suggesting that significant histamine might not be released and that thermal nociceptors might not be activated by this dose of melittin. Meanwhile, melittin produced a visual flare surrounding a wheal near the injection site that disappeared within 2 h. This local inflammatory response was also characterized by an increase in skin temperature monitored by a computer-assisted infrared thermograph. The melittin-induced peak increase in skin temperature was at least 10 min delayed compared to the peak pain sensation and this process was sustained for at least 1 h. Topical lidocaine gel administration markedly blocked the melittin-induced visual flare and the increased skin temperature but not the pain sensation, suggesting that the local inflammatory response induced by melittin is neurogenic and mediated by axonal reflex and sympathetic regulation (Koyama et al., 2000, 2002).

In two other experimental studies on healthy human volunteers, the algogenic effects of two higher doses of melittin (10 µg and 50 µg in 50 µl saline) were also observed (Sumikura et al., 2003, 2006). The peak melittin-induced pain intensity (VAS score) was similar to the above mentioned reports; however, the duration of the pain sensation was much longer, lasting 15 min with the higher dose used and demonstrating a dose-related increase in the duration of the pain sensation with increasing doses of melittin. A similar relationship has been reported in the rat following whole bee venom injection (Lariviere and Melzack, 1996). The doses of melittin used in the human experiments are less than the amount delivered by one bee sting, which is reported to contain about 140 µg of dried bee venom per sting and 40–60% melittin (Schumacher et al., 1992, 1994). It was also found that the melittin-induced pain was different in quality from that induced by intradermal injection of capsaicin, the pungent ingredient of hot chilli peppers (Table 2). The pain sensation for the melittin test was reported as intense and clearly localized, while that of the capsaicin test was a poorly localized, burning sensation. Moreover, intradermal injection of melittin resulted in an area with primary heat and mechanical hyperalgesia as well as a secondary heat hyperalgesia identified in an area remote from the injection site (Sumikura et al., 2003, 2006). In contrast, intradermal capsaicin injection is well known to produce very transient primary hyperalgesia followed by a sustained secondary mechanical hyperalgesia (Baumann et al., 1991; LaMotte et al., 1991, 1992; Koltzenburg et al., 1992; Torebjork et al., 1992; Treede and Cole, 1993; Cervero et al., 1994; Andrews et al., 1999). It is of special interest to note that i.d. injections of melittin and capsaicin in human subjects produce different types of pain and hyperalgesia in

Table 2

Comparisons of nociception, pain and hyperalgesia induced by intradermal (i.d.) or subcutaneous (s.c.) injection of bee venom, melittin and capsaicin in human subjects and rodents.

	Bee venom	Melittin	Capsaicin
Human			
Spontaneous pain			
Quality	Unidentified	Intense pain in a well localized area	Burning pain in a wide area
Intensity and time course	Unidentified	VAS: $64.1 \pm 4.6 < 3$ min ($5 \mu\text{g}$) ¹ ; <15 min ($50 \mu\text{g}$) ² in $50 \mu\text{l}$ saline, i.d.	VAS: $66.4 \pm 4.6 < 15$ min ($10 \mu\text{g}$ in $50 \mu\text{l}$ Tween 80) ²⁻⁴ , i.d.
Heat hyperalgesia			
Primary zone	Unidentified	Yes, long-lasting (60 min) ^{2,5}	Yes, long-lasting (60 min) ²⁻⁴
Secondary zone	Unidentified	Yes, long-lasting (60 min) ^{2,5}	No ^{2,5}
Mirror-image	Unidentified	Unidentified	Unidentified
Mechanical hyperalgesia			
Primary zone	Unidentified	Punctate: distinct (von Frey) ² Stroking: allodynia/paresthesia (cotton swabs) ²	Punctate: unclear (von Frey) ² Stroking: allodynia (cotton swabs) ²⁻⁴
Secondary zone	Unidentified	Area: punctuate = stroking = visual flare ²	Area: punctuate >> stroking > visual flare ²⁻⁴
Mirror-image	Unidentified	Unidentified	Unidentified
Rodents			
Spontaneous pain	Nociceptive score >60 min ⁶ Paw flinches >60 min and paw licking >60 min by 200–300 μg in $50 \mu\text{l}$ saline, s.c. (hindpaw) ^{7,8}	Paw flinches >60 min by 100 μg in $50 \mu\text{l}$ saline, s.c. (hindpaw) ⁹	Paw licking <10 min by 1.6 μg in $20 \mu\text{l}$ 7.5% DMSO, s.c. (hindpaw) ¹⁰ Grooming behaviors <42 min by 1.5 μg in $25 \mu\text{l}$ 7.5% DMSO, s.c. (vibrissa pad) ¹¹
Heat hyperalgesia			
Primary zone	Lasting for 96 h ^{7,8}	Lasting for 2 h ⁹	Unidentified
Secondary zone	Lasting for 48 h ^{7,8}	Unidentified	Unidentified
Mirror-image	Lasting 48–96 h ^{7,8}	No ⁹	Unidentified
Mechanical hyperalgesia			
Primary zone	Lasting for 96 h ^{7,8}	Lasting for >48 h ⁹	Unidentified
Secondary zone	No ^{7,8}	Unidentified	Unidentified
Mirror-image	No ^{7,8}	No ⁹	Unidentified
Primary afferents	Unidentified	<i>In vivo</i> , both thermo- and mechanonociceptors ¹² <i>In vitro</i> , 60–70% small to medium-sized DRG cells, patchclamp recording and Ca ²⁺ influx measured by confocal microscope*	<i>In vivo</i> , capsaicin-sensitive thermnociceptors ^{3,4} <i>In vitro</i> , thermnociceptors ¹⁴⁻¹⁷
Peripheral molecular targets	Multiple	Multiple and remain to be examined. TRPV1 ⁹ , P2X3 ¹³	TRPV1 ¹⁴⁻¹⁷

Notes: VAS, Visual Analog Scale. TRPV1, transient receptor potential vanilloid receptor 1. ¹Koyama et al. (2000); ²Sumikura et al. (2003); ³LaMotte et al. (1991); ⁴Willis (1994); ⁵Sumikura et al. (2006); ⁶Lariviere and Melzack (1996); ⁷Chen et al. (1999b); ⁸Chen and Chen (2000); ⁹Chen et al. (2006b); ¹⁰Sakurada et al. (1992); ¹¹Pelissier et al. (2002); ¹²Cooper and Bomalaski (1994); ¹³Lu et al. (2008) ^{14,15}Caterina et al. (1997, 2000); ^{16,17}Caterina and Julius (1999, 2001); *Chen et al. (unpublished data).

terms of stimulus modalities, time courses of spontaneous pain and hyperalgesia/allodynia, spatial properties of visual flare and hyperalgesia/allodynia, types of primary afferents and molecular targets, etc. Comparisons of nociception, pain and hyperalgesia induced by i.d. or s.c. injection of bee venom, melittin and capsaicin in human subjects and rodents are shown in Table 2.

Hyperalgesia is referred to as an enhanced painful sensation caused by painful stimulation (mechanically or thermally nociceptive), while allodynia is referred to as a painful sensation caused by non-painful stimulation (mechanically or thermally non-nociceptive) (Merskey and Bogduk, 1994; McMahon and Koltzenburg, 2006). Allodynia (also known as stroking hyperalgesia) induced by a cotton swab is a characteristic observed in the capsaicin-treated skin. Human subjects receiving a melittin injection do not report allodynia consistently, highlighting another difference in the perception of these chemical injuries. The discovery of the differences in quality of pain sensation and modality of hyperalgesia between i.d. injections of melittin and capsaicin in human psychophysical experiments provides important cues for understanding the complex molecular basis of pain and pain hypersensitivity of different etiologies. Melittin isolated from whole bee venom has been previously reported to be an allergen itself (Paull et al., 1977; Annala, 2000). It is worthy to note

that all human subjects recruited in the melittin experiments were RAST negative and not hypersensitive to melittin. Because RAST negative individuals might also suffer from allergic reactions (Golden et al., 2001), it is difficult to justify continued human study with melittin as was extensively done with intradermal injection of capsaicin. Use of synthetic melittin may greatly reduce the risk of allergic hypersensitivity reactions. Therefore, further experimental studies of patients receiving BVT or VIT as a treatment of pain or allergic problems are still of interest.

3.2. Experimental animal studies

For the behavioral assays of animals responding to injuries of tissue or nerve origins, pain-related or nocifensive behaviors can be quantitatively evaluated. The use of a pain score in the evaluation of spontaneous nociceptive responses of rodents to peripheral chemical insults was first introduced by Dubuisson and Dennis (1977) in the formalin test. The pain score was divided into 4 grades: grade 0, the chemically injured paw is pressing firmly on the floor and obviously bears the animal's weight; grade 1, the injured paw press slightly on the floor, but little if any weight is placed on the paw, during locomotion there is a definite limp; grade 2, the injured paw is elevated and is not in contact with the

floor surface; grade 3, the animal licks, bites, or shakes the affected paw. Numerical ratings are based upon the time spent in each grade. Moreover, counting the number of paw flinching reflexes or time spent licking or lifting the injured paw at each time-block for 1–2 h has also been validated for pain scoring in rodents (Abbott et al., 1995, 1999; Saddi and Abbott, 2000). To evaluate the animal's hypersensitivity (hyperalgesia) to thermally nociceptive or mechanical stimuli, application of radiant heat or von Frey filaments to the chemically injured paw or non-injured contralateral paw is used and the changes in paw withdrawal thermal latency (PWTL) or paw withdrawal mechanical threshold (PWMT) can be rated quantitatively (for detail see Mogil, 2009).

3.2.1. Nociceptive and inflammatory effects of bee venom

The first experimental animal study on the nociceptive effects of bee venom was published by Lariviere and Melzack (1996). In that study, five doses of lyophilized whole venom of *Apis mellifera* (0.01 mg, 0.05 mg, 0.1 mg, 0.2 mg, and 0.3 mg in 50 μ l saline) were injected subcutaneously into one hind paw of adult Long-Evans hooded rats. Using a modified version of the pain score of Dubuisson and Dennis (1977) in which favoring of the injected paw (grade 1) was excluded, a dose-related increase in the overall mean score of pain-related behaviors was observed. Similar to the human VAS reports, rats responded to the bee venom injection robustly in the first 5–10 min followed by a slow decline in pain scores over 1 h of observation. The duration of spontaneous pain-related behaviors was also dose-related. For example, the lower doses of 0.01 mg and 0.05 mg of bee venom resulted in a period of 20–25 min of mild pain-related behaviors, while the higher doses of 0.2 mg and 0.3 mg produced robust pain responses lasting up to or longer than 1 h, suggesting that the bee venom-induced pain-related behavioral responses are dose-dependent in both time course and response intensity. The bee venom-induced pain-related behaviors were sensitive to pharmacological intervention by morphine and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, demonstrating the effectiveness of conventional analgesics in the treatment of honeybee sting-evoked spontaneous pain.

The response pattern of bee venom-induced spontaneous pain-related behavioral response is tonic and monophasic and as such is significantly different from that of the formalin test. The formalin test is well known to have biphasic nociceptive responses, including an immediate early response (0–5 min) and a late tonic response (20–60 min) with a quiescent phase (10–15 min) in between (Dubuisson and Dennis, 1977; Abbott et al., 1995). Bee venom-induced spontaneous pain-related responses were also studied in different species including cats, albino Sprague-Dawley rats and many strains of mice (Chen et al., 1998, 1999b; Lariviere et al., 2002, 2005). The response pattern and time course are similar to the human response, and thus, there is no evidence for species differences. The consistency of behavioral responses to bee venom among different species reflects common, natural biological processes in mammals in response to bee stings. However, species differences in the behavioral responses to formalin injection have been clearly observed (Chen et al., 1996, 1998, 1999b; Chen and Koyama, 1998; You and Chen, 1999), suggesting biological responses of different mammals to a non-naturally existing chemical structure will not be common.

Pain hypersensitivity, or hyperalgesia, to natural stimulations including heat and mechanical modalities were also carefully studied following subcutaneous bee venom injection into the hind paw of rats (Chen et al., 1999b; Chen and Chen, 2000). In these two separate experimental studies, Chen and his colleagues first described the phenomenon of hyperalgesia following subcutaneous bee venom injection. Spontaneous nociceptive paw flinches have completely disappeared approximately 1 h after bee venom

injection. Reductions in PWTL in response to radiant heat stimuli or in PWMT in response to von Frey filament stimuli were consistently identified at 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, and 96 h after bee venom injection, indicating the occurrence of primary heat and mechanical hyperalgesia in the injured area of the paw following experimental honeybee sting. This result is consistent with what was later observed in experimental human studies (Sumikura et al., 2003). Furthermore, a secondary heat, but not mechanical, hyperalgesia was identified in a remote area from the injection site, which was later demonstrated to also occur in human subjects in response to melittin injection (Sumikura et al., 2006). Moreover, a mirror-image heat, but not mechanical, hyperalgesia was also identified in an area on the contralateral rat hind paw symmetrical to the injected side (Chen et al., 1999b, 2000, 2001, 2003; Chen and Chen, 2000). This is an important clinical phenomenon referred to as a mirror-image pain that occurs on the side of the body contralateral to the injured side (Berman, 1995; Bingel et al., 2007; Cook et al., 2007; Schweinhardt et al., 2006; Tracey, 2008). It is interesting to note that the time course of hyperalgesia to thermal and mechanical stimulus modalities in the primary injury area is different. Primary mechanical hyperalgesia lasts more than 96 h, while primary heat hyperalgesia lasts 48–72 h, implicating distinct underlying mechanisms across stimulus modalities that have been well established later by pharmacological investigations (Chen, 2003, 2007, 2008). Moreover, secondary and mirror-image heat hyperalgesia likely share similar properties that are also different from primary heat and mechanical hyperalgesia (Chen, 2003, 2007, 2008).

Taken together, there are at least five symptomatic 'phenotypes' of bee venom-induced nociception and pain hypersensitivity, including: (1) spontaneous persistent nociception or spontaneous tonic pain-related behavioral response; (2) primary heat or thermal hyperalgesia; (3) primary mechanical hyperalgesia; (4) secondary heat or thermal hyperalgesia; (5) mirror-image heat hyperalgesia (Table 2). These symptomatic features of bee venom-induced spontaneous nociception and pain hypersensitivity reflect several complex characteristics of clinical pathological pain states that make it a useful animal model of pain for studies unraveling the underlying molecular and cellular mechanisms of the transition from early processing to chronicity of pain. These bee venom-induced nociception and pain hypersensitivity phenotypes are also unique due to its relevant chemical constituents. A comparative study of the nociceptive effects of six venoms from the honeybee, bumble bee, yellow jacket and paper wasps, and yellow and white faced hornets on Sprague-Dawley albino rats showed that honey bee venom was the most potent to produce spontaneous persistent nociception (Lariviere and Melzack, 2000).

Bee venom-induced local inflammatory responses were also scored by measurement of increased volume of the injected paw (Lariviere and Melzack, 1996). It was shown that edema developed immediately and reached a maximal plateau at about 15–20 min after injection. The time course of edema outlasted the spontaneous pain-related behaviors and gradually disappeared by 48 h after bee venom injection. The precise relationships between the degrees of nociception and inflammation or between the degrees of pain hypersensitivity and inflammation were quantitatively analyzed in both outbred rats and inbred strains of mice following subcutaneous injection of bee venom (Lariviere et al., 2005). Bee venom-induced edema was found to be highly correlated with spontaneous nociception (paw flinching, licking and lifting), but not with thermal and mechanical hyperalgesia. Indomethacin, an NSAID, could effectively inhibit edema and spontaneous nociception dose-dependently, but could affect hyperalgesia only at the highest dose tested. This correlative analysis of the relationship between inflammation and nociception or hyperalgesia might have therapeutic implications. Namely, effective blockade of local

inflammatory responses may be an indication of effective treatment of spontaneous nociception, but may not serve as an index of effective relief of pain hypersensitivity or hyperalgesia.

It should be proclaimed that no overt necrosis developed in response to bee venom injection and no rat showed signs of an allergic reaction, even when re-tested within 1 month after the first testing (Lariviere and Melzack, 1996).

3.2.2. Nociceptive and inflammatory effects of bee venom peptide constituents

Although earlier studies in the 1950s–1970s did test some pharmacological and toxicological properties of individual components isolated and purified from bee venom, nociceptive or algogenic effects were rarely studied, and not systematically meeting current standards of design and pain measurements. Moreover, the bee venom-induced spontaneous nociceptive paw flinches in mammals is unique and characterized by tonic and long-term sustained paw flinching behaviors lasting 1–2 h, suggesting the existence of unique chemical substances in bee venom responsible for the effects observed. As introduced above, bee venom is a complex composition of more than 20 constituents or ingredients. Although some of the amines found in bee venom and endogenous inflammatory or proinflammatory mediators potentially released by bee venom insult have been demonstrated to be allergens by application to a clantharidin blister base in the human blister test (Armstrong et al., 1951, 1952a,b, 1953, 1954, 1955, 1957; Keele, 1957, 1967; Bleehen et al., 1976), animal studies of subcutaneous injection of several of these compounds did not reveal any long-lasting tonic paw flinching behaviors (Hong and Abbott, 1994; Wheeler-Aceto et al., 1990). Therefore, due to their uniqueness in both chemical and biological properties, polypeptide constituents of venom of *Apis mellifera* are causal candidates of the long-lasting nociception.

To identify the biologically active components of honeybee venom producing inflammation and pain-related behaviors, four major polypeptides including melittin, apamin, MCD peptide and a novel PLA₂-related peptide were separated, purified and structurally identified from whole dried bee venom (Chen et al., 2006b). In animal studies, it was revealed that all four of the polypeptides could produce marked local inflammatory responses (edema) when measured 1 h after subcutaneous injection. However, the nociceptive and hyperalgesic effects differed from substance to substance. Among the four polypeptides tested subcutaneously, melittin, MCD peptide and PLA₂-related peptide were able to produce distinct nociceptive paw flinches; however, only melittin caused pain-related behaviors lasting for nearly 1 h. Because all three polypeptides induce nociception that peaks shortly after injection, it was proposed that they activate nociceptors directly rather than indirectly through the release of proinflammatory or inflammatory mediators and/or endogenous algogenic substances (Chen et al., 2006b). Upon examining the hyperalgesic effects of the four polypeptides, only melittin and apamin were shown to result in heat as well as mechanical hyperalgesia at the primary injury site, and only melittin-induced primary mechanical hyperalgesia that lasted over 48 h. Among the components of bee venom, melittin was, for the first time, demonstrated to be the major polypeptide responsible for the prolonged painful stimulation of bee venom injection, leading to both tonic nociception and hypersensitivity, while the other polypeptides contribute only to the early nociceptive responses within 10–20 min after injection.

Because melittin constitutes about 40–60% of dried whole bee venom, a pairwise study on the dose-effect of bee venom and melittin was conducted in rats. Three doses of melittin (5, 25 and 50 µg) were injected, corresponding to half of the amount of bee venom injected in other rats (10 µg, 50 µg, and 100 µg). The melittin-induced number of rat paw flinches was surprisingly and

particularly similar to the number induced by bee venom during the late period 20–60 min after injection, but less than the bee venom-induced paw flinches during the early period 0–19 min after injection. Moreover, as described above for whole bee venom injection (Lariviere et al., 2005), the melittin-induced spontaneous nociceptive response was dose-dependent and highly correlated with the dose-dependent effect on local inflammatory responses, but not with hyperalgesia to either heat or mechanical stimuli applied at the primary injury site (Li and Chen, 2004; Chen et al., 2006b). It was also surprisingly revealed that melittin-induced nociceptive responses could be partially inhibited by both pre- and post-treatment with capsazepine (CPZ), a potent antagonist of the capsaicin receptor (TRPV1), suggesting involvement of this molecular target in melittin-induced nociception (Chen et al., 2006b). This presumption was further supported by the results from investigation of the anti-hyperalgesic effects of CPZ on melittin-induced primary heat and mechanical hyperalgesia. CPZ reversed the melittin-induced primary heat hyperalgesia, but had no effect on the melittin-induced primary mechanical hyperalgesia, implicating an activation of TRPV1 by melittin that specifically contributes to persistent nociception and primary heat hyperalgesia (Chen et al., 2006b) (for more see below).

Altogether, it becomes clear that during the symphony of bee venom-produced inflammatory pain and hypersensitivity, different components of bee venom play different roles in the entire processing taking place; however, melittin is likely to play a central role in the production of the long-lasting pain, hyperalgesia and local inflammation following honeybee sting in mammals. Whereas early reports suggested that melittin had only non-specific biological functions, the specific biological and pharmacological actions recently reported and the molecular targets of melittin warrant it being re-evaluated with modern biological techniques.

3.2.3. Genetic studies of bee venom-induced nociception

Studies of heritable variability of responses to bee venom injection have been performed and provide further evidence that the phenotypes of bee venom-induced spontaneous nociception versus primary thermal hyperalgesia and mirror-image (contralateral) thermal hyperalgesia are distinct (Lariviere et al., 2002). Using genetic correlation analysis, in which the sensitivity of a panel of standard inbred strains of mice such as C57BL/6J, 129P3/J, and DBA/2J, is compared across traits, one can determine whether the traits have common or distinct underlying genetic mechanisms (Hegmann and Possidente, 1981; Crabbe et al., 1990) even prior to the determination of the precise mechanisms. Twelve standard inbred strains of mice were injected with 50 µg of bee venom and the duration of spontaneous nociceptive behavior of licking of the injected paw was recorded for one hour after injection. From 2 h to 4 h after injection, thermal hypersensitivity was measured in both ipsilateral and contralateral hind paws as decreases in latency to withdraw the paw from a focused hot light beam. Strain means for each of the traits were calculated and 'genetically correlated' with those for 19 other models of nociception and hypersensitivity commonly used in rodents (Lariviere et al., 2002). As for other pain models, marked significant strain differences were observed for all responses to bee venom injection: a 13-fold range of strain means was observed for spontaneous nociception with 37–489 s spent paw licking in the first hour; 30–82% thermal hyperalgesia in the injected paw was observed among strains; and in the contralateral paw, thermal hyperalgesia ranged from none to 70% among strains (Lariviere et al., 2002).

Sensitivity to bee venom-induced spontaneous nociceptive licking behavior was significantly correlated (before Bonferroni correction) with sensitivity to other models of chemical irritant- or inflammation-induced spontaneous nociception including both

first and second phases of the formalin inflammatory pain response (Spearman rank correlation, $r_s = 0.68$ – 0.74) and abdominal constriction evoked by intraperitoneal injection of dilute acetic acid or magnesium sulfate ($r_s = 0.61$ – 0.73). Bee venom-induced licking was less, and not significantly, correlated with capsaicin-induced spontaneous nociceptive behaviors of licking of the injected hind paw ($r_s = 0.51$) (Lariviere et al., 2002), perhaps due to the differences between capsaicin and bee venom-induced pain types discussed above. Thus, strains that are sensitive to the spontaneous nociceptive effects of bee venom are also sensitive to other spontaneous inflammatory nociception assays. Because high positive correlations between traits indicate that the traits have common genetic regulatory mechanisms (Hegmann and Possidente, 1981; Crabbe et al., 1990), these results suggest that studies of the genetic mechanisms of heritable variation in spontaneous inflammatory nociception evoked by bee venom injection will produce results of relevance to several spontaneous inflammatory nociception models and the clinical pains they model.

Further distinguishing the phenotypes of bee venom-induced spontaneous nociception and primary and mirror-image (contralateral) thermal hyperalgesia is the finding that sensitivity to bee venom-evoked spontaneous nociception is not genetically correlated with sensitivity to bee venom-induced thermal hypersensitivity either ipsilateral ($r_s = 0.22$) or contralateral ($r_s = 0.40$) to the injection. In addition, bee venom-induced spontaneous nociception and primary and mirror-image (contralateral) thermal hyperalgesia are also not genetically correlated with thermal hypersensitivity induced by subcutaneous injection of other inflammatory irritants including capsaicin ($r_s = -0.09$ to 0.08) or the seaweed extract carrageenan ($r_s = -0.75$ to -0.15). Because the genetic correlations with capsaicin-induced thermal hyperalgesia are close to zero, and thus dependent on distinct genetic mechanisms, again, the pain-relevant effects of bee venom and capsaicin are further distinguished (also see rat, Table 2).

Although the precise genetic mechanisms responsible for the differences in sensitivity of various rodent strains are not yet known, the expression of several genes has been demonstrated to change in specific tissues following injection or exposure to bee venom. In the dorsal root ganglion (DRG) of the rat, where the cell bodies of peripheral afferent neurons that innervate the injected site are located, expression of mRNA of several serotonin (5-HT) receptors is increased including of 5-HT1A, 5-HT1B, 5-HT2A and 5-HT3 at both 1 h and 4 h after treatment (Liu et al., 2005). mRNA levels of 5-HT2C, 5-HT4, 5-HT6 and 5-HT7 are significantly increased in the DRG at 4 h after bee venom injection, whereas levels of 5-HT1D, 5-HT1F, 5-HT5A remain unchanged (Liu et al., 2005). In the dorsal horn of the spinal cord, especially in laminae I–II and IV–VI, expression of c-Fos protein and presumably its mRNA is increased by peripheral subcutaneous bee venom injection. Similarly, expression of 5-HT1A mRNA and its protein, serotonin 1A receptors is also increased in the ipsilateral lumbar spinal cord by bee venom (Wang et al., 2003b). Spinal administration of 5-HT1A antisense oligonucleotides to block its transcription shows that expression of 5-HT1A contributes specifically to spontaneous nociception and primary heat hyperalgesia, but not to mechanical hypersensitivity (Wang et al., 2003b). These results are discussed in greater detail below.

In addition to having direct effects on neurons, bee venom can also produce its painful consequences via immune cells resident at, or that migrate to, the site of injection. *In vitro* studies have demonstrated that macrophage, fibroblast or synoviocyte cell cultures exposed to bee venom or melittin exhibit increased expression of genes of pro-inflammatory proteins. Cultured RAW264.7 macrophage cells exposed to bee venom in the medium show upregulation of pro-inflammatory genes including IL-1 β encoding for interleukin (IL) 1 beta, Cxcl2 for the chemokine CXC

motif ligand 2 and Ccl7 for chemokine CC motif ligand 7 as determined with genome-wide microarray analysis (Illumina Sentrix Mouse-6 Expression Beadchips) (Jang et al., 2009). See below in Section 4.3.1 for more detailed discussion of these results. Stuhlmeier (2007) demonstrated with real-time polymerase chain reaction (RT-PCR) that in cultured human fibroblast-like synoviocytes from rheumatoid arthritis patients and dermal fibroblasts and mononuclear cells from healthy individuals that bee venom and melittin significantly increases mRNA levels of pro-inflammatory genes including COX-2, TNF- α and IL-8. Thus, immune cells can also contribute to the spontaneous nociception and hypersensitivity evoked by subcutaneous bee venom injection.

3.3. Neural mechanisms of bee venom-induced nociception

3.3.1. Neurochemical substrates

Given that melittin and other algogenic components of bee venom are able to activate nociceptors upon diffusion to peripheral free nerve endings when injected subcutaneously, action potentials (pain 'signals') should be generated and conducted anterogradely to the central terminals of nociceptor cells whose soma are located in the primary sensory ganglia (e.g., DRG), leading to release of neurotransmitters at the dorsal horn of the spinal cord (Willis and Coggeshall, 2004). Moreover, in response to long-term release of neurotransmitters the neuron in the dorsal horn of the spinal cord, the first relay station of the pain ascending pathways, should be activated.

To test this hypothesis, Chen and his colleagues measured release of both excitatory amino acids (EAAs) and inhibitory amino acids (IAAs) and other amino acids involved in recycling of metabolites of those neurotransmitters (Yan et al., 2009). It is well known that glutamate and aspartate are important excitatory neurotransmitters used by the primary afferent to affect the spinal dorsal horn in addition to neuropeptides such as substance P and calcitonin-gene related peptide (CGRP) (Willis and Coggeshall, 2004; McMahon and Koltzenburg, 2006). Correspondingly, glycine and γ -aminobutyric acid (GABA) are important inhibitory neurotransmitters used by local inhibitory interneurons in the dorsal horn acting against over-excitability of the dorsal horn (Willis and Coggeshall, 2004; McMahon and Koltzenburg, 2006). Thus, simultaneous monitoring of both EAAs and IAAs at the spinal level in awake animals experiencing painful stimulation is of particular importance for the understanding of the basic neurotransmitters mediating long-lasting pain. As the first relay station of ascending pain pathways, the dorsal horn neurons can be expected to be activated or sensitized by peripheral painful stimulation. To explore spatiotemporal changes of neural networks (neuronal populations or ensembles), spatial distribution and the time course of expression of c-Fos protein, a distinct biomarker of neuronal activities, were also studied (Luo et al., 1998). These experiments are basic but very important for informing the next stage of enquiry of electrophysiological recordings of the neuronal activities.

3.3.1.1. Spinal release of excitatory and inhibitory amino acids. To test the biological action of bee venom constituents, intrathecal catheterization was performed to place the catheter tip at the level of lumbar segment 4–5 of the spinal cord. Cerebrospinal fluid (CSF) samples were collected by microdialysis in conscious rats every 20 min for a period of 120 min when spontaneous nociceptive responses were induced by subcutaneous bee venom injection (Yan et al., 2009). At least 9 amino acids including excitatory (e.g., glutamate, aspartate), inhibitory (glycine, GABA, taurine) and metabolites (glutamine, alanine, arginine, and threonine) were analyzed by high-pressure liquid chromatography (HPLC). The results showed an immediate increase in the concentration of all

the nine amino acids at 20 min; however, in the remaining period of 100 min, EAAs remained above the baseline levels, while IAAs soon decreased to the baseline levels and thereafter to a level much lower than the baseline for the remaining time. This result provided for the first time a line of evidence showing an imbalance between EAAs and IAAs at the spinal cord that was associated with maintenance of persistent pain-related behaviors. This presumption was further demonstrated by the results of a pre-infiltration with bupivacaine at the bee venom injection site. Following injury site blockade, sustained decreases in bee venom-induced EAAs and IAAs responses at the spinal cord led to disappearance of persistent nociceptive behavioral responses. This result provided a new line of evidence supporting the idea that nociceptive or pain-related behaviors are mediated by ongoing activation of primary nociceptors that use glutamate and aspartate as neurotransmitters in the central terminals projecting to the dorsal horn of the spinal cord (Yan et al., 2009).

Based upon the dynamic changes in EAAs and IAAs in the spinal cord, hypothetical speculation can be formed to explain why nociceptive paw flinching responses can be kept in a tonic lasting state. On one hand, neural activities for both excitatory and inhibitory components at the dorsal horn of the spinal cord are driven by ongoing peripheral input and a homeostatic state can be maintained by a dynamic balance between EAAs and IAAs levels due to an unknown pre-synaptic regulating mechanism. However, on the other hand, under an inflammatory pain state, the spinal homeostatic state will be disrupted due to dramatic increases in long-lasting ongoing input that results in an imbalance between spinal EAAs and IAAs levels probably due to loss of tonic inhibition by glycine and GABA. Although it is still unclear mechanistically, the result at least reminds us of two clinic significances: (1) peripheral nerve block or radiofrequency disruption of primary sensory neurons (DRG or trigeminal ganglia) is a concept-proved therapeutic technique that can be effective pre-, during, and post-pain occurrence due to direct blockade of neurotransmitters release at the spinal level; (2) given that EAAs-IAAs imbalance is more important underlying mechanisms of persistence or chronicity of pain, more attention should be paid to novel therapeutics and medications being able to restore EAAs-IAAs balance. The latter remains to be further confirmed by more pre-clinical studies and clinical trials.

3.3.1.2. Spatiotemporal properties of dorsal horn neural activities. *c-Fos* protein, an immediate early proto-oncogene protein, is widely used as a biomarker of spinal dorsal horn neuronal activities (Harris, 1998; Coggeshall, 2005). It has been clearly shown that neuronal activities associated with nociception in the dorsal horn of the spinal cord can be localized mainly in superficial layers (laminae I–II) and deep layers (laminae IV–VI), but not in lamina III (Hunt et al., 1987). Laminae I–II and IV–VI contain pain-related neurons that receive input from primary nociceptive afferents (for detail see Willis and Coggeshall, 2004). Thus, immunocytochemical localization of *c-Fos*-like immunoreactivity after subcutaneous bee venom injection can reflect spatiotemporal characteristics of the dorsal horn functional state. As shown in a study published by Chen and colleagues (Luo et al., 1998), expression of *c-Fos* protein began to be localized within laminae I–II of the dorsal horn 30 min after bee venom injection into the ipsilateral hind paw, the spatial range of neuronal activities was enlarged with a parallel increase in number of *c-Fos*-positive neurons in both superficial and deep layers after 1 h and reached a peak level at 2 h after bee venom injection. The number of *c-Fos*-positive neurons began to decline in both superficial layers and deep layers at 4 h and completely disappeared at 96 h after bee venom insult. In comparison with the time course of behavioral nociceptive responses and hyperalgesia induced by bee venom, it is likely that *c-Fos* expression reflects

establishment of a sensitized state at the spinal dorsal horn which requires at least 30 min of persistent primary afferent input to the central site (Wu et al., 2002). The sensitized state of the spinal dorsal horn that may be driven by peripheral nociceptor sensitization is responsible for the development and maintenance of hyperalgesia that disappears with the disappearance of spinal *c-Fos* expression (Luo et al., 1998; Chen et al., 1999b; Chen and Chen, 2000). It should be noted that there was clearly increased *c-Fos* localization in superficial and deep layers of the dorsal horn contralateral to the injection side, reflecting the neuronal basis for the development of mirror-image heat hyperalgesia. The underlying neural mechanisms of mirror-image heat hyperalgesia observed in the bee venom test have been well studied and will be described in detail below.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases found in a variety of cells, transducing a broad range of extracellular stimuli into diverse intracellular responses by producing changes in transcriptional modulations of key genes as well as posttranslational modifications of target proteins (Ji, 2004a,b; Ji et al., 2009). There are three main MAPKs family members in mammalian cells: extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinase (JNK), which are primarily demonstrated to conduct different signal transduction and exert various biological functions such as cell proliferation and differentiation, cytokine production during development and cell death induced by a variety of stress stimuli (Widmann et al., 1999; Ji, 2004a,b; Ji et al., 2009). In the past decade, several studies in rodents have elucidated the roles of ERKs, p38 and JNK in generating nociceptive sensitivity and neural plasticity in the pain sensory system, especially in the dorsal horn of the spinal cord (Ji, 2004a,b; Ji et al., 2009). Temporal and spatial features of activated MAPK subtypes ERK and p38 in the spinal dorsal horn in response to subcutaneous bee venom injection were examined using immunocytochemical staining in rats (Cui et al., 2008).

Subcutaneous injection of bee venom into one hind paw of rats results in very quick phosphorylation of ERKs in superficial layer neuronal cell bodies within 2 min that gradually declines within 1 day; however, phosphorylation of p38 MAPK in the superficial layer neurons began 1 h later than ERKs, reached peak at about 2 h and was maintained active until the end of 7 days of observation. There were very few ERK- and p38-labeled neurons observed in the deep layers of the dorsal horn, suggesting that only neurons in the superficial layers use these two types of MAPKs in response to bee venom injection. A dramatic phenomenon was that phosphorylated p38 MAPK began to be localized in microglia across laminae III–IV 1 day after bee venom challenge and reached peak on the 3rd day when bee venom-induced pain hypersensitivity almost disappeared in behavioral observations, implicating appearance of microglia with p38 phosphorylation in the dorsal horn is not likely to be involved in maintenance of pain and hyperalgesia, instead, probably as a scavenger to remove 'dead' cells and repair injury. Throughout the observation period, there was neither ERKs nor p38 MAPK detected in astrocytic cells, suggesting that astrocytes may use other protein kinases in the dorsal horn in response to peripheral injury.

Actually, there are region-related differences in distribution between different isoforms of a given subtype of MAPKs along the somatosensory system (Guo et al., 2007; Liu et al., 2007). For example, under normal state ERK1 and JNK 54 were highly expressed in the spinal dorsal horn with very low level of ERK2 and JNK 46 signal, however, in the primary somatosensory cortex (S1 area) or the hippocampal formation ERK2 and JNK 46 were highly expressed. However, under bee venom-induced inflammatory pain state, ERK2 and JNK 46 phosphorylation were distinctly increased in the dorsal horn, suggesting spinal ERK1 and JNK 54 are

constitutive isoforms, whereas ERK2 and JNK 46 are inducible by peripheral nociception. Thus, targeting inducible isoforms of MAPK subtypes might be more useful in screening novel therapeutic drugs.

TRPV1, also known as the capsaicin receptor and proton-activated ion channel, is well known to be a thermal nociceptor transducing nociceptive heat (>42 °C) stimuli to a series of pain 'signals' (action potentials) at the peripheral terminals of a population of primary nociceptive sensory neurons that further conduct thermal nociceptive information to the dorsal horn of the spinal cord (Caterina et al., 1997, 2000; Tominaga et al., 1998; Caterina and Julius, 1999, 2001). It is interesting to note that TRPV1 immunoreactivity is densely distributed in the superficial layers of the dorsal horn. The exact functions of spinal TRPV1 are still unclear and remain to be studied. The level of TRPV1-like immunoreactivities in the dorsal horn was thus examined by immunocytochemical staining and the results showed that the intensity of TRPV1-containing profiles was significantly increased in response to subcutaneous bee venom injection in rats of young (2–3 months), middle (17–19 months) and old (24–26 months) ages (Li et al., 2004a). Moreover, in comparison with the development of primary heat and mechanical hyperalgesia induced by bee venom, high level expression of TRPV1 in the dorsal horn was revealed to be highly associated with the development of heat hyperalgesia, but not mechanical hyperalgesia (Li et al., 2004a). This is consistent with the significant blockade of primary thermal hyperalgesia without affecting primary mechanical hyperalgesia by local injection of pharmacological TRPV1 receptor antagonists in the periphery (Chen et al., 2006b).

Serotonin (5-hydroxytryptamine, 5-HT) is well known to be an important neuromodulator in the CNS; however, its modulatory actions on pain or nociception are bidirectional depending upon various subtypes of 5-HT receptors (Lopez-Garcia, 2006). It has been shown that 5-HT_{1A} receptor is one of the major subtypes distributed in the dorsal horn of the spinal cord (Lopez-Garcia, 2006). As for the functions of 5-HT_{1A} in the spinal cord dorsal horn, some reports suggest its involvement in mediation of nociception (Zhang et al., 2001; Lindstrom et al., 2009), while others argued for an involvement in anti-nociception of a descending modulatory pathway (Lin et al., 1996; Liu et al., 2002; Bardin et al., 2005; You et al., 2005; Colpaert et al., 2006). By using RT-PCR and Western blot, it was observed that both mRNA and protein levels of 5-HT_{1A} receptors in the ipsilateral dorsal horn could be increased by subcutaneous bee venom injection. Antisense oligodeoxynucleotide knockdown of spinal 5-HT_{1A} receptor expression inhibited the bee venom-induced persistent nociceptive responses as well as primary heat hyperalgesia, but without affecting primary mechanical hyperalgesia, suggesting an involvement of 5-HT in the mediation of bee venom-induced pain and thermal hyperalgesia through spinal 5-HT_{1A} receptors (Wang et al., 2003b). Further examination of mRNA levels of various 5-HT receptors in the DRG showed that 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT₃ were increased by subcutaneous bee venom injection at both 1 and 4 h after treatment (Liu et al., 2005). Moreover, mRNA levels of 5-HT_{2C}, 5-HT₄, 5-HT₆ and 5-HT₇ were significantly increased later at 4 h after bee venom insult, whereas levels of 5-HT_{1D}, 5-HT_{1F}, 5-HT_{5A} remained unchanged and 5-HT_{1E}, 5-HT_{2B}, 5-HT_{5B} were not detected in the DRG (Liu et al., 2005). A careful examination of additional subtypes of 5-HT receptors in the dorsal horn in response to bee venom insult is also expected to reveal further information on the precise role of the 5-HT system.

In summary, subcutaneous injection of bee venom indeed induces long-term activation of spinal dorsal horn neuronal activities that expands from superficial layers to the deep layers in a synchronized way. As a response to the peripheral ongoing

input induced by bee venom, ERKs, a subtype of MAPKs, are quickly phosphorylated and peaked at 2 min in the superficial neurons, followed by appearance of p38 MAPK with peak timing at 2 h in the superficial neurons as well. In contrast, activation of microglia is much later than activation of neurons and phosphorylation of microglial p38 MAPK peaks on the 3rd day after bee venom injection. The modulation of bee venom-induced nociceptive processes at the spinal dorsal horn is likely more complex in nature than previously thought and needs to be systematically studied further.

3.3.2. Electrophysiological recordings along pain pathways

3.3.2.1. In vivo electrophysiology. The spinal cord dorsal horn is structurally and functionally involved as a first synaptic relay in the mediation of nociceptive information from the periphery. There are at least three classes of neurons serving as transducer, encoder and probably filter of various information. The functional classification of dorsal horn neurons is based upon their neuronal response characteristics to natural mechanical stimuli applied to their cutaneous receptive fields (cRF): class 1 (also known as low-threshold mechanoreceptive, LTM) neurons are driven mostly by innocuous stimulation and located mainly in laminae III–IV; class 2 (also known as multireceptive or wide-dynamic-range, WDR) neurons are driven by noxious as well as non-noxious stimulation and located mainly within lamina V; class 3 (also known as nociceptive specific, NS) neurons are driven only by noxious stimulation and located mainly in lamina I (Willis and Coggeshall, 2004). Among the three classes of dorsal horn neurons, there are reliable lines of evidence showing that the majority of dorsal horn class 2 (WDR) neurons, but not class 3 (NS), are intercalated in the circuitry responsible for the nociceptive withdrawal reflex (Schouenborg and Sjolund, 1983; Levinsson et al., 2002; Petersson et al., 2003; Schouenborg, 2004, 2008). Thus, spinal WDR neurons may play very important roles in the mediation of nociceptive responses and hypersensitivity observed in nociceptive behaviors.

The first electrophysiological recording of the response to bee venom injection was made by Chen et al. (1998) on the WDR neurons located mainly within laminae IV–VI of the spinal dorsal horn in anesthetized cats. Similar to what was observed in the behavioral assays, subcutaneous injection of bee venom into the center of the cRF resulted in an immediate increase in neuronal firing that reached peak within 5 s and declined until the end of the 60-min observation period. The bee venom-induced spike discharges of WDR neurons were decreased by morphine and could be completely blocked by application of lidocaine into the sciatic nerve that resulted in blockade of primary afferent input. In that study, responsiveness of spinal WDR neurons to both non-nociceptive (brush, pressure as well as von Frey filaments with bending forces of 0.80 g and 5.00 g) and nociceptive (pinch as well as a von Frey filament with bending force of 20.0 g) mechanical stimuli was also significantly enhanced by bee venom injection 1–2 h after treatment, suggesting that spinal WDR neurons that mediate and encode spinal nociceptive reflex are sensitized by peripheral bee venom injection. Because the response pattern and time course of spinal WDR neuronal activities are quite similar to those of behavioral manifestation induced by subcutaneous bee venom injection and blockade of altered spinal neuronal activities occurs in parallel with disappearance of nociceptive behaviors with many pharmacological investigations (see below), it is rational to believe that the sensitized dorsal horn WDR neurons play a key role in the mediation of paw withdrawal reflex facilitation, displayed as persistent paw flinches and hyperalgesia.

Recordings made of the same class of neurons in the anesthetized rats showed similar altered neuronal responses in the dorsal horn in response to bee venom treatment (You and Chen,

1999; Zheng et al., 2002, 2004a,b). In an excellent study of injection of either bee venom or melittin in the cRF, both the spontaneous spike discharge intensity and the period of time course in spinal WDR neurons recorded were dose-dependent as shown in behavioral observations in conscious rats (Li and Chen, 2004). In the same study, a clear leftward-shift of stimulus-responsiveness functional curves was caused by subcutaneous injection of both bee venom and melittin in comparison with baseline. The stimulus intensities tested for thermal nociception were radiant heat raising from 35 °C baseline body temperature to a series of suprathreshold temperatures as 42 °C, 45 °C, 47 °C or 49 °C, while those for non-noxious mechanical sensation stimuli are brush and pressure, and that for mechanical nociception noxious pinch was used. Both thermally nociceptive and mechanically nociceptive responsiveness of spinal WDR neurons were significantly enhanced by bee venom and melittin measured during the period of 1–4 h after subcutaneous injection. Meanwhile, the mechanically non-nociceptive response was also enhanced. To demonstrate the roles of spinal dorsal horn WDR neurons in the mediation of bee venom-induced withdrawal reflex facilitation, simultaneous pairwise recordings were also made in both WDR neurons and single motor units (SMU) along the spinally organized nociceptive reflex circuitry (You et al., 2003a). The result showed an immediate parallel increase in spike discharges of the paired WDR neuron and SMU that lasted for 1 h in response to injection of bee venom in their common cRF. These paired recordings also showed a parallel enhancement of mechanically nociceptive responsiveness as well as wind-up responses 1–2 h after bee venom treatment (Chen, 2003). These data reliably demonstrate the neuronal basis underlying thermal and mechanical hyperalgesia and allodynia observed in behavioral studies.

Responses of dorsal horn nociceptive neurons within layers VI–VII were also studied in anesthetized rats (You et al., 2008). In contrast to the long-term response patterns of WDR neurons, subcutaneous bee venom injection evoked a short-term (<10 min) firing of this population of neurons. However, the neurons were switched to give a long-term biphasic firing with an early phase (4–13 min) that was followed by a late tonic firing (28–74 min) after a quiescent period (4–11 min) in spinalized rats. The response pattern and time course of WDR neurons were less affected in spinalized rats. The data is interesting and suggests the existence of two separate modulatory systems in the spinal dorsal horn. WDR neurons that serve as encoders of spinal nociceptive withdrawal reflex are not likely tonically controlled by descending anti-nociceptive systems, while the nociceptive dorsal horn neurons within more deep layers (VI–VII) that send nociceptive information along the spino-reticulo-thalamic tract to the medial thalamus are likely tonically controlled by a descending anti-nociceptive system. The response characteristic of lamina I NS neurons to subcutaneous bee venom injection remains uninvestigated and thus the precise role of NS neurons remains to be determined.

In summary, *in vivo* electrophysiological recordings of neurons in the dorsal horn of the spinal cord in both cats and rats revealed similar results of a centrally sensitized state following subcutaneous bee venom injection that is responsible for animal paw withdrawal reflex facilitation and altered pain sensation such as hyperalgesia and allodynia.

3.3.2.2. *In vitro* electrophysiology. To address the role of activation and sensitization of peripheral afferent neurons underlying bee venom-induced nociception and hyperalgesia, *in vitro* whole cell patch-clamp recordings of primary sensory neurons acutely dissociated from DRG of rats are being undertaken by direct application of bee venom peptide constituents, e.g., melittin. Use of this technique has several advantages: (1) the experimental conditions including intra-electrode and extracellular solutions

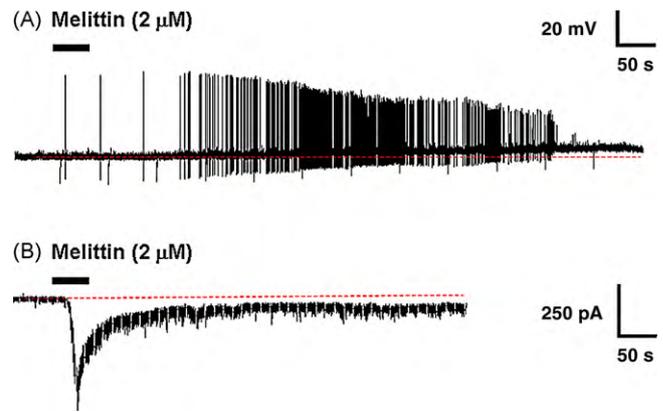


Fig. 2. Activation of dorsal root ganglion (DRG) cells in response to direct application of melittin. (A) Whole cell recording of a DRG cell in response to 2 M melittin under current-clamp. Following 50 s melittin perfusion, the cell is evoked to fire immediately with occasional action potentials that is followed by a tonic period of firing for more than 10 min. Onset latency is 26 s. (B) Whole cell recording of another DRG cell in response to 2 M melittin under voltage-clamp ($V_h = -70$ mV). Following 50 s melittin perfusion, the cell is evoked to produce an inward current with a slow decay time. The onset latency is 20 s. The membrane potentials: cell in A = -60 mV, cell in B = -61 mV.

can be well controlled; (2) endogenous proinflammatory mediators and algogens released from mast cell degranulation and tissue damage following melittin injection *in vivo* can be completely excluded; (3) the samples can be easily prepared and the recordings can be more easily repeated than *in vivo* single fiber recordings; (4) the action potentials and currents evoked by a given reagent can be recorded in alternation which is useful for further pharmacological investigations; (5) a simultaneous Ca^{2+} image and voltage-patch recordings for currents can be reached. Fig. 2 is an unpublished example for whole cell recordings of dissociated DRG cells under current-clamp and voltage-clamp ($V_h = -70$ mV). The data clearly shows a direct activation of the DRG cells to application of 2 μ M melittin in a 50 s period of infiltration (Fig. 2A). The cell began to discharge immediately with action potentials in response to 50 s application of melittin; however, the firing became tonic after a delay of about 100 s when melittin-evoked inward current was almost completely decayed (Fig. 2B). The phenomena are of particular interest because melittin has long been known to be a pore-forming peptide across phospholipids bilayers (Williams and Bell, 1972; Tosteson and Tosteson, 1981; Tosteson et al., 1990; Schwarz et al., 1992; Smith et al., 1994; Fattal et al., 1994; Matsuzaki et al., 1997; Bechinger, 1997; Chen et al., 2007b,c; Klocek et al., 2009). Melittin has also been used as an activator of PLA_2 due to its enhancing effects on the bee venom PLA_2 activities (Mollay and Kreil, 1974; Hassid and Levine, 1977; Nishiya, 1991; Vernon and Bell, 1992; Sharma, 1993). However, in our ongoing experiments, the melittin-evoked inward currents or action potential discharges could only be recorded in 60–70% of the cells studied. Moreover, co-application of capsazepine, a selective capsaicin receptor (TRPV1) antagonist, and A-317491, a potent P2X3/P2X2/3 receptor antagonist, could completely or partially block the occurrence of the melittin-induced inward currents, suggesting a selective action rather than non-selective pore-forming effects (unpublished data). The hypothesis is strongly supported by further results showing that an inhibitor of PLA_2 , but not of PLC, was effective in blockade of melittin-evoked inward current. It was revealed that inhibition of lipoxygenases (LOXs), but not COX-1/2, was also effective. Surprisingly, the cellular data are strongly supported by behavioral pharmacology of the same drugs. It is interesting that a selective activation of the signaling pathway PLA_2 -LOX-metabolites in the DRG cells is likely to be involved in melittin-evoked current that is

probably mediated by activation of TRPV1 via LOX metabolites (unpublished data of Chen's lab). These unpublished data may be of great importance since they challenge a long-standing idea about the biological actions of melittin.

3.3.3. Pharmacological studies of bee venom-induced nociception and hyperalgesia

In the past ten years or more, the pharmacology of the bee venom-induced behavioral nociceptive responses and various symptomatic 'phenotypes' of hyperalgesia and allodynia have been well studied at both the spinal level and the peripheral injury site (Chen, 2003, 2007, 2008). These works were mostly based upon two main ideas: one is specifically associated with an interest in looking at what spinal or peripheral signaling molecules are involved in the mediation of the bee venom-induced nociception and hypersensitivity; the other is generally associated with clinical therapeutic purpose of screening novel symptomatically relevant molecular targets for relief of pain and of validating the bee venom test as an animal model of pathological pain for evaluation of existing or novel drugs that have potential to relieve persistent or chronic pain (for detail see Chen, 2007, 2008). To examine the roles of a given signal transduction molecule in the induction and maintenance of persistent nociceptive responses as well as hyperalgesia, a drug to be evaluated should be administered prior to (pre-treatment) or after full establishment of the pain state (post-treatment). Moreover, because spinal or peripheral nerve blockade is still the most effective way to achieve relief of pain in clinic, the route of intrathecal or subcutaneous administration of a drug was selected to permit the study of the spinal or peripheral pharmacology of the bee venom-induced nociception and hyperalgesia.

3.3.3.1. Glutamate receptors. Antagonism of spinal glutamate receptors: As discussed above, subcutaneous bee venom injection causes a sustained increase in glutamate and aspartate release at the spinal cord dorsal horn (Yan et al., 2009). Thus, it is interesting to see what types of glutamate receptors are activated and involved in bee venom-induced behavioral manifestations and spinal neuronal activities. It is well known that glutamate receptors can be generally divided into two classes: (1) ligand-gated ionotropic glutamate receptors (iGluRs) including NMDA and non-NMDA (AMPA and kainite) subtypes; (2) G-protein coupled metabotropic glutamate receptors (mGluRs) including group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluRs 4, 6, 7, and 8) (Schoepfer et al., 1994; Ozawa et al., 1998). It has been demonstrated that all types of glutamate receptors are distributed in the spinal dorsal horn and involved in mediation of nociception (Ye and Westlund, 1996; Bonnot et al., 1996; Procter et al., 1998; Berthele et al., 1999; Szekely et al., 2002).

In experiments regarding pharmacological examination of the roles of glutamate receptor subtypes in bee venom-induced nociception and hyperalgesia, two major groups of animals were assigned: (1) pre-treatment group, which received i.t. administration of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a competitive non-NMDA receptor antagonist or MK-801, a non-competitive NMDA receptor channel blocker, 5 min prior to bee venom injection; (2) post-treatment group, which received i.t. administration of CNQX or MK-801 either 5 min or 2 h after bee venom injection. Both drugs were effective in suppression of bee venom-induced spontaneous nociceptive responses with either pre- or post-treatment, implicating both NMDA and non-NMDA receptors in the spinal cord mediating the induction and maintenance of tonic nociception (Yan et al., 2009). However, they were not effective in suppressing primary heat and mechanical hyperalgesia with either pre- or post-treatment although secondary and mirror-

image heat hyperalgesia could be inhibited (Chen and Chen, 2000). An earlier report using systemic administration of MK801 also showed it ineffective (Chen and Chen, 2000). Collectively, these results suggest that sufficient amounts of EAAs released to the spinal cord are likely to act on iGluRs to mediate the development and persistence of bee venom-induced tonic pain-related behaviors as well as centrally mediated hyperalgesia (secondary and mirror-image hyperalgesia) (for details see below). The mediating roles of iGluRs in the bee venom-induced sensitized state of the dorsal horn and the efferent effectors SMU were also demonstrated by electrophysiological studies (You et al., 2003b).

To see whether mGluRs are involved in maintenance of primary heat and mechanical hyperalgesia, the same route administration of AIDA (1-aminoindan-1,5-dicarboxylic acid), a group I mGluR antagonist, EGLU ((S)- α -ethylglutamic acid), a group II mGluR antagonist or MSOP ((RS)- α -methylserine-O-phosphate), a group III mGluR antagonist, was applied 2 h after bee venom injection in a separate set of experiments. It was found that i.t. antagonism of different subgroups of mGluRs could effectively block established primary thermal and mechanical hyperalgesia. An important finding of this study was that blockade of spinal group I mGluRs activation by AIDA suppressed primary mechanical hyperalgesia, while inhibition of group II and III mGluRs activity by EGLU and MSOP markedly reduced the extent of primary thermal hyperalgesia. This result suggests that bee venom-evoked primary hyperalgesia is maintained by a centrally sensitized state caused by not only spinal disinhibition but also sustained activation of mGluR subtypes and the sequential signaling cascades (Yan et al., 2009).

Antagonism of peripheral glutamate receptors: The roles of iGluRs including NMDA and non-NMDA receptors at the injury site were also examined by behavioral and electrophysiological assays in cats and rats (Chen et al., 1999a; You et al., 2002; Li et al., 2000a; Luo and Chen, 2000). In cats, peripheral pre-treatment of a single dose of either AP5 (200 μ g/100 μ l), another competitive NMDA receptor antagonist, or CNQX (8.3 μ g/100 μ l) locally in the bee venom injected site equally prevented the dorsal horn WDR neurons from firing; however, for post-treatment only AP5 was effective in suppressing neuron firing (Chen et al., 1999a). In a rat study (You et al., 2002), a single dose of locally applied pre-treatment of AP5 (10 mM, in 50 μ l) or MK-801 (2 mM, in 50 μ l) significantly blocked bee venom-induced dorsal horn WDR neuronal activities; however, only MK-801 was effective with post-treatment. In contrast, neither pre- nor post-treatment with CNQX (5 mM, in 50 μ l) was effective in suppressing neuronal activity. Localized peripheral administration of AP5 with the same dose, but not MK-801 and CNQX, significantly inhibited pressure- and pinch-evoked SMU responses under both normal and bee venom-induced inflammatory states. There is likely a species difference in the pharmacological basis of the roles of peripheral NMDA and non-NMDA receptors in the maintenance of a bee venom-induced centrally sensitized state. However, the difference in dose, volume of the drugs used and the difference in volume of bee venom solution used should be carefully considered. In the cat study, 100 μ l of drug versus 50 μ l of bee venom (0.1–0.2 mg) were used, while in the rat study the volume for both drug and bee venom (0.2 mg) was 50 μ l. As introduced above, bee venom contains hyaluronidase that can degrade tissue matrix and facilitate venom diffusion. Given that a drug can occupy the binding sites on its antagonizing receptors in advance, pre-treatment should be effective in prevention of the bee venom-induced neuronal hypersensitized state if the receptor are involved functionally. In contrast, if the volume of a drug were not large enough the drug could not be quickly brought to the preemptively activated receptors by bee venom, leading to a discrepancy in effectiveness of post-treatment of AP5 and MK-801. The failure in

suppression of bee venom-induced neuronal activities by CNQX in rats might also be a dose problem or volume problem. Borrowing the result of spinal suppressive effects of both MK-801 and CNQX, it could be concluded that both NMDA and non-NMDA glutamate receptors in the peripheral injury site are likely to be activated by bee venom and the release of glutamate might be caused by retrograde dorsal root reflex or axonal reflex (Chen and Chen, 2001; Shin and Kim, 2004; Chen et al., 2006a, 2007a). The involvement of peripheral glutamate receptors in development of primary hyperalgesia is also likely because localized peripheral AP5 administration is effective in suppression of mechanically nociceptive responsiveness enhanced by bee venom (You et al., 2002).

3.3.3.2. Neurokinin receptors. Antagonism of spinal neurokinin receptors: The tachykinin family includes three subtypes: substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) with NK1, NK2 and NK3 as their respective preferred receptors (Regoli and Nantel, 1991; Regoli et al., 1993, 1997). NK1, highly selective to SP, a key member of tachykinin family, has been widely accepted as being involved in nociception (Routh and Helke, 1995). NK2 and NK3, receptors highly selective for NKA and NKB, respectively, are believed not to be involved in nociception under normal physiological states; however, their properties may be changed by peripheral inflammatory states (Xu et al., 1991; Routh and Helke, 1995; Khawaja and Rogers, 1996). Thus, examination of these three types of NK receptors in the spinal cord dorsal horn is necessary to understand the spinal mechanisms of bee venom-induced nociception and hyperalgesia. Intrathecal pre- or post-treatment of spantide ([D-Arg1, D-Trp7, 9, Leu11] substance P), a non-selective antagonist of NK1/2 receptors, or SR14280 ((S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl) piperidin-3-yl) propyl)-4-phenylpiperidin-4-yl)-N-methyl acetamide), a selective NK3 receptor antagonist, was performed in conscious rats (Zheng and Chen, 2001). Pre-treatment with three doses of spantide produced a dose-related suppression of the bee venom-induced flinching reflex compared with the saline control group. Post-treatment of spantide 5 min after bee venom injection also produced a distinct suppression of the paw flinching reflex. Moreover, pre-treatment with the drug partially prevented the primary and secondary thermal hyperalgesia from occurring, while it did not show any influence on the development of primary mechanical hyperalgesia. Neither the established thermal nor mechanical hyperalgesia identified in the above sites was affected by i.t. post-treatment 3 h after bee venom injection. Pre- and post-treatment of SR14280 did not produce any significant effect on the bee venom-induced spontaneous pain and thermal and mechanical hyperalgesia. It was suggested that activation of spinal NK1/2 receptors is involved in both induction and maintenance of the persistent spontaneous nociception, while it is only involved in induction of the primary and secondary thermal, but not primary mechanical hyperalgesia induced by bee venom injection (Zheng and Chen, 2001). The spinal NK3 receptor seems not likely to be involved in the bee venom-induced behavioral response characterized by spontaneous pain and thermal and mechanical hyperalgesia (Zheng and Chen, 2001).

Antagonism of peripheral neurokinin receptors: A study using male Wistar rats receiving co-injection of bee venom and a single dose of FK888, SR48968 and SR14280, acting as NK1, NK2 and NK3 antagonist, respectively, revealed a significant inhibition of bee venom-induced paw edema by FK888, with less effectiveness by SR48968 and SR14280, suggesting a strong involvement of NK1 receptors in bee venom-induced local inflammation (Calixto et al., 2003). The roles of peripheral NK receptors of the three types in bee venom-induced nociception and hyperalgesia have not been studied and remain to be examined.

3.3.3.3. ATP P2X and P2Y receptors. P2 purinoceptors are divided into two families: (1) ionotropic receptors (P2X), including 7 types (P2X1–P2X7); (2) metabotropic receptors (P2Y), including 8 types (P2Y1, 2, 4, 6, 11, 12, 13 and 14) (Boeynaems et al., 2005; Sawynok, 2007; Inoue, 2008). P2X3 and P2Y1 are expressed mainly in primary nociceptive sensory neurons, implicating a particular role in the mediation of nociception (Burnstock, 2000; Chizh and Illes, 2001; Kennedy et al., 2003; North, 2003; Boeynaems et al., 2005; Kennedy, 2005; Koles et al., 2007; Sawynok, 2007; Wirkner et al., 2007).

Antagonism of spinal P2X receptors: A great body of evidence shows that P2X receptors are involved in facilitation of glutamate release at the central terminals of primary nociceptive sensory neurons and affect spinal synaptic transmission (Bardoni et al., 1997; Gu and MacDermott, 1997; Gu et al., 1998; Li et al., 1998; Tsuda et al., 2000; Nakatsuka and Gu, 2001; Nakatsuka et al., 2002; Gu and Heft, 2004). Antagonism of spinal P2X receptors by a selective antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) through i.t. pretreatment (5 and 10 μ g) resulted in suppression of the bee venom-induced flinching reflex in a dose-related manner (Zheng and Chen, 2000). However, i.t. PPADS at a higher dose (30 μ g) failed to produce any inhibitory effect (Zheng and Chen, 2000). It was suggested that activation of the P2X-purinoceptor in the spinal cord contributes to the induction of bee venom-induced persistent nociception; however, more work remains to be done for clarification of its role in BV-induced hyperalgesia.

Antagonism of peripheral P2X and P2Y receptors: Besides the actions of P2X and P2Y in the spinal cord dorsal horn, a role of peripheral actions of these two types of ATP receptors in nociception has also been strongly suggested (North, 2004; Stucky et al., 2004; Waldron and Sawynok, 2004). To test the roles of P2X and P2Y at the peripheral injury site in the maintenance of bee venom induced nociception and hyperalgesia, A-317491, a potent P2X3/P2X2/3 receptor antagonist and Reactive Blue 2, a potent P2Y receptor antagonist, were administered locally (Lu et al., 2008). Because melittin is the key polypeptide of bee venom in production of long-lasting nociception and hyperalgesia as well as local inflammation, the use of melittin is preferable for studying the underlying peripheral mechanisms of the bee venom-induced pain and hyperalgesia (Li and Chen, 2004; Chen et al., 2006b). Briefly, post-treatment of the primary injury site with local A-317491 could significantly suppress the melittin-evoked persistent nociception within 30 min after treatment. Moreover, both primary heat and mechanical hypersensitivity were significantly reversed by antagonizing P2X3/P2X2/3 receptors. Localized peripheral administration of two doses of Reactive Blue 2 also resulted in a significant attenuation of nociceptive paw flinches and the inhibition was most significant within 30 min after administration. However, dose-dependence was not detectable between 4 mg and 9 mg in 20 μ l. Similar to the effects of A-317491, both primary heat and mechanical hyperalgesia were relieved by two doses of Reactive Blue 2. Taken together, these data indicate that activation of P2X and P2Y receptors in the injury site might be essential to the maintenance of melittin-induced primary thermal and mechanical hyperalgesia as well as on-going pain (Lu et al., 2008).

3.3.3.4. Voltage-dependent calcium channels. Voltage-dependent calcium channels (VDCCs) are a group of voltage-gated ion channels found in excitable cells. Pharmacologically, they were classified into four groups: (1) the dihydropyridine-sensitive L-type; (2) ω -conotoxin GVIA-sensitive N-type; (3) ω -agatoxins-sensitive P/Q-type; (4) the R-type channels (Catterall, 1991; Takahashi and Momiyama, 1993; Birnbaumer et al., 1994; Ertel et al., 2000; Catterall et al., 2003, 2005). Among the VDCCs, L-type, N-type and P/Q-type are demonstrated to be involved in mediation

of spinal synaptic transmission and nociception both pre- and post-synaptically (Chaplan et al., 1994; Neugebauer et al., 1996; Omote et al., 1996; Chaplan, 2000; Vanegas and Schaible, 2000; Matthews and Dickenson, 2001; Snutch et al., 2001; Field et al., 2006). Thus, spinal effects of verapamil, ω -conotoxin GVIA and ω -agatoxins were evaluated by i.t. administration pre- or post-injection of bee venom. The results showed that N- and P/Q-subtypes seem to be co-activated by subcutaneous bee venom and be involved in both the induction and maintenance of persistent nociception, and primary and secondary (and mirror-image) heat hyperalgesia; however, they seem not to play a role in either inducing or maintaining primary mechanical hypersensitivity. In contrast, L-subtype of VDCC is activated in the maintaining process of the BV-induced primary and secondary (and mirror-image) heat hyperalgesia, but it is not activated in any other processes (Chen, 2007, 2008; and Chen's lab unpublished data).

3.3.3.5. MAPKs. Roles of spinal ERK, p38 MAPK and JNK: As described above (Cui et al., 2008), different types of MAPKs, such as ERKs and p38 MAPK in the spinal cord dorsal horn are phosphorylated during different processes, at different times, and in different neural cell types (neurons, microglia and astrocytes) in response to subcutaneous bee venom injection. Three doses of U0126 (1,4-diamino-2,3-dicyano-1, 4-bis-[o-aminophenylmercapto]butadiene), a widely used specific MAP kinase kinase (MEK) inhibitor, were administered through chronic i.t. catheterization prior to or after injection of melittin. It was found that (Yu and Chen, 2005): (1) the induction of melittin-induced persistent spontaneous nociception, mechanical and heat hypersensitivity could be suppressed by U0126 in a dose-related manner; (2) specific inhibition of ERK pathway suppressed the maintenance of melittin-induced nociception and heat hypersensitivity, while the established mechanical hypersensitivity was not reversed; and (3) i.t. administration of U0126 had no effects on peripheral inflammation induced by melittin.

To further evaluate the roles of spinal p38 and JNK in production of bee venom-induced nociception and hyperalgesia, i.t. pre-treatment of a p38 inhibitor SB239063 and a JNK inhibitor SP600125 was conducted in the conscious rat (Cao et al., 2007). The results showed that i.t. pre-treatment with either SB239063 or SP600125 resulted in a significant prevention of the bee venom-induced persistent paw flinches. Moreover, i.t. pre-treatment of SB239063 and SP600125 prevented primary heat and mirror-image heat hyperalgesia from occurring; however, the same treatment had no effect on bee venom-evoked mechanical hyperalgesia.

Collectively, the data demonstrate that activation of both ERK and p38 MAPK in the spinal cord contributes to bee venom-induced persistent nociception and mirror-image heat hyperalgesia, but not primary mechanical hyperalgesia, while spinal JNK signaling pathway is likely to play more important roles in the induction of primary heat hyperalgesia rather than being involved in other processes (Yu and Chen, 2005; Cao et al., 2007; Chen, 2007, 2008).

Roles of peripheral ERK, p38 MAPK and JNK: Pre- and post-administration of three MAPK inhibitors, namely U0126 for ERK, SB239063 for p38 MAPK, and SP600125 for JNK, into the melittin-injected area of one hind paw of rats, significantly suppressed the occurrence and maintenance of melittin-evoked persistent spontaneous nociception and primary heat hyperalgesia, with little antinociceptive effect on mechanical hyperalgesia (Hao et al., 2008). In another study using bee venom as an irritant, localized peripheral administration of more selective ERKs inhibitor (PD98059) and p38 inhibitor (SB202190) was performed. The results showed that pre-inhibition of ERK in the injury site blocked the bee venom-induced persistent nociception but

without affecting either primary mechanical hyperalgesia or local inflammation, while inhibition of p38 MAPK was not effective in prevention of persistent nociception but remains effective in prevention of primary mechanical hyperalgesia and local inflammation (Chen et al., 2009).

To confirm the local effects of ERK inhibition by U0126, extracellular recordings of dorsal horn WDR neurons were further made in anesthetized rats. The results showed a distinct suppression of melittin-induced long-lasting spike discharges and of enhanced thermally nociceptive responsiveness by peripheral inhibition of ERKs; however, melittin-associated enhancement of mechanical responsiveness was not influenced at all although electrically evoked wind-up and after-discharges of dorsal horn neurons was significantly suppressed (Li et al., 2008; Yu et al., 2009).

Taken together, activation of peripheral MAPKs, including ERK, p38 MAPK and JNK, might contribute to the induction and maintenance of persistent ongoing pain and primary heat hyperalgesia following both melittin and bee venom injection. However, they are not likely to be involved in the processing of melittin-induced primary mechanical hyperalgesia, implicating a mechanistic separation between mechanical and thermal hyperalgesia in the periphery. Use of more selective inhibitors of some types of MAPKs for localized peripheral administration may improve the effectiveness in prevention or suppression of nociception, hyperalgesia and inflammation.

3.3.3.6. Protein kinases. Protein kinases (PKs), such as diacylglycerol (DAG)-protein kinase C (PKC) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), are likely to play important roles in the phosphorylation of ligand-gated receptor channels (e.g., NMDA/non-NMDA receptors), G-protein-coupled receptors (e.g., mGluRs and NK1/2 receptors) and other enzymes, which have been demonstrated to be essentially involved in the establishment and maintenance of central sensitization (Chen and Huang, 1992; Mayer et al., 1995; Basbaum, 1999; Petersen-Zeit and Basbaum, 1999; Malmberg, 2000). Some subtypes of PKC (e.g., PKC γ) and PKA [type I regulatory β subunit (RI β)] have been demonstrated to be localized only in the CNS and peripheral tissue or nerve injury can induce an increase in mRNA or immunoreactivity of RI β of PKA or PKC γ in the dorsal horn of the spinal cord (Boland et al., 1991; Malmberg et al., 1997a,b; Aley and Levine, 1999; Dina et al., 2000; Martin et al., 1999). Mice lacking RI β of PKA or PKC γ show a decrease in some type of pain, hypersensitivity and inflammation (Malmberg et al., 1997a,b). On the other hand, both PKC and PKA are shown to mediate nociception in the periphery and may be involved in peripheral sensitization of nociceptors (Cesare and McNaughton, 1997; Aley et al., 1998; Aley and Levine, 1999; Cortright and Szallasi, 2004; Suh and Oh, 2005; Zhang et al., 2007; Cang et al., 2009; Mizumura et al., 2009).

Roles of spinal PKC and PKA: Targeting at spinal PKC or PKA was achieved by i.t. administration of chelerythrine chloride (CH), a PKC inhibitor and H89 (N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride), a PKA inhibitor (Li et al., 2000b; Li and Chen, 2003). SQ22536 was also administered to inhibit adenylyl cyclase (AC), which produces cAMP that is required by PKA to function (Li and Chen, 2003). For the bee venom-induced persistent nociception, pre-treatment of spinal cord with three drugs of CH, H89 or SQ22536 at three separate doses significantly prevented the paw flinches from occurring in a dose-related manner, while only CH and H89 produced effective suppression following post-treatment of the highest dose used (Li et al., 2000b; Li and Chen, 2003). Primary heat hyperalgesia was only significantly eliminated by CH, but not by H89 and SQ22536 regardless of either pre- or post-treatment (Li and Chen, 2003). However, in contrast, primary mechanical hyperalgesia was

distinctly eliminated by H89 and SQ22536 with pre-treatment, while only by H89 when post-treated (Li and Chen, 2003). Mirror-image heat hyperalgesia was completely blocked by spinal inhibition of PKC, PKA and AC, respectively, without time difference in drug administration (Li et al., 2000b; Li and Chen, 2003). This result clearly shows a differential role of spinal DG-PKC and cAMP-PKA in the mediation of different symptomatic 'phenotypes' of bee venom-induced nociception and hyperalgesia, namely, spinal PKC is mainly involved in development and maintenance of primary heat hyperalgesia, while spinal PKA is mainly involved in development and maintenance of primary mechanical hyperalgesia. Both PKC and PKA are equally involved in development and maintenance of persistent nociception and mirror-image heat hyperalgesia. This mechanistic phenomenon has also been found in other pain models (Basbaum, 1999; Petersen-Zeit and Basbaum, 1999; Yajima et al., 2003).

Roles of peripheral PKC and PKA: In another behavioral study, peripheral roles of PKC and PKA were also evaluated for the bee venom-induced persistent nociceptive response, mechanical hyperalgesia and inflammation in rats (Chen et al., 2008). Three doses of CH and H-89 were administered locally in the bee venom injection site. As for spinal administration, localized pre-administration of CH, but not H89, dose-relatedly suppressed bee venom-induced spontaneous nociception and local inflammation, without any suppressive effect on the mechanical hyperalgesia. In contrast, H-89 dose-dependently inhibited the BV-induced mechanical hyperalgesia, but without affecting persistent nociception and inflammation. Unfortunately, the local effects of these two drugs on the bee venom-induced primary heat hyperalgesia were not observed. However, peripheral PKC and PKA are also likely to differentially mediate different symptomatic 'phenotypes' of bee venom-induced pain behaviors.

3.3.3.7. Cyclooxygenases. Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins from arachidonic acid and has two isoforms, COX-1 and COX-2 (Vane et al., 1998; Smith et al., 2000; Rouzer and Marnett, 2009). It is believed that COX-1 is constitutive and COX-2 is inducible, and both types are important in mediation of pain, inflammation and fever (Vane et al., 1998; Hoffmann, 2000; Bennett, 2001; Bertolini et al., 2002; Burian and Geisslinger, 2005). Spinal COX-1 and COX-2 have been demonstrated to participate in facilitation of neurotransmitter release from primary afferents to the dorsal horn that can alter synaptic transmission (Kaufmann et al., 1997; Hoffmann, 2000; Vanegas and Schaible, 2001; Leith et al., 2007; Li et al., 2009). Inhibition of spinal COXs was achieved by i.t. administration of three doses of a selective COX-2 inhibitor (etodolac, 1, 10, and 50 μ g) and a dually acting non-steroidal anti-inflammatory drug (indomethacin, 30 μ g, 50 μ g, and 100 μ g), respectively (Lariviere et al., 2005; Li and Chen, 2000). The results showed that both etodolac and indomethacin dose-dependently suppressed the bee venom-induced persistent nociception; however, neither primary heat nor primary mechanical hyperalgesia was affected by these two drugs. Upon examination of the effects of these two drugs on bee venom-induced c-Fos expression, only those c-Fos positive neurons localized within the superficial layers were significantly decreased by etodolac and indomethacin, whereas expression in deep layer c-Fos positive cells was not altered. Taken together, it is likely that spinal COX-2 and probably COX-1 are involved in primary afferent-driven persistent nociception, but contribute little to the hyperalgesia.

3.3.3.8. Nitric oxide. Nitric oxide (NO) or nitrogen monoxide is an important signaling molecule in mammals. NO is biosynthesized endogenously from arginine and oxygen by various nitric oxide synthase (NOS) enzymes. NO acts through the stimulation of the

soluble guanylate cyclase (sGC), a heterodimeric enzyme, with subsequent formation of cyclic GMP. Cyclic GMP activates protein kinase G to perform various physiological and pathophysiological functions (Millan, 1999; Luo and Cizkova, 2000). In the spinal cord dorsal horn, NO is involved in facilitation of neurotransmitters release from primary afferent input and plays important roles in the mediation of pathogenesis of pathological pain of various origins (Dickenson et al., 1992; Haley et al., 1992; Zhuo et al., 1993; Dohrn and Beitz, 1994; Goettl and Larson, 1996; Qian et al., 1996; Roche et al., 1996; Ferreira et al., 1999; Sousa and Prado, 2001; Vetter et al., 2001; Tao and Johns, 2002; Hoheisel et al., 2005; Zhang et al., 2005). Unpublished data showed that release of NO in the spinal cord can be induced by subcutaneous bee venom injection (Chen, 2003, 2007, 2008). It was found that inhibition of cGC and NOS could prevent and inhibit both persistent nociception and primary mechanical hyperalgesia. Moreover, the same treatment of the drugs targeting at cGC and NOS also significantly reversed the primary and secondary (and mirror-image) heat hypersensitivity, while there was no influence upon their induction. The results suggest that NO plays an important role in the mediation of persistent nociception and primary mechanical hypersensitivity during the whole process, while only in the late processing NO is necessary for maintenance of primary and secondary (and mirror-image) heat hypersensitivity (Chen, 2003, 2007, 2008).

3.3.3.9. Other pharmacological studies. To validate the bee venom test as an animal model of pain, currently used and non-approved anti-nociceptive or anesthetic drugs were evaluated. It is interesting to find that the various symptomatic 'phenotypes' of bee venom-induced nociception and hyperalgesia are beneficial to drug discovery and screening.

Nociceptin: Nociceptin (also known as orphanin FQ) is an active endogenous heptadecapeptide ligand for the orphan opioid receptor-like (ORL1) receptor (Meunier et al., 1995; Reinscheid et al., 1995; Calo' et al., 2000; Xu et al., 2000). Using the bee venom test (Sun et al., 2004b), i.t. administration of three doses (3, 10, 30 mmol) of synthetic nociceptin was shown to dose-relatedly block the bee venom-induced persistent nociceptive behavioral responses with both pre- and post-treatment. However, the drug did not have any anti-hyperalgesic effects against the primary heat and mechanical hyperalgesia. There was no beneficial effect on the bee venom-induced local inflammation. This implies that nociceptin has anti-nociceptive effects, but not anti-hyperalgesic and anti-inflammatory effects (Sun et al., 2004b).

Propofol and etomidate: By using the bee venom test, spinally and peripherally anti-nociceptive effects of propofol were evaluated in both behavioral and electrophysiological recordings of dorsal horn WDR neuronal activities (Wang et al., 2003a; Sun et al., 2005). Anti-nociceptive effects of etomidate given via either i.v. or i.t. routes of administration were also evaluated and it was found that etomidate was effective in suppressing both persistent nociception and hyperalgesia when cumulative doses were given (Sun et al., 1999, 2001). The anti-nociceptive effects of etomidate were also confirmed by using other models of pain such as the formalin test; and the oral route of administration is safe, effective and promising for clinical use for the relief of pain when morphine or NSAIDs are not appropriate. The spinal mechanisms of etomidate analgesia are likely to be due to its enhancing effects upon glycine and GABA receptors or restoration of EAAs-IAs balance in the place of decreased IAAs level in persistent or chronic pain (Li et al., 2004b).

Compounds or TCM medicinal compositions: Anti-nociceptive and anti-inflammatory effects of some compounds (e.g., paeoniflorin), TCM medicinal compositions (e.g., Radix Angelicae dahuricae), and others were well evaluated by the bee venom

test in comparison with the other existing pain models (Peng et al., 2003, 2004a,b; Yu et al., 2007).

3.3.4. Proposed mechanisms of bee venom-induced nociception and inflammation

3.3.4.1. Peripheral mechanisms. Until now, the underlying peripheral mechanisms of bee venom-induced nociception and hyperalgesia have not yet been fully elucidated. The major difficulty lies in the fact that bee venom is complex in composition. However, the results from several pharmacological studies in humans and animals provide a scheme of proposed mechanisms (Fig. 3). In general, two sets of mechanisms are likely to be involved: (1) direct actions of the bee venom constituents on the free nerve terminals that contain various membrane proteins and transmembrane signal transducing molecules mediating nociception; (2) indirect actions of the bee venom constituents on the free nerve terminals through allergens or proinflammatory and inflammatory mediators released from mast cell degranulation, tissue damage and immune responses.

Induction of pain or nociception via direct actions: There are several lines of solid evidence to support the direct action of bee venom on neurons. Phenomenologically, the temporal character-

istics of responses to bee venom injection in mammals are immediate and fast with the occurrence of both spontaneous pain sensation in humans and nociceptive responses in animals 10–15 min earlier than the occurrence of visual flare in humans and paw edema in animals (Lariviere and Melzack, 1996; Chen et al., 1999b; Chen and Chen, 2000; Koyama et al., 2000, 2002; Sumikura et al., 2003). Visual flare and edema are known to be a result of the neurogenic inflammatory response. Electrophysiological recordings of nociceptive neurons in both DRG and spinal dorsal horn neurons also revealed immediate spike discharges (Chen et al., 1998; Li and Chen, 2004; You and Arendt-Nielsen, 2005; You et al., 2008; Li et al., 2008; Yu et al., 2009, also see Fig. 2). Moreover, among the more than 20 bee venom constituents, melittin, MCD peptide, apamin, histamine, 5-HT and other substances present in small amounts have been demonstrated to be pain-producing substances. All of these substances can activate nociceptors through known or as yet unknown molecular targets or mechanisms to produce pain sensations immediately and at the sight of insult.

Given that 50% of dry bee venom is the unique polypeptide melittin (Fig. 3), prerequisite roles of this substance in the induction of pain should be taken into account. As demonstrated in both human and animal studies, melittin is the major

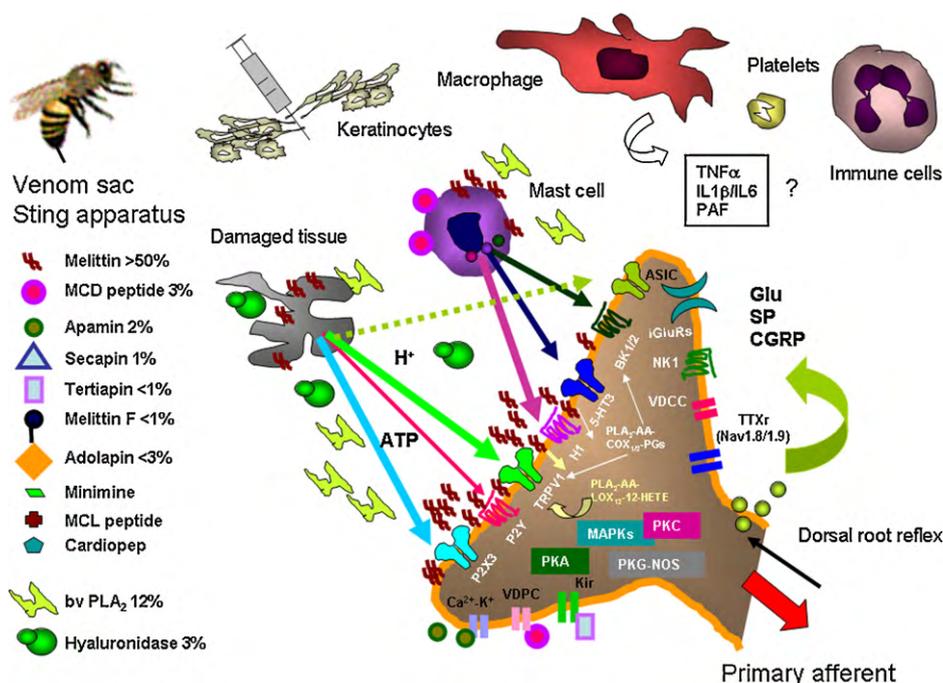


Fig. 3. A schematic drawing of proposed underlying mechanisms of the bee venom induced persistent nociception and primary hyperalgesia to heat and mechanical stimuli applied in the periphery. On the left column, venom sac and sting apparatus is shown at the tip of a honeybee abdomen. The major polypeptides and enzymes of the bee venom are listed below (also see Table 1). Subcutaneous injection of bee venom by a syringe is shown on the left top, while a nerve terminal of primary afferent is shown on the bottom right. The direct and indirect actions of each ingredient of the bee venom are proposed. Color symbols representing each ingredient of the bee venom (left) can be clearly seen on the right where melittin (dark red double strand), MCD peptide (red colored circle), apamin (green colored circle) and tertiapin (light blue rectangle) bind directly to the membrane of a nociceptor cell leading to activation of it. Meanwhile, melittin, MCD peptide, bv PLA₂ (light green 'H'), and hyaluronidase (dark green double balls) cause tissue damage (grey) and release ATP and H⁺ that activate P2X3 (thick blue arrow and paired channel)/P2Y (thin red arrow and paired channel), TRPV1 (green arrow and paired channel) and ASiC (light green dashed arrow and paired channel). Indirect actions of melittin, MCD peptide and bv PLA₂ cause degranulation of mast cells (purple) and release histamine, BK and 5-HT that activate H₁ receptor (pink thick arrow and paired receptor), 5-HT₃ receptor (blue arrow and paired receptor) and BK_{1/2} receptors (dark green arrow and paired receptor). The firing of nociceptor terminals will be mediated by voltage-dependent sodium channels (TTXr Nav1.8/1.9), VDCC, VDPC, Kir and Ca²⁺-K⁺. Dorsal root reflex and axon reflex may cause release of glutamate and neuropeptides (SP and CGRP) that further activate their autoreceptors on the nociceptor terminals or blood vessels causing inflammatory extravasation (neurogenic) with infiltration of macrophage, immune cells and platelets and many cytokines (TNF α , IL1 β , etc.). The syringe indicates transcutaneous injection of bee venom. **Abbreviations:** 5-HT₃, 5-hydroxytryptamine receptor 3; 12-HETE, 12-hydroxyeicosatetraenoic acids; AA, arachidonic acid; ASiC, acid-sensing ionic channel; ATP, adenosine triphosphate; BK_{1/2}, bradykinin receptors 1/2; bv PLA₂, bee venom phospholipase A₂; Ca²⁺-K⁺, calcium-dependent potassium channel; CGRP, calcitonin-gene related peptide; COX-1/2, cyclooxygenases 1/2; Glu, glutamate; H₁, histamine receptor type 1; iGluRs, ionotropic glutamate receptors; IL1 β , interleukin 1 β ; IL6, interleukin 6; Kir, inward-rectifier potassium channel; LOXs, lipoxygenases; MAPKs, mitogen-activated protein kinases; MCD peptide, mast cell degranulating peptide; MCL peptide, mastocytolytic peptide; NK1, neurokinin 1; NOS, nitric oxide synthase; P2X₃, P2-purinoreceptor X₃; P2Y, P2-purinoreceptor Y; PAF, platelet-activated factor; PGs, prostaglandins; PKA, protein kinase A; protein kinase C; protein kinase G; SP, substance P; TNF α , tumor-necrosis factor α ; TRPV1, transient receptor potential vanilloid receptor 1; TTXr, tetrodotoxin-resistant; VDCC, voltage-dependent calcium channel, VDPC, voltage-dependent potassium channel.

pain-producing substance among the list of constituents of bee venom (Koyama et al., 2000, 2002; Sumikura et al., 2003, 2006; Chen et al., 2006b). On one hand, due to the nature of its amphipathic and cationic charge-bearing structure, melittin has long been shown to have an ability to insert itself into the phospholipid bilayers of cells where it may interact with various proteins (Schoch and Sargent, 1980; Vogel, 1981; Kempf et al., 1982; Strom et al., 1982; Talbot et al., 1982, 1987; Blumenthal et al., 1983; Vogel et al., 1983; Dufourcq et al., 1986; Hermetter and Lakowicz, 1986; Beschiaschvili and Seelig, 1990; Frey and Tamm, 1991; Nishiya and Chou, 1991; Ohki et al., 1994; Hristova et al., 2001; Chen et al., 2007c; Raghuraman and Chattopadhyay, 2007; van den Bogaart et al., 2007; Lundquist et al., 2008; Wessman et al., 2008). Although there is no direct evidence showing this dynamic characteristic of melittin in the mammalian DRG cell membrane, we propose that it interacts directly with ionic channels associated with nociception for example, TRPV1 or P2X3, to open the cation channels (Fig. 3). The blocking effects of pre-treatment with TRPV1 and P2X2/P2X3 inhibitors on the melittin-induced persistent nociception are supportive of this presumption.

On the other hand, melittin has a pore-forming effect that may lead to Na^+ and Ca^{2+} influx (Williams and Bell, 1972; Tosteson and Tosteson, 1981; Tosteson et al., 1990; Schwarz et al., 1992; Fattal et al., 1994; Smith et al., 1994; Bechinger, 1997; Matsuzaki et al., 1997; Allende et al., 2005; Becucci and Guidelli, 2007; Chen et al., 2007b; Klocek et al., 2009), which, in turn, may depolarize the membrane potential of DRG cells to a threshold giving rise to action potentials. However, unlike the artificial model of lipid bilayers in which melittin readily forms pores, the stability of the membrane is highly dependent upon the density of lipid-rafts, the ratio between phosphatidylcholine and phosphatidylethanolamine, the amount of cholesterol and the density of membrane proteins (Allende et al., 2005; Chen et al., 2007b).

Based upon previous and our preliminary data, the non-specific pore-forming action of melittin in the mammalian DRG cell membrane is unlikely to be the major effect for the induction of pain because: (1) *in vivo* electrophysiological recordings of single fibers showed that with localized peripheral melittin injection into the cRF of a single fiber, only mechanonociceptors and mechanonociceptors are activated (Cooper and Bomalaski, 1994); (2) *in vitro* patch-clamp recordings of DRG cells revealed that only a subpopulation (60–70%) of small- to medium-sized DRG cells studied could be evoked to produce inward current and intracellular rise in Ca^{2+} concentration in response to direct application of melittin; (3) according to the electrophysiological feature of nociceptor cells that possess a hump or a shoulder on the falling phase of the action potential (Harper and Lawson, 1985; Djouhri et al., 1998), all the DRG cells that could be evoked to fire in response to melittin are nociceptor cells; (4) the action potential firing, inward current and Ca^{2+} transients can be blocked by selective blockers or inhibitors of specific membrane proteins in about 40–60% of melittin-sensitive cells, excluding a mediation by non-selective pore-forming action of melittin. On the other hand, because melittin is an activator of PLA_2 (Mollay and Kreil, 1974; Hassid and Levine, 1977; Nishiya, 1991; Vernon and Bell, 1992; Sharma, 1993), it can activate nociceptors through PLA_2 -catalyzed metabolites. This presumption is the most possible way for melittin to induce nociception, because the inward current evoked by melittin in DRG cells can be blocked by inhibition of endogenous PLA_2 activity (Chen's lab unpublished data).

The direct actions of other bee venom constituents in the induction of pain are not clear, but may include inhibition of various potassium channels (Fig. 3). For instance, MCD peptide has been demonstrated to be a high affinity inhibitor of voltage-dependent potassium channels (VDPC) (Bidard et al., 1987a,b; Rehm et al., 1988; Rehm and Lazdunski, 1988; Gandolfo et al.,

1989), apamin, to be a selective inhibitor of calcium-dependent potassium channels (Ca^{2+} - K^+) (Hugues et al., 1982; Wadsworth et al., 1994), and tertiapin, an inhibitor of inward-rectifier potassium channel (Kir) (Kanjan et al., 2005; Felix et al., 2006; Ramu et al., 2008). Histamine and 5-HT found in bee venom can induce pain through direct action on their receptors, but are not likely responsible for the long lasting pain due to their small amounts in bee venom (Lariviere and Melzack, 1996) (see Table 1).

The two enzymes PLA_2 and hyaluronidase constitute 12% and 3% of dry bee venom, respectively. The direct actions of these two enzymes on nociceptor cells are not clear and remain to be tested. However, they are major allergens and may be involved in the catalysis of membrane phospholipids or causing matrix tissue damage that may induce inflammatory responses or facilitate the penetration of pain-producing substances to the free nerve terminals (Fig. 3, Table 1).

Induction of pain or nociception via indirect actions: There are at least two mechanisms underlying indirect actions of bee venom constituents for the induction of pain or nociception (Fig. 3). One alternative mechanism is via mast cell degranulation caused by MCD peptide (or melittin) (Jasani et al., 1979; Banks et al., 1990) and tissue damage caused by hyaluronidase (Habermann, 1957; Barker et al., 1963; Kemeny et al., 1984). Following the above actions, many pain-producing substances such as histamine, bradykinin, 5-HT, ATP, K^+ and protons can be released and act on their respective receptors and channels, leading to persistent activation of nociceptor cells (Fig. 3). During this process, we hypothesize that there are many proinflammatory mediators, including $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 and platelet-activated factor (PAF) and pain-enhancing substances such as prostaglandins released from macrophages, immune cells and mast cells, involved in the mediation of nociception and hyperalgesia (Millan, 1999). As a line of evidence, Calixto et al. (2003) investigated the roles of endogenous proinflammatory mediators and their receptors in the processing of the bee venom-induced paw edema that was shown to be highly correlated with persistent nociception (Calixto et al., 2003; Lariviere et al., 2005). In that study, localized peripheral histamine deletion by Compound 48/80, antagonism of histamine and 5-HT receptors by pyrilamine and cyproheptadine, and inhibition of COX-2 by rofecoxib resulted in almost complete inhibition of paw edema from 0.5 to 4 h. Inhibition of NOS and antagonism of BK1, BK2, and NK1 receptors were only significantly effective from 1 to 4 h, suggesting that mast cell degranulation is more important in mediation of the early induction process, while proinflammatory mediators from other sources are more important in the maintenance of the bee venom-induced local inflammation and nociception.

The other alternative mechanism is neurogenic inflammation that can enhance nociceptive responses. Neurogenic inflammation is a process mediated by the release of neuropeptides (e.g., CGRP and SP) from peripheral free nerve endings of capsaicin-sensitive primary afferents evoked by the local propagation of action potentials through the peripheral terminals and by the dorsal root reflex, in which retrograde impulses come from primary afferent depolarization in the spinal cord dorsal horn (Willis, 1999; Lin et al., 1999, 2000). This process probably also causes the release of EAAs (Chen et al., 1999a; You et al., 2002). The released neuropeptides and EAAs subsequently enhance the inflammatory responses and nociception. In the human study, melittin-induced visual flare and increase in skin temperature were shown to be eliminated by topical lidocaine blockade of membrane depolarization (Koyama et al., 2000). In the animal studies, bee venom-induced nociceptive responses, hyperalgesia and local inflammatory responses were all shown to be mediated by capsaicin-sensitive primary afferents and the dorsal root reflex (Chen and Chen, 2001; Chen et al., 2006b, 2007a). Moreover, melittin-induced

nociceptive responses and spinal dorsal horn neuronal activities are also mediated by capsaicin-sensitive primary afferents (Shin and Kim, 2004; Chen et al., 2006b). These results support the involvement of neurogenic inflammation in bee venom-induced nociception, hyperalgesia and inflammation (Fig. 3). Furthermore, elimination of capsaicin-sensitive primary afferents by topical capsaicin treatment of the ipsilateral sciatic nerve 1 day prior completely blocked bee venom-induced paw edema, but did not have any significant influence on the widely used models of CFA-, carrageenan-, and formalin-induced paw edema, suggesting specific involvement of neurogenic inflammation in bee venom-induced inflammatory responses (Chen et al., 2007a). However, elimination of capsaicin-sensitive primary afferents suppressed only the late period (11–60 min), but not the early period (0–10 min) of the bee venom-induced persistent nociception response, further implicating an important role of neurogenic responses in the late period, but not the early period, of the nociceptive processing (Chen et al., 2007a). These data strongly support a pivotal role of neurogenic inflammation in enhancement of the bee venom-induced nociception and hyperalgesia as indirect actions of the bee venom constituents.

In summary, compared with the inflammatory pain state of other tissue injuries, bee venom-induced nociceptive processing is more complicated due to its complex composition. Through direct and indirect actions of bee venom constituents, nociceptors can be activated for a long-term through activation of membrane-bound 'pain' sensors that lead to increased intracellular Ca^{2+} concentration and phosphorylation of various subtypes of MAPKs and PKs (e.g., PKC and PKA). The phosphorylated forms of MAPKs and PKs in turn hypersensitize ionic nociceptor molecules (e.g., TRPV1, P2X3, ASICs, and 5-HT3) and G-protein coupled receptors (e.g., P2Y1, H1, BK1 and BK2), leading to up-regulation of TTX-resistant voltage-

dependent sodium channels (Nav1.8 and Nav1.9) (Chen's lab unpublished data). This process has been well established and referred to as peripheral neural plasticity or sensitization (Woolf and Salter, 2000; Julius and Basbaum, 2001).

3.3.4.2. Spinal mechanisms. The spinal dorsal horn is the first relay of synaptic transmission for nociceptive information between primary afferent input and pain-related central neuronal cells. The functional state of the spinal dorsal horn has been shown to be activity-dependent and is changed by peripheral persistent neural sensitization or plasticity induced by bee venom injection (Chen, 2007, 2008). However, the neural mechanisms of various symptomatic 'phenotypes' of bee venom-induced pain and hyperalgesia are likely to be separate according to different stimulus modalities (chemical, thermal and mechanical). Generally, bee venom-induced nociception are composed of four modality-related processes as aforementioned: (1) chemically relevant persistent nociception; (2) thermally relevant primary hyperalgesia; (3) mechanically relevant primary hyperalgesia; (4) thermally relevant secondary or mirror-image hyperalgesia. The schematic neural mechanisms underlying these different modality-related nociceptive reflexes and pain or hyperalgesia are shown separately in Figs. 4–6.

Chemically relevant persistent nociception (CRPN): As shown in Fig. 4, the induction and maintenance of bee venom-induced CRPN is dependent upon the functional state of the synaptic connections between the primary afferent nerve terminals and dorsal horn neuronal cell bodies (Chen, 2007, 2008). Astrocytes and microglial cells can also be activated by long-term impulse barrages from the periphery. The most striking phenomenon is that the levels of EAAs (glutamate and aspartate) and IAAs (glycine and GABA) at the spinal cord are disrupted by bee venom-induced persistent nociceptive

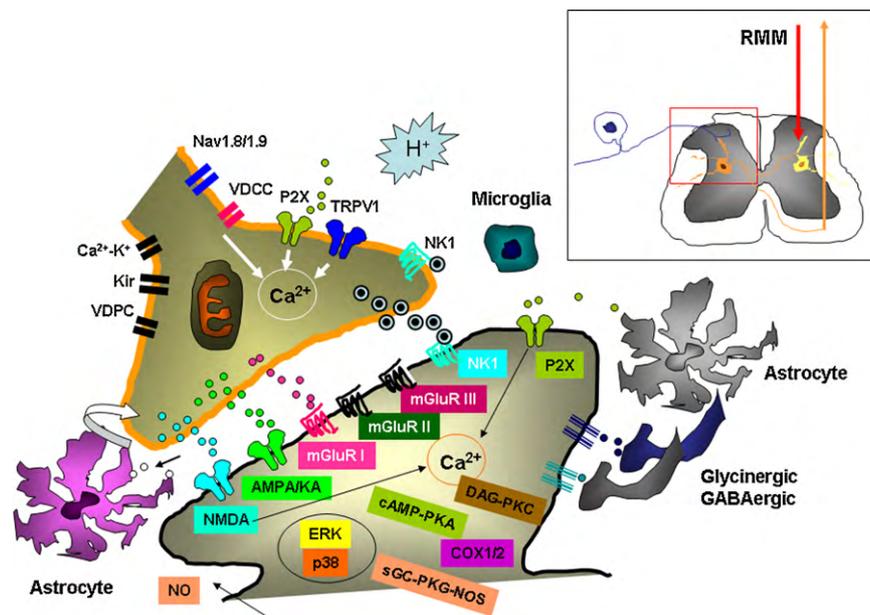


Fig. 4. A schematic drawing of proposed underlying mechanisms of the bee venom (BV)-induced chemically relevant persistent nociception (CRPN) in the spinal cord dorsal horn. The BV-induced thermally relevant secondary hyperalgesia (TRSH) and thermally relevant mirror-image hyperalgesia (TRMIH) are proposed to share the mechanisms similar to the CRPN. Shown is a synaptic structure between a pre-synaptic component (central terminal of primary afferents, left) and a post-synaptic component (membrane of a pain-signaling neuron, right). Astrocytes and microglia are not fully activated at this stage. At the synaptic cleft, in response to the coming of tonic firing action potentials, excitatory amino acids (EAAs, glutamate and aspartate, small colored clear vesicles) are selectively activating ionotropic glutamate receptors (NMDA and AMPA/KAR) and metabotropic glutamate receptor group I (mGluR I), while substance P (large dense-cored vesicles) are activating neurokinin 1 (NK1) receptors. At this process, mGluR II and III are not activated. At pre-synaptic component, vanilloid receptor TRPV1, voltage-dependent calcium channel (VDCC), and NK1 are activated. ATP (green clear vesicles) released from glial cells or damaged cells due to cytotoxic effects are activating ATP P2X receptors. On the other hand, glycinergic and GABAergic modulations become weak due to lack of inhibitory amino acids. Intracellularly, extracellular signaling-regulated kinase (ERK) and p38 MAPK, protein kinases (PKA and PKC and PKG) are phosphorylated and recruited as enhancing modulators of membrane receptors and ion channels. Cyclooxygenases 1/2 (COX-1/2) are also recruited as enzymes catalyzing arachidonic acids to prostaglandins. The inset on the right top shows involvement of descending nociceptive facilitatory pathway from rostral medial medulla (RMM) in the development and maintenance of TRMIH due to the centrally sensitized state.

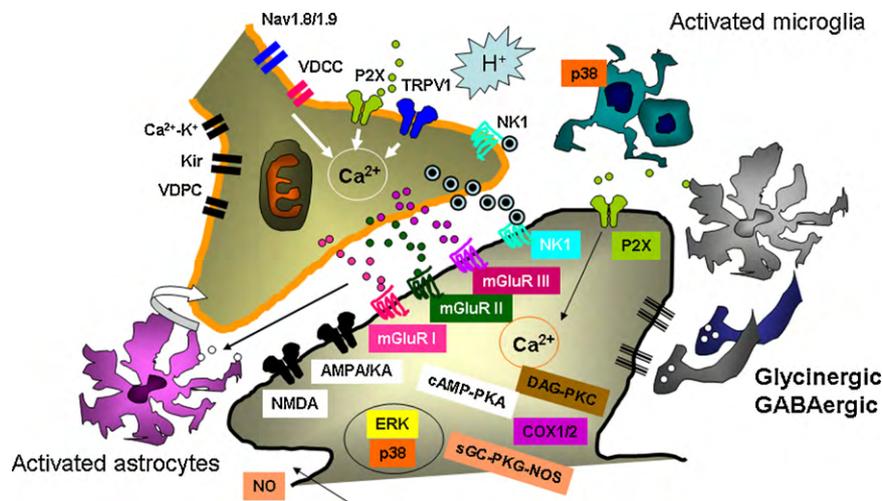


Fig. 5. A schematic drawing of proposed underlying mechanisms of the bee venom (BV)-induced thermally relevant primary hyperalgesia (TRPH) in the spinal cord dorsal horn. Shown is a synaptic structure between a pre-synaptic component (central terminal of primary afferents, left) and a post-synaptic component (membrane of a pain-signaling neuron, right). Astrocytes and microglia are fully activated at this stage. At the synaptic cleft, in response to thermally nociceptive heat stimuli, excitatory amino acids (EAAs, glutamate and aspartate, small colored clear vesicles) are selectively activating group I, II and III of metabotropic glutamate receptors but without activation of ionotropic glutamate receptors (NMDA and AMPA/Ka). Substance P (large dense-cored vesicles) are also activating neurokinin 1 (NK1) receptors. At pre-synaptic component, vanilloid receptor TRPV1, voltage-dependent calcium channel (VDCC), and NK1 are activated. ATP (green clear vesicles) released from glial cells or damaged cells due to cytotoxic effects are activating ATP P2X receptors. Intracellularly, extracellular signaling-regulated kinase (ERK) and p38 MAPK, protein kinases (PKC and PKG, but not PKA), are phosphorylated and recruited as enhancing modulators of membrane receptors and ion channels. Cyclooxygenases 1/2 (COX-1/2) are also recruited as enzymes catalyzing arachidonic acids to prostaglandins.

input, leading to a sustained increase in EAAs release and decrease in IAAs release (Yan et al., 2009). The sustained release of EAAs from primary afferents is playing a key role in co-activation of both AMPA/Ka and NMDA iGluR receptors (You et al., 2003b; Yan et al., 2009), and, as a consequence, leads to increase in intracellular Ca^{2+} concentration. On the other hand, release of EAAs and SP also activates mGluR group I and NK1, but not mGluR groups II and III (Zheng and Chen, 2001; Yan et al., 2009). This transsynaptic signal transduction via G-protein mediated signaling results in phosphorylation of various MAPKs (e.g., ERK and p38 MAPK) (Yu and Chen,

2005; Cao et al., 2007; Cui et al., 2008; Li et al., 2008) and PKs (PKC, PKA and PKG) (Li et al., 2000b; Li and Chen, 2003) as well as enzymes COX-1/2 (Li and Chen, 2000). ATP P2X receptors are activated by extracellular ATP leakage (Zheng and Chen, 2000). Meanwhile, at pre-synaptic component, TRPV1, P2X3, VDCC, Nav1.8 and Nav1.9 are also likely to be activated or up-regulated by tonic persistent primary afferent that in turn facilitate neurotransmitter release. Pre-synaptic localization of NK1, as autoreceptors of SP released from primary afferent terminals, might be further activated and has been demonstrated to enhance both TRPV1 and TTX-resistant Nav1.8

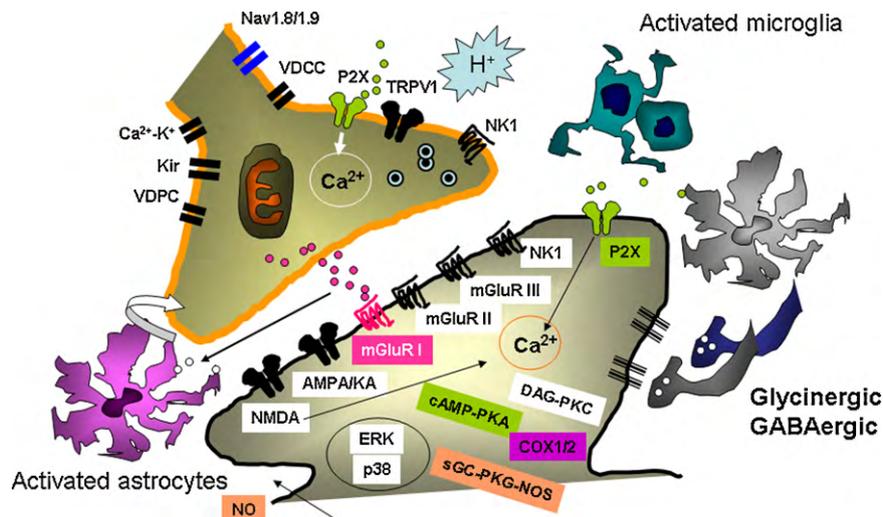


Fig. 6. A schematic drawing of proposed underlying mechanisms of the bee venom (BV)-induced mechanically relevant primary hyperalgesia (MRPH) in the spinal cord dorsal horn. Shown is a synaptic structure between a pre-synaptic component (central terminal of primary afferents, left) and a post-synaptic component (membrane of a pain-signaling neuron, right). Astrocytes and microglia are fully activated at this stage. At the synaptic cleft, in response to mechanically von Frey filament stimuli, excitatory amino acids (EAAs, glutamate and aspartate, small colored clear vesicles) are selectively activating metabotropic glutamate receptors (mGluR) group I, but without activation of ionotropic glutamate receptors (NMDA and AMPA/Ka) and mGluR group II and III. Neurokinin 1 (NK1) receptors are not activated. At pre-synaptic component, vanilloid receptor TRPV1, voltage-dependent calcium channel (VDCC), and NK1 are inactivated. ATP (green clear vesicles) released from glial cells or damaged cells due to cytotoxic effects are still activating ATP P2X receptors. Intracellularly, only protein kinase A (PKA) and PKG, but not PKC, are phosphorylated. Cyclooxygenases 1/2 (COX-1/2) are recruited as enzymes catalyzing arachidonic acids to prostaglandins.

through PKC ϵ (Zhang et al., 2007; Cang et al., 2009). However, the ongoing spinal dorsal horn neural activities are peripherally dependent and the establishment of the centrally sensitized state in the spinal cord was shown to require a certain minimum time of peripheral input before dorsal horn sensitization can be established (Chen et al., 1996, 1998, 1999a, 2000, 2001; You et al., 2002). The maintenance of this centrally sensitized state was also shown to require activation of peripheral EAAs receptors, P2X and P2Y receptors, PKs and MAPKs (Chen et al., 1999a, 2008; You et al., 2002; Hao et al., 2008; Lu et al., 2008; Yan et al., 2009; Yu et al., 2009).

Thermally relevant primary hyperalgesia (TRPH): The spinal mechanisms underlying bee venom-induced TRPH are likely to be different from other modality-relevant pain-related events (see above and below). When bee venom-induced persistent nociceptive responses decline to the baseline level for both behavioral and neuronal activities, a modality of radiant heat stimuli is used and a long-term TRPH can be identified in the local injury site. As shown in Fig. 5, the iGluRs (NMDA and AMPA/KA) in the dorsal horn are not likely to work (Chen and Chen, 2000; Yan et al., 2009). Instead, G-protein coupled receptors including mGluRs (groups I, II and III) and NK1 play important roles in induction and maintenance of the TRPH (Zheng and Chen, 2001; Yan et al., 2009). As a consequence, intracellular DG-PKC, but not cAMP-PKA, is activated (Li et al., 2000b; Li and Chen, 2003). Meanwhile, ERK and p38 MAPK and sCG-PKG-NOS and COX-1/2 in the spinal cord are also recruited as important factors in the mediation of TRPH (Li and Chen, 2000; Yu and Chen, 2005; Cao et al., 2007; Cui et al., 2008; Chen, 2008; Li et al., 2008). Similar to what is shown in Fig. 4, the TRPH shares similar spinal pre-synaptic facilitation process with the CRPN (Fig. 5). At this stage, however, microglial cells become activated and express p38 MAPK that is probably necessary for production of cytokines and other substances (Cui et al., 2008). The roles of astrocytes have not yet been well studied following bee venom injection. However, preliminary results did show activation of astrocytes in the dorsal horn since 2 h after bee venom injection (Chen's lab unpublished data).

Mechanically relevant primary hyperalgesia (MRPH): Through intensive pharmacological studies on the spinal mechanisms underlying bee venom-induced MRPH, it was surprising to note that the MRPH is insensitive to many drugs although the doses used were highly effective in suppression of other pain-related behaviors including the CRPN, the TRPH and the TRSH-MIH (Chen, 2007, 2008). Thus we have reasons to believe that the bee venom-induced MRPH is mediated by a unique spinal as well as peripheral mechanism. Although the spinal mechanisms of the MRPH induced by bee venom are not fully known yet due to its pharmacologically intractable features, we also worked out a working hypothesis for reference according to previous parallel studies with other pain-related behavioral and neuronal activities. As clearly shown in Fig. 6, group I of mGluRs was demonstrated experimentally to be involved in induction and maintenance of the MRPH, while other membrane receptors including iGluRs (NMDA and AMPA/KA), groups II and III of mGluRs and NK1 are not likely to be involved (Yan et al., 2009). Antagonism or inhibition of TRPV1 and VDCC were not effective in suppression as well as prevention of the MRPH (Li et al., 2004a,b; Chen, 2007, 2008). Because inhibition of ATP receptors P2X3/P2X2 and P2Y in the periphery were shown to be effective (Lu et al., 2008) and the types of ATP receptors have well been demonstrated to be involved in facilitation of spinal synaptic transmission (Bardoni et al., 1997; Gu and MacDermott, 1997; Gu et al., 1998; Li et al., 1998; Nakatsuka and Gu, 2001; Nakatsuka et al., 2002; Gu and Heft, 2004), involvement of P2X receptors are highly possible. As for the intracellular cascades, on the other hand, cAMP-PKA and CG-PKG-NOS, but not DG-PKC, are phosphorylated and responsible for induction and maintenance of the MRPH (Li et al., 2000b; Li and Chen, 2003). Activation of COX-1/

2 is also required for spinal mechanisms of the MRPH (Li and Chen, 2000). Taken together, further biological and pharmacological investigations of the spinal and the peripheral mechanisms of bee venom-induced MRPH might be of particular interest and importance, because unraveling of its mechanisms can be helpful for deep understanding of some intractable features of pain in clinic as well as for the searching for novel therapeutic approaches.

Thermally relevant secondary hyperalgesia (TRSH) or mirror-image hyperalgesia (TRMIH): Through intensive pharmacological studies, the TRSH and TRMIH are known to share similar spinal mechanisms and therefore referred to as TRSH-MIH for simplicity (Chen, 2007, 2008). It is intriguing to note that bee venom-induced TRSH-MIH also share mostly similar spinal mechanisms to the CRPN described above (Fig. 4). Thus, briefly, we pay special attention to the TRMIH due to its special usefulness for studies of the central mechanisms of pain. It has been well established that the induction of the TRMIH is a behavioral manifestation that results from an unequivocal centrally sensitized state (Chen et al., 2000). However, the sustained state of central sensitization was shown to require a short-term time window for summation of intensely ongoing primary afferent impulses to the dorsal horn of the spinal cord (Chen et al., 2001; You and Arendt-Nielsen, 2005). Moreover, intra-brain chemical disruption of rostral medial medulla (RMM), where the descending anti-nociceptive pathway to the dorsal horn originates, prevented the persistent behavioral nociceptive responses (also referred to as CRPN) as well as TRMIH from occurring, without influences upon the occurrence of the TRPH and the MRPH (Chen et al., 2003). This result provided a further line of evidence supporting an involvement of facilitatory action of the descending pathway in setting-up of the TRMIH. The facilitatory nociceptive descending pathway from the brain stem has also been found to mediate the mirror-image pain in other animal models (Urban and Gebhart, 1999), suggesting a similar mechanism-based feature of the bee venom-induced nociception to other sources of injury or etiology. The spinal mechanisms of bee venom-induced TRSH-MIH is shown in Fig. 4 (see inset on the upper right for TRMIH).

In summary, the bee venom-induced multiple symptomatic 'phenotypes' of nociception and hyperalgesia have separate spinal mechanisms. The spinal mechanisms of these nociception and hyperalgesia are highly associated with the stimulus modalities applied in the periphery. Since the spinal block of one molecular target for relief of clinic pain might not be equally effective for all types of pain, discovery of common signaling pathways or setting-up of mechanism-based therapeutic approaches is necessary and important.

4. Anti-nociceptive and anti-inflammatory effects of BVT

Despite the known nociceptive and inflammatory effects of bee stings, BVT has been used since ancient times and continues to be used by proponents of the method in Asia, Eastern Europe and increasingly across the globe including in the United States of America to produce analgesia for an ongoing pain and other ailments. Several case reports have been published, societies of proponents exist, and numerous testimonials can be found on the internet and elsewhere. Nonetheless, the experiential evidence for the effectiveness of BVT to relieve chronic ongoing pain in people is tentative at best. The evidence from studies of animal models is somewhat more consistent and robust, but often lacks critical controls that bias the results to conclude that bee venom injection has only beneficial effects.

4.1. Experimental human studies

Numerous studies published in Korean and Chinese scientific journals have been performed to examine the beneficial effects of

bee venom injection for osteoarthritis (OA), rheumatoid arthritis (RA), and several other types of musculoskeletal pain in human patients. According to English language reviews of the human studies (Son et al., 2007; Lee et al., 2005a; Lee et al., 2008), the majority of studies are of bee venom acupuncture (BVA) therapy, also referred to as apipuncture, in which dilute bee venom is injected into acupuncture points. The vast majority of the reports are case reports or trials without controls (Lee et al., 2008). In the 11 randomized clinical trials (RCTs) published to September 2007, BVA involves multiple injections at frequencies ranging from “over 2 times” (Lee et al., 2008, p. 292), to once a week for several weeks or months to daily for a month. No standard frequency or dose or concentration of bee venom is used across studies. Almost unanimously, a statistically significant effect with an effect size from 0.3 to 1.56 is reported regardless of whether the control group received only saline injection, traditional acupuncture without bee venom injection, or saline injection and traditional acupuncture therapy (Lee et al., 2008). No specificity of the analgesic effect is discernible as the types of pain relieved included neck, low back, acute ankle or wrist sprain, rheumatoid arthritis and osteoarthritis, pain of a herniated lumbar disc and post-stroke shoulder pain.

Enthusiasm is greatly reduced, though, as the overall quality of the RCT studies was too poor to permit firm conclusions (the average mean Jadad score was 3 of a maximum of 5) (Lee et al., 2008). Sample sizes were extremely small, with as little as 10 subjects per experimental and control groups, usually less than 20 per group and a maximum of 40 subjects per group. Even in the highest powered studies, other factors that plague the group of studies decreased a study's validity including open label instead of double blind study design and suspected nonuniformity of acupuncture method and quality. In addition, concomitant treatments included unspecified herbal medicine, infrared therapy, hot and/or ice packs, physical therapy and disease modifying antirheumatic drugs, making interpretation of significant findings difficult to impossible. Furthermore, no information regarding duration of analgesic effects was provided in the reviews precluding assessment of whether follow up was adequate and whether the risks and adverse effects outweigh the benefits of the treatment, which overall was a significant decrease of 14.0 mm greater—on a 100 mm visual analogue pain rating scale—with BVA and standard acupuncture compared to saline injection and standard acupuncture (Lee et al., 2008).

Critical to the risk/benefit assessment, it is important to note that the BVA RCTs that mentioned adverse effects reported pain or skin hypersensitivity in bee venom injected patients and high dropout rates greater than 20% (Lee et al., 2008). Four RCTs of bee venom injection in non-acupuncture points are also reported in the Korean and Chinese literature, finding that in three of the RCTs bee venom injection significantly reduced the pain of osteoarthritis of the knee, acute ankle sprain or a range of musculoskeletal pains compared to standard acupuncture, a NSAID, or several types of control (Lee et al., 2008). Although insufficient evidence exists to conclude whether bee venom injection at a non-acupuncture point is effective, it is important to note that in one study (Won et al., 1999) at least one adverse event was reported by each subject, again cautioning against the widespread use of bee venom injection to treat pain.

4.2. Experimental animal studies

The antinociceptive effects of bee venom injection are demonstrated somewhat more clearly in animal studies. Despite the numerous demonstrations reviewed above that subcutaneous injection of bee venom produces significant spontaneous nociceptive behaviors, hypersensitivity to thermal and mechanical stimuli and marked signs of inflammation in rodents, cats and people,

paradoxically, bee venom injection has also been shown to decrease inflammation and pain behaviors associated with several rodent pain models.

The majority of the more recent animal studies that demonstrate an antinociceptive and anti-inflammatory effect of bee venom have been performed by a single group of investigators and have focused on injection of bee venom or venom components into an acupuncture point (or apipuncture), followed by examination of pain and inflammatory measures in a second pain model. For instance Kwon et al. (2001d) injected a bee venom solution bilaterally into the Zusanli acupuncture points located 5 mm distal and lateral to the anterior tubercle of the tibia of the Sprague–Dawley rat daily for 3 weeks beginning the day after induction of a systemic (adjuvant-induced) rheumatoid arthritis model induced by unilateral intraplantar injection of a suspension of heat-killed bacteria. Bee venom injection in the bilateral acupoints significantly reduced the ipsilateral adjuvant-induced increases in paw volume, vocalizations upon flexion and extension of the inflamed ankle, thermal and mechanical sensitivity, and Fos-like immunoreactivity in the neck of the spinal cord dorsal horn (Kwon et al., 2001d). Injection of bee venom at the midline of the back was reportedly ineffective against arthritic responses in the adjuvant-injected paw, leading the authors to conclude that apipuncture is more effective than bee venom injection alone in this study and several others (Kwon et al., 2001a,b,c,d, 2002, 2003, 2005; Lee, 2003; Kim et al., 2003). However, the same study (Kwon et al., 2001d) demonstrated that bee venom injected at the midline of the back is able to significantly reduce the same indicators of adjuvant-induced arthritis that develop after 12 days in the hind paw contralateral to adjuvant injection. Studies by other groups have also successfully inhibited signs of adjuvant-induced systemic polyarthritis with repeated bee venom injections at non-acupuncture points in the paws and on the back of rats (Chang and Bliven, 1979; Yiangou et al., 1993) indicating that injection at the acupuncture point is not critical to observe significant effects. Nonetheless, the same group has demonstrated that repeated bee venom injection (at acupuncture points) is anti-inflammatory in other rodent pain models including the prolonged type-II collagen-induced polyarthritis model (Lee et al., 2004).

They also demonstrated in the more acute inflammatory nociception models of intraplantar carrageenan injection, intraplantar formalin injection and intraperitoneal acetic acid injection that a single bee venom injection (at the corresponding acupuncture point) given 30 min prior is anti-inflammatory (in the former) and antinociceptive in the first hour after formalin or acetic acid injection and at 3 h after carrageenan injection (Kim et al., 2003, 2005; Kwon et al., 2001a,b, 2005; Lee et al., 2001, 2003). In a neuropathic pain model of chronic constriction injury (CCI) induced by tight ligatures of the sciatic nerve, thermal hyperalgesia was reduced 5–45 min, but not 60 min, after a single bee venom injection in the Zusanli acupoint (Roh et al., 2004). Antinociception has been reported in the formalin test initiated up to 2 h after bee venom injection, and up to 12 h after acupoint injection of poly (lactic-co-glycolic acid) nanoparticle-encapsulated bee venom (Jeong et al., 2009). Currently, it is not known how the transient antinociceptive effect of bee venom injection is related to a more prolonged (and anti-inflammatory) effect. Unfortunately, the time of sensory testing relative to the repeated bee venom injections in adjuvant-induced arthritic rodents was not reported (Kwon et al., 2001d, 2002) nor was the time of pain relief assessment in knee osteoarthritis (OA) patients, other than “at the end of the therapeutic period”, in the uncharacteristic report in which “OA symptoms completely disappeared after therapy” in 15 of 40 patients after 4 weeks of bee venom acupuncture twice each week (Kwon et al., 2001c). It is important to note that Chang and Bliven (1979) showed that a single injection of bee venom was most

effective in inhibiting the development of paw swelling after subcutaneous adjuvant injection when administered the day prior, with decreasing effectiveness of a single bee venom injection out to 14 days post-adjuvant injection. Daily bee venom injection in arthritic rats initiated 16 days after adjuvant injection when adjuvant arthritis is established was effective in reducing paw inflammation for approximately seven days, after which paw swelling increased to the level of daily saline injected rats, despite continued daily bee venom injections (Chang and Bliven, 1979). Thus, it is currently unknown if acute or repeated injections can have long-term effects on fully developed pain and inflammation.

4.3. Proposed mechanisms of bee venom-induced antinociception

4.3.1. Anti-inflammatory mechanisms

Reports from several research groups have demonstrated that bee venom injection can indeed reduce the inflammation evoked in a second inflammatory pain model including adjuvant-induced and type-II collagen-induced arthritis models and intraplantar injection of carrageenan (Chang and Bliven, 1979; Yiangou et al., 1993; Kwon et al., 2001d, 2002; Lee et al., 2004; Park et al., 2004). Although reproducible, this is paradoxical as bee venom injection itself produces signs of inflammation including marked edema, redness and nociception and hypersensitivity in rats and mice (Lariviere and Melzack, 1996; Chen et al., 1999b; Chen and Chen, 2000; Lariviere et al., 2005) and reports of pain and flare responses in human subjects receiving injections of bee venom components (Koyama et al., 2000, 2002), despite many claims from BVT proponents to the contrary.

To try to explain the anti-inflammatory effects, bee venom and melittin have been tested for their ability to inhibit lipopolysaccharide (LPS)-induced expression of inflammatory mediators in cultures of murine macrophage and microglial cells and of synoviocytes from rheumatoid arthritis patients. Both whole bee venom and melittin were reported to decrease LPS-induced production of PGE₂, nitric oxide (NO) measured as nitrite, and expression of COX-2, inducible NOS (iNOS), and cytosolic PLA₂ (cPLA₂) (Park et al., 2004; Jang et al., 2005, 2009; Yin et al., 2005; Han et al., 2007; Moon et al., 2007). The effect of bee venom alone was not studied. Other reports, including by some of the same authors, also reported that induced COX-2 activity (but not COX-1 activity) and production of proinflammatory TNF- α and IL-1 β are inhibited *in vitro* by aqueous subfractions of bee venom and *in vivo* after repeated apipuncture (Nam et al., 2003; Lee et al., 2004). Bee venom and melittin were proposed, with supporting evidence, to have anti-inflammatory effects by direct binding to the p50 subunit resulting in inhibition of LPS-induced I κ B release and p50 translocation and, hence, blocking of LPS-induced activation of NF- κ B, a transcription factor that up-regulates several proinflammatory genes (Park et al., 2004). Lee et al. (2009) also showed that NF- κ B and AP-1 transcription factors likely mediate their demonstration of inhibition of LPS-induced iNOS, COX-2 and IL-1 β in C6 glioma cells. Again, the effects of bee venom or melittin alone (without LPS treatment) were not studied.

Others have failed to replicate these findings in related models, instead showing that bee venom and melittin applied to cultured fibroblast-like synoviocytes of rheumatoid arthritis patients and dermal fibroblasts and mononuclear immune cells of healthy subjects did not compete for p50-DNA interactions, nor did they inhibit IL-1 β -induced and/or TNF- α -induced pro-inflammatory responses of phosphorylation or degradation of I κ B α , activation of NF- κ B, or increased expression of COX-2, IL-6, TNF- α , IL-1 β or other NF- κ B activation-dependent genes (Stuhlmeier, 2007). Stuhlmeier (2007) has highlighted several methodological issues that question the validity and interpretation of findings of the Park et al. (2004) study that are beyond the scope of this review.

Critically, and unlike the vast majority of studies advocating the use of BVT for the relief of pain and inflammation, Stuhlmeier (2007) tested the effects of bee venom and melittin alone in the same tissue cultures and demonstrated that several MAPK kinases including p38 MAPK, ERK and JNK are phosphorylated and the proinflammatory genes COX-2, TNF- α and IL-8 are significantly upregulated (albeit much less than by IL-1 β or TNF- α). Note that administration of inhibitors of p38 MAPK, ERK, and JNK into the melittin-injected hind paw of rats significantly decreases spontaneous nociception and primary heat hyperalgesia (Hao et al., 2008) suggesting their critical role in peripheral tissue in the production of responses to peripheral injection of bee venom and melittin. In one of the few other studies examining both the anti-inflammatory and inflammatory effects of bee venom, a microarray analysis study of gene expression profiles of cultured macrophage cells reported that LPS treatment of the cells reportedly caused 1.5-fold or greater upregulation of 158 and 383 genes with 30 min or 1 h treatment with LPS alone, respectively, and down-regulation of 20 genes (Jang et al., 2009). Curiously, only 17 and 1 genes were up- and down-regulated, respectively, by treatment with 20 μ g/ml bee venom alone (Jang et al., 2009), which is at least twice the concentration shown to produce robust cell lysis of red blood cells and apoptosis and necrosis of synovial and lung cells (Jang et al., 2003; Hong et al., 2005; Stuhlmeier, 2007) and 20–200 times the concentration shown to affect viability of chondrosarcoma and synovial cells in culture (Yin et al., 2005; Hong et al., 2005). The genes reportedly upregulated by bee venom included pro-inflammatory genes and compared to LPS treatment alone bee venom plus LPS treatment is reported to “reverse” LPS induced up-regulation of several inflammatory genes (Jang et al., 2009). However, the microarray study omits many methodological details and lacks any biological replicates of samples from each group, and hence any statistical analysis, precluding conclusions regarding group differences. Thus, yet another study falls short of providing sound evidence regarding the relative beneficial and adverse effects of bee venom treatment and possible underlying mechanisms.

Another more carefully designed and more thoroughly described microarray study simultaneously examined expression of 344 genes by human chondrosarcoma cells in culture exposed for 12 h to bee venom or LPS alone and LPS and bee venom combined using the cDNA TwinChipTM Human Cancer 0.4 K chip of Digital Genomics, Korea (Yin et al., 2005). In that relatively early study, a fourfold change in expression was considered significant based on related literature. LPS exposure caused the upregulation of 16 genes including arthritis- and inflammation-related genes for the IL-6 receptor, matrix metalloproteinase 15 (MMP-15), TNF (ligand) superfamily (TNFSF) 10, caspase 6, and tissue inhibitor of metalloproteinase-1 (TIMP-1), and downregulation of 7 genes compared to vehicle treatment. Combined bee venom and LPS exposure caused the downregulation of 32 genes, including those specifically listed just above, compared to treatment with LPS alone. Compared to vehicle treatment, cells exposed to bee venom only showed downregulation of 35 genes including of IL-6 receptor, IL-1 α , IL-8 and IL-12A, TNFSFs 4, 8, and 12 and TNF receptor superfamily (TNFRSF) members 7 and 8, and others related to inflammation. Not a single gene was reported to be upregulated by bee venom exposure alone or in combination with LPS. This is unexpected based on bee venom's known pro-inflammatory effects. However, the concentration of 10 ng/ml used throughout the study is among the lowest ever studied and the conservative fourfold change threshold for consideration may have overlooked significant increases in expression that were less than fourfold that were discussed for LPS exposure alone but not for bee venom alone or LPS and bee venom. Current standards of microarray design and analysis may detect more changes. Thus, it

remains possible—but remains to be demonstrated—that bee venom concentration is critical in determining the net effect; that is, very low concentrations of bee venom may have a net anti-inflammatory effect on relative pro-inflammatory gene expression due to a second highly robust inflammatory insult.

At present, it is difficult to determine with certainty what is occurring to evoke anti-inflammatory effects following a subcutaneous injection of bee venom or its components. Anti-inflammatory subcutaneous injections can contain 300 µg/ml in humans (Kwon et al., 2001c), and although injection volumes are usually not specified in the rodent studies, if common injection volumes of 20–100 µl were used, initial local concentrations of bee venom at the site of injection can be 1 mg/ml (Kim et al., 2003), 1.5–7.5 mg/ml (Kwon et al., 2001d), and even 10 mg/ml (Kwon et al., 2001a). These initial concentrations are up to several orders of magnitude more concentrated than what has been shown to be toxic in cell cultures. The immediate response to bee venom injection is significant edema at the site of injection (Lariviere and Melzack, 1996) which will immediately reduce the local concentration and facilitate absorption. Further research is needed to determine concentrations of bee venom and its components at the site of inflammation and in the CNS sites of microglia, for instance, to be able to place the *in vitro* studies in the context of effects of local or distant bee venom injection on local or systemic inflammatory disease.

Currently, it cannot be explained precisely how a distant bee venom injection has an anti-inflammatory effect, nor why injections given earlier in the development of adjuvant-induced arthritis are more effective. Effects of bee venom components could be mediated, at least in part, through the activation of the hypothalamic–pituitary–adrenal axis and the release of corticosteroids with anti-inflammatory effects (Ovcharov et al., 1976; Vick and Shipman, 1972; Vick et al., 1972). In response to intraperitoneal melittin injection, rats show elevated plasma corticosterone levels for as much as 48 h after injection compared to saline injected rats (Dunn and Killion, 1988). This time course is similar to that of edema and hyperalgesia after intraplantar bee venom or melittin injection (Lariviere and Melzack, 1996; Chen et al., 1999b; Chen and Chen, 2000). When a second melittin injection was administered 3 d later, the elevation lasted as much as 8 d after injection (Dunn and Killion, 1988). This time course is similar to that of the anti-inflammatory effect of repeated injections of whole bee venom in adjuvant-induced arthritic rats (Chang and Bliven, 1979). Thus, corticosteroids likely play a significant role in the observed anti-inflammatory effects, especially when live bee stings are administered to rodents (Lee et al., 2005b). Other possible mechanisms include central muscarinic type 2 receptors and activation of sympathetic preganglionic neurons (Yoon et al., 2005) and the release of catecholamines from the adrenal medulla (Kwon et al., 2003).

4.3.2. Counter-irritation-induced antinociception

Reduction of inflammation, be it via local suppression of pro-inflammatory genes or other mechanisms, is expected to contribute to antinociception in inflammatory nociception assays. Bee venom injection also likely reduces inflammatory (and other) pain behaviors independently of anti-inflammatory effects as antinociception has been reported in the mouse hot plate test of thermal nociception which does not involve inflammation at the time of testing (Chen et al., 1993). Antinociception following injection of whole bee venom or bee venom peptides was observed in the hot plate test 0.5–4 h after injection (Chen et al., 1993), which is consistent with the time course observed across bee venom-induced antinociception studies using acetic acid, formalin or carrageenan injection (Kim et al., 2003, 2005; Kwon et al., 2001a,b, 2005; Lee et al., 2001). This is one reason why it is critical

to report the time of sensory testing after injection of bee venom or its components: to know whether observed effects are acute antinociceptive effects or prolonged anti-inflammatory effects.

Bee venom injection also reduces inflammatory (and other) pain behaviors via counter-irritation, a general phenomenon and approach in which a second nociceptive stimulus is used to reduce the pain reported or pain behaviors observed in a primary painful or nociceptive condition. Although spontaneous nociception after bee venom injection has almost universally not been investigated (and even claimed to not occur) by proponents of BVT, ample evidence reviewed above shows that significant nociception occurs beginning immediately after injection. This nociceptive input reaches the spinal cord and then the brainstem, where descending noxious inhibitory controls (DNIC) can inhibit further transmission of ascending inputs from spinal cord dorsal horn cells. The descending inhibitory systems originating from the rostral ventromedial medulla (RVM) are serotonergic, and those from the locus coeruleus are noradrenergic. In the rodent intraplantar formalin and intraperitoneal acetic acid tests, in the rat model of collagen-induced arthritis, and in a chronic nerve constriction injury neuropathic pain model, bee venom-induced antinociception has been shown to be mediated by descending adrenergic and serotonergic pathways and spinal α_2 -adrenoceptors (Kwon et al., 2001a, 2005; Lee, 2004; Roh et al., 2004; Kim et al., 2005; Baek et al., 2006). Intrathecal injections of the α_2 -adrenoceptor antagonists yohimbine and idazoxan, but not the α_1 -adrenoceptor antagonist prazosin or β -adrenoceptor antagonist propranolol, significantly inhibited bee venom-induced antinociception. In addition, the serotonin receptor antagonist methysergide inhibited antinociception in the formalin test, but the opioid receptor antagonists naltrexone and naloxone were ineffective at reversing the antinociception in all of the nociception assays (Kwon et al., 2001a, 2005; Roh et al., 2004; Kim et al., 2005; Baek et al., 2006). Somatotopic organization of the descending inhibitory system might explain why bee venom-induced antinociception appears to have an effectiveness that is a function of distance from intraplantar formalin injection (Zusanli > gluteal > back; back not effective) or intraperitoneal acetic acid injection (Kwon et al., 2001a,b).

Roh et al. (2006) further suggest that capsaicin-insensitive peripheral afferent neurons destroyed by resiniferatoxin pretreatment mediate bee venom-induced antinociception (tested in the formalin test), which led them to conclude that BV acupuncture produces antinociception “without nociceptive behavior in rodents”. In the same study, they report that injection of up to 10 mg/kg bee venom (0.20–0.25 mg/mouse) into the Zusanli acupuncture point does not produce pain behaviors of licking or biting, showing a maximum of 10–15 s of the behaviors per 5-min period 16–30 min after injection of only bee venom (Roh et al., 2006). This same amount of bee venom injected in the rat hind paw produces near maximal mean pain scores (of paw shaking, licking, and lifting or guarding combined) for about 1 h in rats (Lariviere and Melzack, 1996) and 0.05 mg produces significant licking of the injected paw in inbred mouse strains for 0.5–8 min of the 60 min after injection (or up to 13% of the time spent paw licking) (Lariviere et al., 2002).

4.3.3. Anti-inflammatory and antinociceptive bee venom components

Melittin has been shown to have anti-inflammatory or antinociceptive effects in several of the same models and studies of bee venom's effects (Billingham et al., 1973; Park et al., 2004; Kwon et al., 2005), but not unanimously (Stuhlmeier, 2007). The mode of anti-inflammatory action of melittin is not yet known with great certainty due in part to the equivocal results between studies of expression of pro-inflammatory transcription factors and genes (Park et al., 2004; Stuhlmeier, 2007). Melittin,

comprising half the dry weight of whole bee venom and producing pain and inflammatory responses (Stuhlmeier, 2007) similar to that of twice the dose of bee venom, could produce antinociception via the same descending inhibitory mechanisms as whole bee venom. Boiling melittin for 10 min, but not whole bee venom or the water-soluble subfraction of bee venom containing melittin, inactivates the anti-inflammatory effects, demonstrating that there are other anti-inflammatory components of bee venom (Kwon et al., 2005). Other components have been shown to have anti-inflammatory properties, including apamin, MCD peptide and adolapin. Although comprising only 1–3% of dry bee venom each, they could contribute to the overall anti-inflammatory effect of whole bee venom since apamin has been shown to inhibit inflammation in models including subcutaneous serotonin- and dextran-evoked paw edema (Ovcharov et al., 1976), and subcutaneous injection of MCD peptide (previously peptide 401) can effectively inhibit carrageenan-induced paw edema and turpentine induced protein plasma extravasation (Billingham et al., 1973). Adolapin has been shown to be antinociceptive in the intraperitoneal acetic acid visceral inflammatory nociception model and in the Randall-Selitto mechanical pressure nociception model and to be anti-inflammatory in models of carrageenan-, prostaglandin-, and adjuvant-induced paw edema (Shkenderov and Koburova, 1982). Interestingly, the same study showed that adolapin was more effective at the lower dose tested, 20 $\mu\text{g}/\text{kg}$, than at the higher dose of 100 $\mu\text{g}/\text{kg}$.

Overall, the experiential evidence for the effectiveness of bee venom injections to relieve chronic ongoing pain in people is tentative at best. The evidence from studies of animal models is somewhat more consistent and robust, but often lacks critical controls that bias the results towards interpretation and conclusion that bee venom injection has only beneficial effects.

5. Conclusions

5.1. Efficacy and effectiveness

In rodent models of nociception and inflammation, bee venom injection is clearly capable of inhibiting inflammation and nociceptive behaviors. In patients, the degree of effectiveness still requires further study with larger scale, better designed trials. Nonetheless, the practice of BVT for pain relief continues despite inherent risks of the procedure.

5.2. Safety

The recently well-characterized adverse effects of bee venom injection on nociceptive mechanisms in the peripheral and central nervous systems reviewed above should further dissuade widespread use of BVT due to the risk of development of prolonged pathological pain states. In addition, a pain syndrome dubbed beekeeper's arthropathy (Cuende et al., 1999) has been reported to develop over 2–14 days after repeated bee stings to beekeepers in Spain harvesting honey in the month of August. Although not all beekeepers develop the signs and symptoms, monoarticular arthritis developed every episode in this study, with occasional oligoarticular disease developing. The acute reaction subsides 15–30 days later, but repeated acute episodes are suspected to result in chronic symptoms. Further systematic study is required since this report has an obvious selection bias, studying only beekeepers who have reported a previous acute arthritis episode (Cuende et al., 1999). However, because the pathogenesis in these beekeepers is poorly understood, caution should be taken before widespread use of bee venom injection as a pain relief method. The components of bee venom are not without cytotoxic effects as proponents of BVT often claim. Components of, and whole, bee venom have been

shown to evoke signs of cell membrane instability and cellular apoptosis and necrosis. Concentrations of bee venom less than those claimed to be completely innocuous (usually without showing supporting data) have been reported to cause severe disruption of red blood cell membranes and signs of apoptosis and necrosis of several cell types, contrary to bee venom therapy supporters' unsupported claims of a complete lack of toxicity of bee venom. Thus, more information and improved evaluation in clinical trials is required of the frequency and relative risk to patients of adverse side effects prior to widespread adoption of this technique.

The possibility of a severe anaphylactic reaction should be a strong deterrent to widespread use of BVT for pain relief. As indicated above, stings by bees may cause allergic reactions in sensitized individuals, including systemic or anaphylactic reactions (Golden, 1989, 2006; Kay and Lessof, 1992; Bernstein et al., 2008; Simons et al., 2008). Allergy to bee venom is dangerous and life-threatening. Bee venom immunotherapy (VIT, also termed as allergen-specific immunotherapy) is a medical treatment of patients with hypersensitivity to bee venom by vaccination with increasingly larger doses of an allergen (substances to which they are allergic) to induce immunologic tolerance. VIT began to be in use in the 1970s and has been demonstrated to be an effective medical practice to treat bee sting allergic disorders (Busse et al., 1975; Muller et al., 1979; Bousquet et al., 1988; Kay and Lessof, 1992; Youlten et al., 1995; Blaauw et al., 1996; Muller, 2003; Winther et al., 2006; Bilo and Bonifazi, 2007). Allergen-specific immunotherapy is the only treatment strategy which treats the underlying cause of the allergic disorder. The most important early clinical studies of VIT set out to identify allergens from patients with hypersensitivity to bee venom (Busse et al., 1975; Paull et al., 1977; Muller et al., 1979, 1985; Bousquet et al., 1988; Schumacher et al., 1992; Adamek-Guzik, 1994; Blaauw et al., 1996) or to identify plasma antisera from beekeepers or VIT-treated patients with bee venom tolerance (El and Habermann, 1956; Franklin and Baer, 1975; Bar-Sela and Levo, 1981; Kemeny et al., 1983; Ferrante et al., 1986; Urbanek et al., 1986; Pastorello et al., 1987; Eich-Wanger and Muller, 1998; Konno et al., 2005a,b, 2006). On one hand, it was found that enzymes including phospholipase A2 (PLA₂) and other substances contained in bee venom are major allergens to cause systemic reactions (Blaser et al., 1998; Devey et al., 1989; Hoffman et al., 1977; Kammerer et al., 1997; Kettner et al., 2001). On the other hand, increased IgG4 levels and decreased IgE levels were also found in patients with effective VIT or bee venom tolerance (Kemeny et al., 1983; Ferrante et al., 1986; Urbanek et al., 1986; Pastorello et al., 1987; Jutel et al., 1995; McHugh et al., 1995). It was also found that VIT resulted in decreased IL-4 and IL-5 and increased IFN- γ secretion in allergen-specific T cells, implicating a change in this T cell activity evoked by VIT (Jutel et al., 1995). These clinical discoveries of the major allergens and mechanisms underlying VIT-associated immunological tolerance led to the current safe and effective use of the therapy in clinic. Moreover, the intensive studies of allergens from bee venom ingredients provided enormous lines of solid evidence supporting a possibility to use BVT with other nonallergenic and effective bee venom ingredients to treat rheumatic arthritis and other autoimmune disorders in clinical settings.

5.3. Recommendations

Mechanisms of nociception and hypersensitivity following bee venom injection are now extremely well characterized. The mechanisms of anti-inflammation and antinociception are much less well characterized. Significant methodological issues persist and do not permit widespread use of bee venom therapy for pain

relief without caution regarding relative effectiveness or benefit versus potential risks for allergic reactions and significant iatrogenic changes of nociceptive processing. Although bee venom injection may be a last resort therapeutic option for those not helped by currently available mainstream methods, and for whom chronic arthritis pain produces debilitation that is highly disruptive, further research is warranted.

5.4. Future research directions

One hurdle to progress in the field has been the study of either deleterious or beneficial effects with little regard for the opposing effects. The field is severely lacking research intended to bridge the understanding that bee venom injection is both acutely pro-inflammatory and pro-nociceptive, but can also be anti-inflammatory and antinociceptive. Proponents of BVT continue to produce findings that are interpreted erroneously as suggesting that bee venom has only beneficial effects. Further mechanistic studies are needed of the beneficial effects, while heeding and simultaneously examining the possible adverse effects that will ultimately determine the clinical utility of widespread use the method of bee venom injection for pain relief. These intersections may occur where nociceptive and immune systems intersect, for instance.

The results of these two camps of scientific research could probably reflect the whole processing of long-existing medical practices, BVT. Moreover, because BVT is characterized by its effectiveness upon autoimmune diseases (e.g., rheumatic arthritis), there may be a biological link between VIT-induced immunological tolerance and BVT-induced pain relief. What are the factors linking immunological and neurological domains for the effectiveness of BVT? What is the role of pain-signaling systems in mediating bee venom-induced immunological responses (disease processing) and immunological tolerance (disease healing)? Is there parallel processing between nociceptive responses (pain-activation) and immunological responses (anaphylaxis and local inflammation) or between nociceptive tolerance (pain-inactivation) and immunological tolerance (disappearance of allergy and local inflammation) after repeated bee stings? Testing a new working hypothesis highlighting that interactions between pain and immunological system are required for a beneficial response to BVT may prove to be a valuable next step of study.

Genetic studies of the relative sensitivity to the deleterious and beneficial effects may also be informative. Are the mechanisms of relative sensitivity different suggesting that some individuals may be more sensitive to the beneficial effects than the adverse effects or vice versa? Are there distinct or overlapping dose–response relationships for the various effects? Do the effects on different tissues contribute more to the beneficial or adverse effects? Is BVT effective in the long term? Are there any adverse consequences of long term BVT? Many fundamental questions remain regarding the ability of BVT to benefit patients with chronic pain that future research needs to address. As Western medicine becomes more amenable to include alternative and complementary treatments such as BVT, it is critical that future research be as strict in design and interpretation as possible in the immediate future.

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