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Bai-Chuang Shyu · Makoto Tominaga
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Chapter 1
Molecular Mechanisms of the Sense of Touch: An Overview of Mechanical Transduction and Transmission in Merkel Discs of Whisker Hair Follicles and Some Clinical Perspectives

Jianguo G. Gu

Abstract The Merkel disc is a main type of tactile end organs for sensing gentle touch and is essential for sophisticated sensory tasks including social interaction, environmental exploration, and tactile discrimination. Recent studies have shown that Merkel cells are primary sites of mechanotransduction using Piezo2 channels as a molecular transducer in Merkel discs. Furthermore, tactile stimuli trigger serotonin release from Merkel cells to excite their associated whisker Aβ-afferent endings and transmit tactile signals. The tactile transduction and transmission at Merkel discs may have important clinical implications in sensory dysfunctions such as the loss of tactile sensitivity and tactile allodynia seen in patients who have diabetes and inflammatory diseases and undergo chemotherapy.

Keywords Merkel disc · Tactile sensation · Whisker hair follicles · Mechanical transduction and transmission · Sensory dysfunction

1.1 Introduction

Tactile end organs, including Merkel discs, Pacinian corpuscles, Meissner's corpuscles, and Ruffini endings, are specialized microstructures in the periphery of mammals [2, 43, 44, 77]. These tactile end organs are crucial to sensing mechanical stimuli including a gentle touch and performing sophisticated sensory tasks such as environmental explorations, social interactions, and tactile discrimination [44]. The Merkel disc is a main type of tactile end organs clustered at touch-sensitive areas

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(touch domes) of glabrous and hairy skin as well as hair follicles of mammals [39, 77]. Structurally, Merkel discs are composed of Merkel cells and their associated Aβ-afferent nerve endings to form a structure of disc-shaped expansion [32, 72]. Merkel discs have high tactile acuity and are very sensitive to skin indentation, pressure, hair movement, and other tactile stimuli. Tactile stimuli to Merkel discs in the touch domes of the skin and whisker hair follicles result in slowly adapting type 1 (SA1) responses, the characteristic Aβ-afferent impulses for tactile encoding [8, 9, 39, 44, 72]. Functionally, SA1 responses in fingertips and whisker hair follicles are essential for tactile discrimination of an object's texture, shape, and other physical properties [9, 13, 44]. Although Merkel discs were discovered more than 140 years ago [54] and numerous studies tried to understand mechanisms underlying tactile functions of Merkel discs [12, 25, 29, 31, 40, 53, 55, 56, 66], only recently have we started to uncover how Merkel discs convey tactile stimulation into sensory impulses to result in tactile behavioral responses [41, 52, 73]. This chapter will provide a brief overview of our studies on tactile transduction and transmission in whisker hair follicles of rodents and also other studies on this topic using touch tomes of rodent skin preparations. In addition, some clinical perspectives in sensory disorders from these studies will be discussed.

1.2 Whisker Hair Follicles and Merkel Discs

Whisker hair follicles are one of the most important tactile organs in nonhuman mammals. Whisker hair follicles in rodents are mostly located on whisker pads, the two sides of the facial regions above the upper lip (Fig. 1.1a). There are five rows of whisker hair follicles or whisker hairs that are present on each whisker pad. They are designated as rows A to E. In addition, four very large whisker hair follicles bearing large whisker hairs are located at the caudal site of each whisker pad and are designated as α to δ (Fig. 1.1b) [10]. Each whisker hair follicle is innervated by a bundle of whisker afferent fibers that are derived from the V2 branch of trigeminal afferent nerves. Most of whisker afferent fibers (~70%) are large myelinated Aβ-afferent fibers, but there are also other types of afferent fibers including Aδ-fibers and unmyelinated c-afferent fibers in each whisker afferent bundle [22].

Sensory inputs following tactile stimuli to whisker hairs are mainly conveyed by whisker Aβ-afferent fibers polysynaptically to the barrel cortex to produce tactile sensations. Whisker hair follicles can be dissected out from rodent whisker pads easily (Fig. 1.1) and used to make recordings from whisker afferent bundles and to study tactile responses. Whisker hairs and whisker hair follicles are present in almost all nonhuman mammals but not humans. However, human fingertips and whisker hair follicles are functionally similar in that they both are important for performing tactile tasks. Structurally, tactile transducing machinery in whisker hair follicles also well resembles those in human fingertips. Therefore, whisker hair follicles provide an excellent model to study tactile transduction and transmission with high relevance to the human.

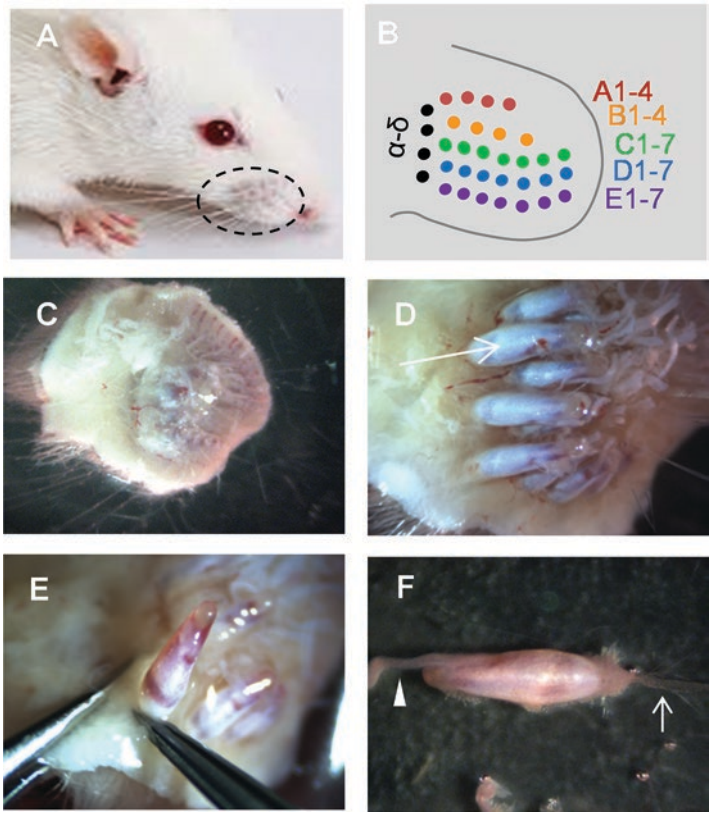

Fig. 1.1 Whisker hair follicles in rat whisker pads. (**a**) A circle outlines the location of the whisker pad in a rat. (**b**) Diagram illustrates the organization of whisker hairs in a rat whisker pad. Each whisker hair is represented by a round dot and designated according to its location in a whisker pad. (**c**) A whisker pad was cut off from the face of a rat and placed in a dish with tissue side (inside) up. (**d**) Images show the tissue side after the removal of fat tissues. Individual whisker hair follicles can be clearly seen. One of them is indicated by an arrow. (**e**–**f**) One whisker hair follicle is pulled out from the whisker. In (**f**) arrow indicates a whisker hair and arrowhead indicates a whisker afferent bundle

General structures of a whisker hair follicle relevant to our electrophysiological studies include a tough whisker follicle capsule, a whisker afferent nerve bundle, a cavernous sinus, a ring sinus, glassy membranes, an outer root sheath, and a whisker hair shaft (Fig. 1.2). Detailed structures of whisker hair follicles in different species have been described elsewhere [10]. There are a number of tactile nerve endings that have been identified in whisker hair follicles, including Merkel disc endings, circumferential and longitudinal lanceolate endings, and other morphologically distinct tactile endings [10]. Merkel cells are mainly located in the enlargement part of a whisker hair follicle underneath the ring sinus. In addition, there are also some Merkel cells that are located at the ridge collar of whisker hair follicles. Merkel cells

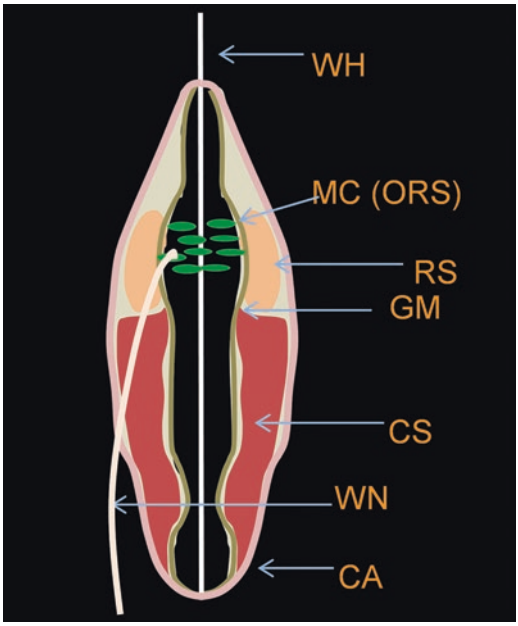

Fig. 1.2 Schematic diagram shows some main structures of a rat whisker hair follicle. *WH* whisker hair shaft, *MC* Merkel cells, *ORS* outer root sheath, *RS* ring sinus, *GM* glassy membranes, *CS* cavernous sinus, *WN* whisker nerve, *CA* capsule

reside in the outer root sheath (ORS), a cell layer underneath the glassy membranes in a whisker hair follicle (Fig. 1.2). Merkel cells can be specifically labeled by the fluorescent dye quinacrine and ready to be identified under a fluorescent microscope [27]. Under electronic microscopy, majority of Merkel cells were found to be closely contacted by whisker Aβ-afferent nerve endings in whisker hair follicles. Merkel cells and their associated Aβ-afferent nerve endings form a synaptic-like structure termed Merkel discs [39, 54]. Whole whisker hair follicles with attached afferent nerve bundles (Fig. 1.1) have been previously used to record whisker afferent nerve impulses in responses to whisker hair movement [7]. The most prominent response induced by whisker hair movement is the slowly adapting type I (SA1) responses. SA1 responses are believed to be transduced by Merkel discs. In addition to whisker hair follicles and human fingertips, Merkel discs are also located at other touch-sensitive spots called touch tomes throughout mammalian body [39, 56]. Using nerve fiber recordings, previous studies found that mechanical stimulation to Merkel discs of the skin led to afferent nerve impulses [39, 69]. Merkel discs were found to be most sensitive to sustained indentation of the skin with very small receptive field, and the sustained indentation generated SA1 responses, similar to the SA1 impulses of whisker afferents following tactile stimulation to whisker hair follicles [39, 43]. The SA1 responses of Merkel discs are believed to be sensory encodings essential for tactile discrimination for the texture and shape of an object [13, 19, 47].

1.3 Mechanical Transduction and Transmission at Merkel Discs of Whisker Hair Follicles

Molecular mechanisms underlying tactile-induced SA1 responses were not known for long time. There had been long debates about roles of Merkel cells at Merkel discs as whether these cells were tactile transducing cells or simply supportive cells. In fact, Merkel cells were believed to be merely supportive tissues for nerve endings' functions rather than actively transducing tactile signals [29]. Early studies on this issue using chemical deletion of Merkel cells generated controversial results [40, 63]. It has recently been shown that SA1 responses to light touch were lost in the mice with Merkel cells genetically deleted [53]. However, trying to demonstrate tactile sensitivity in Merkel cells were failed in some previous studies [74]. Using the in situ patch-clamp recordings on Merkel cells of rat whisker hair follicles [42], Ikeda et al. [41] recently first observed that mechanical stimulation of Merkel cells evoked rapid adapting mechanically activated (MA) currents (Fig. 1.3). In the same time period, two other groups also recorded MA currents from cultured Merkel cells from mouse skin touch tomes [52, 73]. Interestingly, MA currents in Merkel cells of rat whisker hair follicles were found to be able to drive Merkel cells to fire Ca^{2+} action potentials, indicating that Merkel cells not only are mechanically sensitive but also electrically excitable [41]. However, in the studies with cultured Merkel cells [52, 73], these cells fail to fire action potentials. This probably is due to an alteration of the expression of voltage-gated ion channels under culture conditions rather than species differences. This is because out study using in situ patch-clamp recordings from Merkel cells of mouse whisker hair follicle preparations also showed action potential firing in Merkel cells in response to membrane depolarization [16]. Alternatively, Merkel cells in touch tomes of the skin and in whisker hair follicles may be different in their excitability.

Seeking for molecular identities for Merkel cell's tactile transducers turned out to be challenging. Mechanical transducing currents were recorded earlier in invertebrates' touch-sensing nerves, and molecules mediating these mechanically activated currents were defined in previous studies [15, 46, 75]. In *Caenorhabditis elegans*, DEG/ENaC channels transduce touch stimuli to excite touch-sensing neurons [21, 38]. Mammalian homologues to *C. elegans* DEG/ENaC channels are expressed in mammalian sensory neurons [26, 58], but deletion of these channels in mice either does not lead to touch defects [20] or only produces modest defects [58]. In Drosophila larvae, no mechanoreceptor potential C (NOMPC) channels have been shown to be touch transducers, and their activation by light touch directly excites Drosophila mechanosensory neurons [75]. However, mechanically activated currents in Merkel cells were found to be kinetically different from those invertebrates' mechanical transducers. While Ikeda et al. were seeking for mammalian mechanical transducers mediating MA currents in Merkel cells [41], Coste et al. cloned and identified Piezo ion channels (Piezo1 and Piezo2) as mechanically activated ion channels (MA) in several mammalian tissues [17]. Piezo2 channels are found to be highly expressed in dorsal root ganglion (DRG) neurons, mediating rapidly adapting

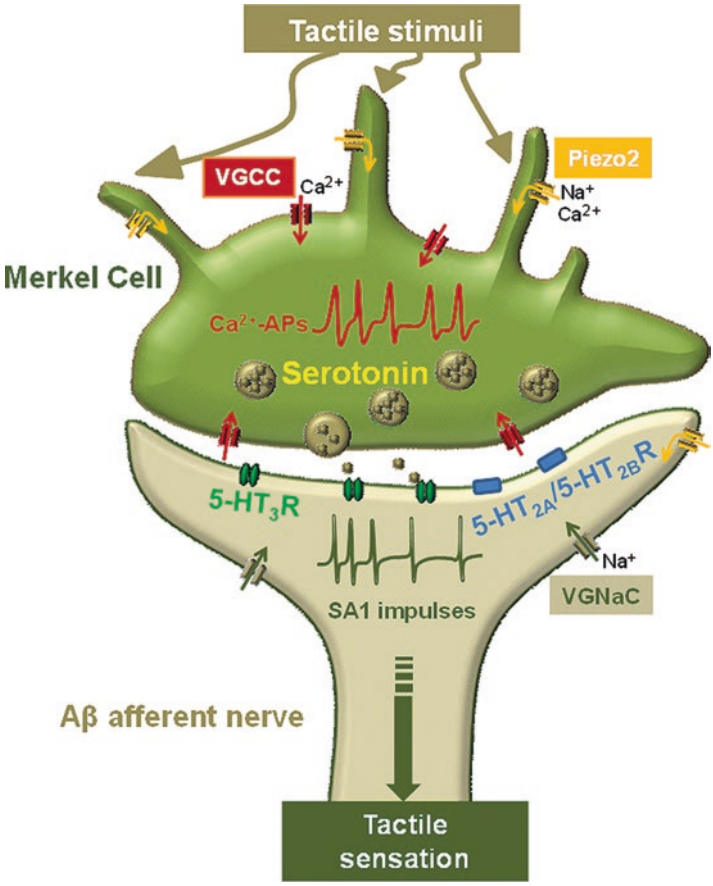

Fig. 1.3 Illustration of transduction and transmission of tactile stimuli at Merkel discs of whisker hair follicles. Tactile stimuli to whisker hairs stretch Merkel cells to activate Piezo2 channels expressed on Merkel cells. This leads to Na^+ and Ca^{2+} ions entering into Merkel cells to cause membrane depolarization and subsequently to fire Ca^{2+} action potentials. Elevation of Ca^{2+} levels following the Ca^{2+} action potentials triggers the vesicular release of serotonin from Merkel cells to the endings of whisker Aβ-afferent fibers. Serotonin then activates 5-HT_3, 5-HT_{2A}, and 5-HT_{2B} receptors to excite the nerve endings and evoke slowly adapting type 1 (SA1) responses. The SA1 impulses are conveyed by the whisker afferent fibers to the CNS to produce tactile sensations

MA currents in these sensory neurons [17, 23, 49]. However, the original study showed that there was minimal expression of Piezo2 transcripts in the skin of mice [17]. Nevertheless, in rat whisker hair follicles, Ikeda et al. [41] showed that Peizo2 channels were expressed in Merkel cells but not in epidermal keratinocytes. The authors further showed that Merkel cells are primary sites of tactile transduction with the Piezo2 ion channel as the key Merkel cell mechanical transducer. Piezo2

channels transduce tactile stimuli into MA currents, which depolarize Merkel cell membrane and elicit Ca^{2+} action potentials in Merkel cells. The Ca^{2+} action potentials on Merkel cells then drive Aβ-afferent nerve endings to fire slowly adapting impulses. Furthermore, Piezo2 channels and Ca^{2+} action potentials in Merkel cells were shown to be essential for behavioral tactile responses (Fig. 1.3) [41]. Consistent with the study of Ikeda et al. [41], two other studies using Piezo2 knockout mice demonstrated that Piezo2 channels were also mechanical transducers in mouse touch tome Merkel cells involving SA1 impulses and tactile behavioral responses [52, 73]. In addition, these studies also suggest that Piezo2 channels are expressed in Aβ-afferent endings of Merkel discs in skin touch tomes and are involved in early phase (dynamic phase) of SA1 responses.

Merkel cells using Piezo2 channels for tactile transduction [41, 52, 73] raises a question as how tactile signals can be further transmitted from Merkel cells into SA1 impulses on whisker Aβ-afferent fibers. One hypothesis was that tactile signals on Merkel cells are transmitted to whisker Aβ-afferent nerve endings by chemical messengers or transmitters [18, 32, 51]. This hypothesis challenges the classical model of somatosensory transmission which occurs first in the dorsal horn of the spinal cord and brain stem [6, 76]. However, several lines of evidence support the chemical transmission hypothesis. For example, earlier studies showed that Merkel cells contain dense-core vesicles, and these vesicles were thought to store glutamate, ATP, serotonin, substance P, enkephalin, and other chemical messengers [32, 39]. Furthermore, molecular profiling of Merkel cells identified transcripts of synaptic release machinery such as synapsin, synaptotagmin, and vesicular glutamate transporter 2 in Merkel cells [30]. Unfortunately, earlier pharmacological experiments testing this hypothesis did not reach a clear conclusion since these studies used very high concentrations of pharmacological reagents that very like produced no-specific effects [24, 34, 57]. In addition, previous studies also did not identify any excitatory receptors for the proposed transmitters at Aβ-afferent endings of Merkel discs [67]. Thus, the chemical messengers stored in Merkel cell vesicles were thought at one time to be autocrine and/or paracrine to modify Merkel disc's functions [32, 51, 68] rather than to be transmitters to directly elicit tactile impulses at Aβ-afferent endings in Merkel discs. Recently, by using whisker hair follicle preparations, Chang et al. [16] have convincingly shown that Merkel cells release 5-HT in responses to mechanical stimulation (Fig. 1.3). The 5-HT release from Merkel cells is Ca^{2+}-dependent and shows synaptic transmitter release properties. Furthermore, Aβ-afferent nerve endings that are associated with Merkel cells express ionotropic $5\text{-}HT_3$ receptors and metabotropic $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors, and activation of these receptors by endogenously released 5-HT excites Aβ-afferent nerve endings to result in SA1 impulses [16] (Fig. 1.3). Thus, the mechanisms underlying both mechanical transduction and transmission in Merkel discs of whisker hair follicles are now elucidated following years of exploration.

1.4 Perspectives in Sensory Disorders Including Pain and Numbness from the Studies with Whisker Hair Follicles

The elucidation of tactile transduction and transmission mechanisms at Merkel discs may have pathological implications in sensory abnormalities including tactile allodynia and numbness. Tactile allodynia is an exaggerated pain state triggered by innocuous stimuli such as a gentle touch. Tactile allodynia occurs following peripheral nerve injury, tissue inflammation, and other pathological changes in a number of disease conditions including diabetic neuropathy, chemotherapy-induced peripheral neuropathy, traumatic nerve injury, and arthritis. Experimentally, tactile allodynia can be induced by peripheral injection of capsaicin to activate nociceptive c-afferent fibers. Capsaicin-induced tactile allodynia is thought to be caused by central sensitization in the spinal cord dorsal horn so that tactile inputs carried by non-nociceptive Aβ-afferent fibers can lead to the activation of nociceptive pathway in the spinal cord dorsal horn [62, 71]. Ikeda et al. [41] showed that following capsaicin injection into whisker pads Piezo2 channels in Merkel cells could transduce tactile stimuli into nocifensive behavioral responses. This result suggests that Piezo2 channels on Merkel cells can be involved in tactile allodynia and Piezo2 channel blockers may be used to prevent the transduction of allodynic tactile inputs.

Another aspect of pathological implication of Merkel disc transduction and transmission is the loss of tactile sensitivity or numbness in patients following chemotherapy or other disease conditions. Chemotherapy drugs including taxanes (e.g., paclitaxel), vinca alkaloids (e.g., vincristine), and platinums (e.g., oxaliplatin) are essential anticancer drugs for treating a wide range of solid and hematological malignancies [4, 14], and they are indispensable in saving lives of many cancer patients. However, more than 80% of cancer patients who receive chemotherapies develop chemotherapy-induced peripheral neuropathy (CIPN) that manifested with numbness, tingling sensation, and pain [4, 45, 48, 64]. These sensory dysfunctions occur soon after the start of a chemotherapy regimen and persist from months to years beyond the completion of chemotherapy [48, 50]. CIPN negatively impacts function and quality of life and is a significant clinical problem. Double-blinded and placebo-controlled clinical trials show that clinical management of CIPN with currently used drugs such as gabapentin, lamotrigine, baclofen, ketamine, and amitriptyline are not better than placebos [5, 28, 50, 60, 61]. Interestingly, duloxetine, a serotonin and norepinephrine transporter inhibitor branded as Cymbalta by Eli Lilly [11], has recently been shown to be effective in alleviating pain and recovering tactile sensitivity from numbness in a 5-week clinical trial with daily oral dose at 60 mg [65]. Since Merkel discs are serotonergic synapses, it raises a possibility that serotonin transporter inhibitors such as duloxetine may potentiate tactile transmission at Merkel discs to alleviate numbness in CIPN. Thus, a deep understanding of tactile transduction and serotonergic transmission may help for the development of highly effective management for CIPN [14].

Numbness in hands and/or feet is typically the first symptom of CIPN that persists for months to years [3]. Interestingly, numbness in hands and feet is largely confined in fingertips and toes [70], where Merkel discs are highly abundant [32]. Animal models of CIPN well recapitulate the painful CIPN that manifests with tactile allodynia and cold allodynia [1, 33, 36, 37], but numbness aspect of CIPN has not been well studied. Whisker hair follicles are functionally equivalent to human fingertips in that they have high tactile sensitivity and acuity [13, 35, 59]. Whisker hair follicles are also structurally similar to human fingertips in that they also have high abundance of Merkel discs [10, 41]. Thus, whisker hair follicles may offer a new and highly relevant model system to study whether Merkel discs are targeted by chemotherapy drugs to contribute to the numbness aspect of CIPN.

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Chapter 2
TRP Channels in Nociception and Pathological Pain

Chen-Yu Hung and Chun-Hsiang Tan

Abstract Thermal and noxious stimuli are detected by specialized nerve endings, which transform the stimuli into electrical signals and transmit the signals into central nervous system to facilitate the perception of temperature and pain. Several members within the transient receptor potential (TRP) channel family serve as the sensors for temperature and noxious stimuli and are involved in the development of pathological pain, especially inflammatory pain. Various inflammatory mediators can sensitize and modulate the activation threshold of TRP channels and result in the development of inflammatory pain behaviors. A brief review of the role of TRP channels in nociception and the modulatory mechanisms of TRP channels by inflammatory mediators, focusing on TRPV1, TRPA1, and TRPM2, will be presented. Recent advances in the development of therapeutic strategies targeting against TRP channels will also be reviewed.

Keywords Nociception · Pain · TRP · TRPV1 · TRPA1 · TRPM2

2.1 Introduction

"Pain," the word that comes from Greek goddess of revenge, Poine, describes an unpleasant experience that is elicited by noxious stimuli. Such experience often serves as a warning flag and reminds an individual of avoiding or eliminating the encountered threats. Pain can be divided into three categories including nociceptive

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pain, inflammatory pain, and neuropathic pain [66]. Nociceptive pain is generated by specialized nerve endings (nociceptors) with a relatively high activation threshold compared with those responsible for the sensation of light, sound, smell, and taste. Nociceptors serve as sensors for strong mechanical stimuli, chemical irritants, and noxious thermal stimuli, and only stimuli that are potentially capable of causing tissue injuries can reach the threshold to activate nociceptive nerve terminals and generate actions potentials, which are then transmitted and perceived as pain signals [21]. Transformation of these stimuli into electrical signals and transduction of the action potentials involve the participation of multiple receptors and channels located on free nerve endings and synaptic terminals. As a group of multimodal cation-permeable channels that depolarize the cells, TRP channels are involved in various aspects of physiological function, including nociception [70].

TRP channels were first discovered in *Drosophila melanogaster* and viewed as mutant structures exhibiting only a transient receptor potential (TRP) rather than normal sustained potential in response to light [16]. Later on, mammalian homologs of TRP channels were discovered [89], which then opens the opportunity for further investigation on the functional roles of TRP channels. To date, 28 mammalian TRP channels have been identified and are divided into 6 subfamilies according to their sequence homology: canonical or classic (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML), and ankyrin (TRPA) [77]. Among them, several types of TRP channels have been found to be involved in the generation of pain, including TRPV1 [13], TRPV2 [93], TRPV3 [58], TRPV4 [80], TRPA1 [60, 91], TRPM2 [30], and TRPM8 [27]. Each channel has its unique characteristics and contributes to the generation of various pain behaviors including heat hyperalgesia, mechanical hyperalgesia, cold allodynia, and inflammatory hyperalgesia.

As for the roles of TRP channels in inflammatory pain, inflammatory mediators can sensitize or alter the threshold of TRP channels, leading to pain behaviors including thermal hyperalgesia, mechanical allodynia, and spontaneous pain [41]. In this review, we will focus on the molecular mechanisms of TRP channel modulation in the generation of nociception and the development of inflammatory pain, focusing on TRPV1, TRPA1, and TRPM2.

2.2 TRPV1

Among the members within the TRP channel family, TRPV1 is the one that has been most thoroughly investigated, and its pivotal role in sensing noxious stimuli and generating pain in primary afferent nociceptors has also been demonstrated across different studies [41]. Since the successful cloning of TRPV1 in 1997, follow-up studies have identified TRPV1 as a cation-permeable channel which is responsive to thermal stimuli in the range of noxious heat (over 43 °C) [82] and changes in pH [19]. As a polymodal channel, TRPV1 can also be activated by vanilloids (e.g., capsaicin from chili peppers or anandamide from inflammation process)

[72], vanillotoxins [73], and protons [3]. The finding of TRPV1 being expressed almost exclusively in C-fibers indicates its role as a sensor for noxious stimuli [46]. Furthermore, the activity of TRPV1 can be enhanced by a variety of inflammatory mediators, including bradykinin, ATP, and nerve growth factor (NGF), through second messenger-signaling pathways such as phospholipase C (PLC) and protein kinase A (PKA) [56]. The sensitization and activation of TRPV1 in peripheral nociceptors lead to the transmission of the noxious signals to the central nervous system and, hence, the production of unpleasant and painful sensation warning the body of potentially harmful threat [41, 66].

The success in the application of cryo-microscopy to understand the molecular structure of TRPV1 has enabled us to gain deeper understanding in the gating mechanism of TRP channels [12]. TRPV1 is composed of four identical protein subunits assembled into a functional and cation-permeable channel [12]. Each subunit contains six transmembrane segments, a loop constructing pore helix between segment five and six, and intracellular N- and C-termini with two restriction points in the pore helix defined as the selectivity filter and the lower gate [12, 28]. During inactive state, both the selectivity filter and the lower gate are constricted, and the pathway for ion conduction is blocked. An intracellularly located hydrophobic pocket, the so-called vanilloid pocket, is composed of the external surface of the S3–S4 helices, S4–S5 linkers, and S6 helix [12, 32]. It allows small vanilloid molecules, such as resiniferatoxin (RTX) and capsaicin, to cross the plasma membrane to bind and allosterically modulate the pore, more precisely, expanding the lower gate of the pore domain. As for the extracellular outer pore region, the binding of chemicals, such as double-knot toxin (DkTx), or stimulation with thermal stimuli cause substantial conformational changes of TRPV1, resulting in marked change in the relative position of the pore helix in the outer pore region. The change of the relative position of the pore helix may also break down the potential hydrogen bonding formed between the amino acids on the chains within the outer pore region in resting state resulting in the widening of the selectivity filter. It is rather remarkable that the upper and lower gates could be allosterically coupled and regulate the activation of the channel. Such synergy between different levels of gates could contribute to the coordination of disparate physiologic signals [12].

Under physiological condition, TRPV1 has been shown to be co-expressed with PKCβII in a subset of sensory neurons. In these neurons, TRPV1 binds directly to PKCβII, which in return markedly enhances the responses of TRPV1 by phosphorylating TRPV1 at T705 [52]. The differences in the basal phosphorylation of TRPV1 at T705 may explain the differences in the threshold of TRPV1-expressing neurons to heat stimuli [45]. In addition, TRPV1-PKCβII complex-containing neurons have been suggested to represent a subset of hypersensitive nociceptive neurons [95]. Not only does TRPV1 play a pivotal role in generating proportionate pain under physiological condition, it also contributes to the generation of action potentials during inflammation, leading to pathological pain behaviors such as thermal hyperalgesia, spontaneous pain, and mechanical allodynia [7, 13, 56]. In response to tissue damages, numerous inflammatory mediators such as eicosanoids (e.g., prostaglandin E2), neuropeptides (e.g., substance P and bradykinin), excitatory amino acids (e.g.,

glutamate), leukotrienes, and cytokines (e.g., TNF-α, IL-6and INF-γ) [90] are released, and the inflammatory mediators lower the mechanical and thermal thresholds of the exposed sensory neurons, a process called "sensitization" [41]. Besides acting as a downstream target of proalgesic factors, TRPV1 itself can also trigger the secretion of neuropeptides, including substance P and calcitonin gene-related peptide (CGRP) upon activation [39, 91]. The neuropeptides secreted then bind to specific receptors expressed on the surrounding cells, such as lymphocytes, dendritic cells, mast cells, and macrophages, which then trigger a series of reactions involved in immune responses [4]. This process is known as neurogenic inflammation, and the involvement of TRPV1 in the development of inflammatory pain is clearly demonstrated by the significantly reduced thermal hyperalgesia in TRPV1 knockout mice after tissue injury [13, 18].

The mechanisms responsible for the exaggerated response of TRPV1 during inflammatory state are associated with protein kinases [8], which modulate the activities of proteins through phosphorylation. Phosphorylation of TRPV1 can be facilitated by inflammatory mediators through multiple protein kinases including cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC), phosphatidylinositol-3 kinase (PI3K), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), and extracellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK/MAPK) [56]. Receptors of several inflammatory mediators, including prostaglandin receptors (e.g., prostaglandin E2 receptor 2, prostaglandin E2 receptor 4, I prostanoid receptor, and prostaglandin D2 receptor 1), 5-hydroxytryptamine(5-HT) receptors and endothelin ET_A receptors, are G protein-coupled receptors (GPCRs) which are coupled to the Gs type of Gα subunit [54]. When these inflammatory mediators bind to GPCRs, adenylyl cyclases are activated and cause increase of intracellularly cAMP level and full activation of PKA [78].

Another mechanism modulating the function of TRPV1 is through the PLC/PKC pathway. Receptors for inflammatory mediators such as histamine H1, bradykinin B2, protease-activated receptor-2 (PAR2), prostaglandin E2 receptor 1, substance P, neurokinin 1 (NK1), and purinergic P2Y are also GPCRs. Instead of being coupled to Gs type of Gα subunit, they are coupled to Gαq type of Gα subunit which then initiate the activation of phospholipase C (PLC). The activation of PLC leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and results in the production of two second messengers: 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [25]. Some studies showed results suggesting that PIP_2 causes inactivation or desensitization of TRPV1, and the hydrolysis of PIP_2 by PLC results in the activation of TRPV1 [25, 69]. However, there are conflicting results showing that direct application of PIP_2 causes TRPV1 activation and absence of PIP_2 results inTRPV1 inactivation [71]. Another study showed that TRPV1 can be fully functional in the absence of PIP_2, suggesting that PIP_2 contributes to the sensitization of TRPV1 by disinhibiting the channel [11]. In addition, PIP_2 was shown to activate TRPV1 even in the absence of capsaicin, though the current intensity was much smaller than that elicited by capsaicin. The result suggests that PIP_2 is a positive regulator of TRPV1 and the difference of amplitude caused by the presence of capsaicin may

indicate that PIP_2 serves as a cofactor rather than pure agonist of TRPV1 [44]. The lack of conclusion for the role of PIP_2 on the regulation of TRPV1 indicates the sophisticated modulation of TRPV1. Meanwhile, DAG stimulates PKC and subsequently leads to the activation of TRPV1 [25, 35]. However, one study showed that 1-oleoyl-2-acetyl-sn-glycerol (OAG), an analog of DAG, causes TRPV1 activation in rat dorsal root ganglion neurons in the presence of chelerythrine, a PKC inhibitor, suggesting that DAG is a direct endogenous ligand of TRPV1 and the TRPV1 activation induced by OAG is independent of PKC. In addition, the study also showed that the binding site of DAG is similar to that of capsaicin, though the effect of DAG on TRPV1 is much smaller than that of capsaicin [92]. All these results indicate the involvement of Gαq-PLC pathway in the modulation of TRPV1 by inflammatory mediators.

The TRPV1 modulatory mechanisms mentioned above are basically at post-translational level, which includes phosphorylation and proteolysis of protein subunits, resulting in the change of the activity of TRPV1. However, inflammatory mediators have a more comprehensive effect on TRPV1. For example, NGF, a neurotrophic factor that binds to tropomyosin receptor kinase A (trkA) [74], modulate TRPV1 through several different aspects, including level of transcription, translation, and posttranslation, and such interactions are also suggested to be responsible for the development of thermal hyperalgesia during inflammation [51]. One study showed an increased level of NGF and higher percentage of neurons expressing TRPV1 following inflammation induced by intraplantar injection of Freund's complete adjuvant, and inhibiting the effect of NGF with anti-NGF was shown to prevent the increased TRPV1 expression within trk-A positive neurons and lessen the thermal hyperalgesia induced by inflammation [1]. These results indicate the mechanisms modulating TRPV1 at transcription or translation level.

2.3 TRPA1

As a member of TRP channel family, TRPA1 and TRPV1 share some common features, including the similarity in the structures consisting of six transmembrane domains with intracellular N- and C-termini and ion permeability to cation nonselectively. Within vertebrate TRP channel family, TRPA1 is characterized by a long N-terminus with multiple ankyrin repeats along with three critical cysteine residues. These three cysteine residues are located in the linker region connecting the ankyrin-rich domain to the transmembrane domain and are involved in the channel activation by electrophiles [34, 41, 67]. In addition, TRPV1 and TRPA1 are co-expressed in a specific subgroup of dorsal root ganglion neurons that is responsible for the detection and transduction of noxious stimuli [23]. Interestingly, TRPA1 was shown recently, to work together with TRPV1 and TRPM3 for acute noxious heat sensing in mice [84].

Like TRPV1, TRPA1 is also a polymodal receptor and has been shown to be activated by numerous natural pungent chemicals, including allyl isothiocyanate

(AITC), cinnamaldehyde, and allicin, which are all electrophiles [85]. One intriguing question is the mechanism by which these structurally diverse electrophiles serve as specific agonists for TRPA1 activation and modulation. Instead of structural specificity, the activation of TRPA1 by the pungent chemicals depends on the covalent modification of cysteine residues on the N-terminal of the channel. The covalent modification causes conformational change in protein structure and modulates the channel permeability [34, 57].

Furthermore, presence of calcium ions may also play a pivotal role in TRPA1 modulation. TRPA1 currents evoked by some agonists, such as AITC and cinnamaldehyde, were shown to be potentiated in the presence of extracellular Ca^{2+} [40, 63]. Meanwhile, TRPA1 activation by icilin requires the presence of calcium, and adding BAPTA (an intracellular calcium chelator) to the pipette solution significantly reduces icilin-evoked currents. This indicates that intracellular calcium serves as a co-agonist of TRPA1 [20, 87]. Modulation of intracellular calcium on TRPA1 through direct binding to an EF-hand-like motif within intracellular N-terminus has been suggested by several studies [20, 96]. However, some other studies suggest that the activation might be contributed by Ca^{2+}-binding protein calmodulin [31]. Both suggestions have been demonstrated in genetic deletion model with both positive and negative results [20, 31, 87]. These divergent results implie that the underlying mechanism is complicated and further revaluation is needed.

Besides pungent chemicals, TRPA1 can also be activated by environmental irritants, such as acrolein [5], formalin [60], and metabolic by-products of chemotherapeutic agents. The activation of the TRPA1-expressing C-fibers in the respiratory tract and bladder was proposed to be associated with the development of airway and urinary tract symptoms, as evidenced by the findings showing that TRPA1 agonists evoke coughing in both guinea pig and human volunteers and TRPA1 antagonists attenuate symptoms of cyclophosphamide-induced hemorrhagic cystitis [9, 61].

Apart from the activation by the chemicals mentioned above, TRPA1 is activated or potentiated during inflammatory state as well. During tissue injury, the reactive oxygen species generated cause superoxidation of membrane phospholipids and result in the production of 4-hydroxy-2-nonenal (HNE), which then causes activation of TRPA1 [2, 83]. The inhibition of the pain-related behaviors elicited by 4-HNE injection with TRPA1 antagonists and the absence of the pain-related behaviors in TRPA1-deficient mice demonstrate the importance of TRPA1 in mediating the effect of 4-HNE in the development inflammatory pain [2, 83]. In addition, the binding of bradykinin to bradykinin receptor B2 causes activation of PLC and PKA, and results in the enhancement of the TRPA1 current activated by AITC or cinnamaldehyde [86]. All the results above indicate the involvement of TRPA1 in the induction of acute pain and hyperalgesia during inflammation.

2.4 TRPM2

Interactions between neurons and immune cells contribute substantially to the initiation of pathological pain, in which neurogenic inflammation and generation of reactive oxygen species and reactive nitrogen species (ROS/RNS) are of fundamental importance. TRPM2 is a member within TRP channel family that plays a crucial role in serving as the downstream target of ROS/RNS [42] and can be activated by micromolar levels of H_2O_2 and agents producing ROS/RNS [29, 88]. TRPM2 is a cation channel characterized by a nudix hydrolase (NUDT9) homology region in the intracellular C-terminus, which was suggested to be responsible for channel activation and modulation by intracellular adenosine diphosphate ribose (ADPR) [48, 68]. In addition, cADPR and NAADP have synergistic effect with ADPR, as evidenced by the finding showing that the EC_{50} for cADPR and NAADP decrease significantly from 44 to 3 μM and from 95 to 1 μM, respectively, in the presence of subthreshold levels of ADPR (100 nM) [49]. However, whether they bind directly to the Nudix box motif as ADPR or to distinct synergetic sites or had been converted to ADPR beforehand remains unclear. ADPR can also be produced extracellularly. Extracellular NAD^+ can be catalyzed into ADPR, cADPR, and NAADP with the enzymatic activity of nicotinamide adenine dinucleotide nucleosidase, such as CD38 [64] and CD157 [38] that are extensively expressed on hematopoietic and non-hematopoietic cells [55, 77]. However, as the binding site of ADPR for the activation of TRPM2 seems to be located inside the plasma membrane, whether and how these extracellularly formed ADPR crosses the membrane and activates TRPM2 channels is not entirely known. Nevertheless, extracellular ADPR has been reported to modulate the activity of TRPM2 indirectly from the extracellular area through activation of P2Y receptors [50] and PLC [36]. In either pathways, the activation of P2Y receptors and PLC results in an increase in intracellular calcium concentration and leads to the enhancement of TRPM2 channel sensitivity toward ADPR. Intracellular Ca^{2+} has also been shown to serve as a coactivator of TRPM2, and a minimum of 30 nM intracellular calcium concentration is required to cause partial TRPM2 activation with ADPR in the absence of extracellular Ca^{2+} [17, 76]. In addition to the metabolites and ROS/RNS mentioned above, TRPM2 can also be activated by thermal stimuli with an activation threshold at temperature above 35 °C [81]. Meanwhile, the temperature threshold for TRPM2 activation has also been shown to be lowered in the presence of H_2O_2, a phenomenon termed “sensitization” [43].

TRPM2 is ubiquitously expressed among various tissues (e.g., central nervous system, peripheral nervous system, bone marrow, and heart) and in different cell types (e.g., pancreatic β-cells, endothelial cells, microglial cells, neurons, and immune cells) [33, 37, 47, 65]. Importantly, the expression of TRPM2 in the phagocytic lineages (e.g., neutrophils and monocytes/macrophages) of immune cells enables the cells to respond to signals of ROS/RNS [49, 94].

The TRPM2 expressed in sensory neurons in dorsal root ganglion plays an important role in thermosensation and nociception. In the experiment investigating

the effect of TRPM2 on thermal preference, wild-type male mice showed preference for a 33 °C plate over a 38 °C plate, while TRPM2 knockout male mice showed no such preference. The results indicate that the genetic deletion of TRPM2 causes a remarkable behavioral change in the thermal preference [79]. In addition to the crucial role of TRPM2 in the warmth sensation, TRPM2 has also been shown to be involved in the pathogenesis of various chronic pain in several studies. When tested with von Frey filament test for mechanical sensitivity and Hargreaves and hot plate test at 52 °C and 55 °C for noxious heat sensitivity, wild-type and TRPM2 knockout mice showed no difference in their basal sensitivity. However, TRPM2 knockout mice showed attenuated nocifensive responses when injected intraplantarly with formalin. In carrageenan-induced inflammatory pain and sciatic nerve injury-induced neuropathic pain models, in which the expression of TRPM2 mRNA in the inflamed paw and the area around the injured sciatic nerve were found to be increased, mechanical allodynia and thermal hyperalgesia were attenuated in TRPM2 knockout mice [30]. In addition, the mechanical allodynia in the monosodium iodoacetate-induced osteoarthritis pain model, the mechanical allodynia in paclitaxel-induced peripheral neuropathy and streptozotocin-induced painful diabetic neuropathy models have all been shown to be significantly attenuated in TRPM2 knockout mice [75]. In addition, econazole, a TRPM2 inhibitor, was shown to reduce the visceromotor response to noxious colorectal distention in rats in both baseline condition and trinitrobenzene sulfonic acid-induced colitis model. Furthermore, TRPM2 knockout mice showed significantly reduced visceral hypersensitivity induced by trinitrobenzene sulfonic acid. The results mentioned above all demonstrate the crucial role of the TRPM2 expressed in both sensory neurons and immune cells for the development of various types of pain.

2.5 TRP Channel and Analgesic Drug Development

TRP channels have attracted much attention for the development of analgesic agents. Huge effort has been attempted in the development of antagonists of TRPV1 to treat inflammatory pain and cancer-related pain since the results demonstrating the attenuation of thermal hyperalgesia in inflammatory pain model in TRPV1 knockout mice [13, 18]. A TRPV1 antagonist, AMG 9810, was shown to be effective at preventing capsaicin-induced eye wiping and reversed thermal and mechanical hyperalgesia induced by intraplantar injection of complete Freund's adjuvant [26]. One study evaluated the effects of a TRPV1 antagonists, SB-705498, in humans and showed that SB-705498 reduced the area of capsaicin-evoked flare, increased the heat pain threshold on non-sensitized skin, and increased heat pain tolerance at the site of UVB-evoked inflammation [15]. The results all demonstrate the great potentials of TRPV1 as a therapeutic target for treating chronic pain. However, most previous TRPV1 antagonist programs have now been put on hold, due to the unwanted on-target side effects. One side effect was the development of marked hyperthermia after TRPV1 blockade, which caused the early termination of

phase I clinical trials with AMG 517 for dental pain in humans [15]. Furthermore, TRPV1 antagonists also elevate noxious heat sensation threshold and cause higher risk of burn injuries in individuals receiving TRPV1 antagonists. Several TRPV1 antagonists (e.g., MK-2295 [62], SB-705498 [15] and JNJ-39439335 [59]) have been reported to have such adverse effect in human studies. Although direct blockade of TRPV1 causes the adverse effects mentioned above, an alternative strategy of developing therapeutic agents disrupting the sensitization of TRPV1 is showing promising effect. By disrupting the interactions between TRPV1 and AKAP79, the sensitization of TRPV1 under pathological conditions can be inhibited without changing the normal physiological function of TRPV1 [10]. An effective cell permeable peptide capable of preventing TRPV1-AKAP79 interaction was shown to be analgesic in three mouse models of inflammatory hyperalgesia without causing hyperthermia or decreased sensitivity to noxious heat [24]. The approach demonstrates the potentials for developing therapeutic agents targeting against TRPV1.

Meanwhile, TRPA1 antagonists also show some promising results in treating pathological pain. When applying TRPA1 selective antagonists, attenuation in mechanical hypersensitivity was shown in animal inflammatory and neuropathic pain models [14, 22]. Adverse effect regarding body temperature regulation (such as hyperthermia) is not common after TRPA1 antagonist application. However, another concern that has been brought up is whether TRPA1 antagonism will compromise the ability to elicit protective actions against harmful hazards, such as coughing, sneezing, and generation of nociception to eliminate foreign irritants. These protective responses were reported to be absent in TRPA1 knockout mice [6]. Whether TRPA1 antagonists will cause the loss of such protective reflexes will be challenges for the development of therapeutic agents targeting against TRPA1. Furthermore, TRPM2 channel has been proposed to be a therapeutic target for a wide variety of oxidative stress-related diseases including cardiovascular and cerebrovascular diseases. However, effects of therapeutic strategies targeting against TRPM2 selectively have not been reported, and future efforts are needed for the development of such therapeutic agents for clinical use [53].

2.6 Conclusions

We have gained much deeper understanding in the functions of TRP channels in nociception and chronic pain in the last two decades. However, the modulatory mechanisms of TRP channels are still not entirely known, which can have enormous effects on the chronic pain state. However, it is also due to this sophisticated and complex design that provides us the chance to develop therapeutic strategies for pain relief without affecting the physiological functions of TRP channels. Meanwhile, analgesic agents targeting against TRP channels without side effects are still under development. Hopefully, in the future, medication targeting against TRP channels and related pathways will be brought into clinical use with fewer side effects to fight against refractory pain and other associated disorders.

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Chapter 3
Involvement of TRPV1-ANO1 Interactions in Pain-Enhancing Mechanisms

Y. Takayama and Makoto Tominaga

Abstract Primary sensory neurons detect potentially dangerous environmental situations via many "sensor" proteins located on the plasma membrane. Although receptor-type cation channels are thought to be the major sensors in sensory neurons, anion channels are also important players in the peripheral nervous system. Recently, we showed that transient receptor potential vanilloid 1 (TRPV1) interacts with anoctamin 1 (ANO1, also called TMEM16A) in primary sensory neurons and that this interaction enhanced TRPV1-mediated pain sensation. In that study, we induced ANO1 currents by application of capsaicin to small DRG neurons and showed that ANO1-dependent depolarization following TRPV1 activation could evoke more action potentials. Furthermore, capsaicin-evoked pain-related behaviors in mice were strongly inhibited by a selective ANO1 blocker. Together these findings indicate that selective ANO1 inhibition can reduce pain sensation. We also investigated non-specific inhibitory effects on ion channel activities to control ion dynamics via the TRPV1-ANO1 complex. We found that 4-isopropylcyclohexanol (4-iPr-CyH-OH) had an analgesic effect on burning pain sensations through its inhibition of TRPV1 and ANO1 together. Additionally, 4-iPr-CyH-OH did not have clear agonistic effects on TRPV1, TRPA1, and ANO1 activity individually. These results indicate that 4-iPr-CyH-OH could function globally to mediate TRP-ANO1 complex functions to reduce skin hypersensitivity and could form the basis for novel analgesic agents.

Keywords TRP channel · Anoctamin 1 · Isopropylcyclohexanol · Acute pain

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3.1 Introduction

Transient receptor potential (TRP) channels are involved in a diverse range of physiological functions, including pain sensation. TRP channels expressed in primary sensory neurons can be activated by several different physical and chemical stimuli, including temperature changes and irritants [34]. However, the output phenotypes produced by these stimuli are not solely dependent on TRP channel activities. As is well known, almost all TRP channels have high calcium permeability [9]. This calcium influx could affect other calcium-dependent proteins located within a micrometer range of the channel pore [22]. Anoctamin (ANO) is one of the calcium-dependent proteins [1, 26, 37]. We recently showed that the calcium-activated chloride channel ANO1 (also known as TMEM16A) can be strongly activated by calcium influx through TRPV1 activation and that TRPV1-ANO1 interaction is involved in pain enhancement [30]. This chapter reviews the recent findings concerning TRP interactions in sensory systems and potential strategies for pharmacological control of the ion dynamics.

3.2 TRPV1: ANO1 Interaction

Both TRPV1 and ANO1 are expressed in primary sensory neurons and are involved in acute pain sensation [30]. TRPV1 is activated by various natural ligands, including capsaicin, resiniferatoxin, bivalent tarantula toxin, acid, and noxious heat [13]. Rat TRPV1 is phosphorylated at Ser502 and Ser800 by protein kinase C epsilon (PKCε) activated in response to signaling by G protein-coupled receptors (GPCR), including the bradykinin receptor and P2Y receptor [32, 38]. This PKCε phosphorylation is mediated by A-kinase anchoring proteins [38]. Because phosphorylation reduces the threshold for TRPV1 activation, phosphorylated TRPV1 can be activated at temperatures lower than core body temperature [32]. This characteristic is thought to be involved in molecular mechanisms that cause inflammatory pain. Therefore, TRPV1 is a primary target for pain therapy. However, the chloride channel ANO1 is also thought to play a major role in generating pain signals in primary sensory neurons due to its heat sensitivity and immediate activation following GPCR activation [3, 18]. ANO1 directly interacts with the IP_3 receptor on the endoplasmic reticulum (ER) membrane [11]. Interestingly, TRPV1 and ANO1 are also co-expressed in small dorsal root ganglia (DRG) neurons [2]. We previously demonstrated an interaction between TRPV4 and ANO1 in choroid plexus epithelial cells [29]. Similar to TRPV1, TRPV4 has high calcium permeability (Na^+:Ca^{2+} = 1:10). Therefore, calcium entering the cell rapidly induces ANO1 activation followed by secretion of fluids such as cerebrospinal fluid, saliva, and tears [6, 29]. We thus investigated whether TRPV1-ANO1 interaction occurs in DRG neurons and the physiological relevance of this interaction.

Typically, we began by conducting an electrophysiological analysis using whole-cell patch-clamp recording in HEK293T cells expressing TRP channels and ANO1. The main composition of the bath and pipette solutions in these assays is N-methyl-D-glutamine chloride (NMDG-Cl), and the free calcium in the pipette solution was maintained at 100 nM using 5 mM O,O′-Bis (2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid (BAPTA). To study TRPV1-ANO1 interactions, we activated TRPV1 by applying 300 nM capsaicin, which is approximately the half effective concentration, although in DRG neurons the concentration is 1 μM [15]. Under these conditions, large chloride currents that could induce cell shrinkage at −60 mV holding potential were observed in cells expressing TRPV1 and ANO1, but not cells expressing TRPV1 or ANO1 alone. Moreover, these currents were abolished in a calcium-free bath solution and a reversal potential shift occurred in NMDG-aspartate bath solution. These results clearly suggest that calcium influx through TRPV1 activation strongly induces ANO1 activation. Furthermore, immunoprecipitation results indicated that TRPV1 and ANO1 directly interact. Thus, TRPV1 directly and functionally interacts with ANO1 although ANO1 alone could be activated by global calcium increases depending on ER calcium stores and voltage-gated calcium channels on plasma membrane [12].

3.3 Pain-Enhancing Mechanisms in DRG Neurons

The physiological activity of ANO1 is dependent on concentration differences in extracellular and intracellular chloride. Interestingly, in many DRG neurons, the intracellular chloride concentration is reportedly higher than in other neurons, such as those in the central nervous system [20]. The equivalent potential in DRG neurons containing high chloride can reach −20 mV, and the resting potential is approximately −60 mV. Therefore, ANO1 activation should induce depolarization due to chloride efflux and neuronal excitations. To examine this possibility, we performed the same experiments as those for HEK293T cells using isolated small DRG neurons. In whole-cell patch-clamp recordings, capsaicin-induced currents decreased by half following application of the selective ANO1 inhibitor T16Ainh-A01 with a physiological ion concentration in the bath solution (NaCl base solution containing 2 mM $CaCl_2$). The capsaicin-induced current is composed of cations and chloride movements, even though capsaicin-mediated neuronal excitation in DRG neurons was thought to depend only on TRPV1 function. Moreover, action potentials evoked by capsaicin applications were almost completely inhibited by T16Ainh-A01. Together, these results indicate that a TRPV1 and ANO1 interaction should also occur in DRG neurons in the presence of high intracellular chloride concentrations.

However, the efficacy of this interaction remained unclear because some DRG neurons have low concentrations of intracellular chloride. In these neurons, ANO1 could induce hyperpolarization with TRPV1 activation. To clarify whether ANO1 activation following TRPV1 activation is involved in pain generation but not pain

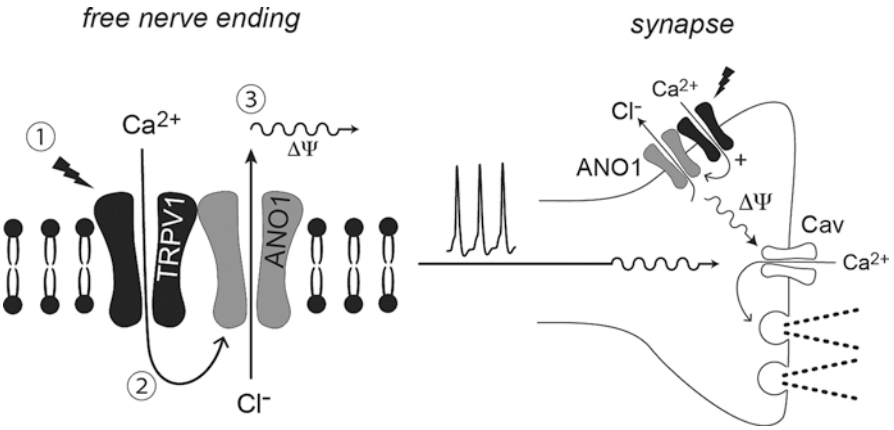

Fig. 3.1 Schematic model of interactions between TRPV1 and ANO1. TRPV1 interacts with ANO1 on both free nerve endings and synapses of DRG neurons. TRPV1 is initially activated and ANO1 is also immediately activated in calcium nano-domains. The ANO1 activation enhances action potential generation (ΔΨ). TRPV1 also interacts with ANO1 on the central side, and the depolarization activates voltage-gated calcium channels. These two pathways are involved in neurotransmitter release from presynaptic regions in secondary neurons of the spinal cord

reduction, we analyzed the effect of T16Ainh-A01 on capsaicin-induced pain-related behaviors in mice. We found that pain-related behaviors were significantly ameliorated by concomitant administration of T16Ainh-A01. Thus, the TRPV1 and ANO1 interaction appears to be involved in pain enhancement, and TRPV1 and ANO1 behave as irritant detector and signal amplifier, respectively, although ANO1 could act as a suppressor in some DRG neurons (Fig. 3.1).

3.4 Analgesic Agents to Target TRPV1-ANO1 Interactions

The specificity of channel antagonists might not always be an important property in pain reduction because selective drugs often have strong side effects that discourage their use in vivo. Moreover, complete reduction of pain is not always desirable in clinical applications because pain pathways can have a protective effect in certain situations, such as avoiding bone destruction in *Candida* infection [21]. An alternative strategy would be to identify an agent that can inhibit several ion channels involved in pain sensation in peripheral regions. For instance, TRPV4 is also thought to be involved in pain sensation, and the weak-specific antagonist, compound 16-8, is more effective at reducing pain than the TRPV4-specific antagonist GSK205 [14]. While investigating the interaction between TRPM8 and ANO1, we fortuitously found that menthol inhibits ANO1 [31]. Although in that study we were unable to characterize the physiological role of the TRPM8-ANO1 interaction, the menthol-related findings were nonetheless interesting because menthol can also

inhibit the TRPV1 activation [28]. However, the ability of menthol to inhibit both ANO1 and TRPV1 is puzzling given the differences in the structures of these channels. TRPV1 and ANO1 have six and ten transmembrane regions, respectively, and TRPV1 is a tetramer, whereas ANO1 is a dimer [5, 17, 23]. We first assessed the effects of other menthol analogues, including menthone, 1,4-cineole, and 1,8-cineole, on ANO1 currents. In whole-cell patch-clamp recordings of HEK293T cells expressing ANO1, only 1,8-cineole lacked a strong inhibitory effect on the ANO1 current induced by high free calcium concentration. Because the chemical structure of 1,8-cinaole is the most divergent among the three analogues tested, we surmised that potential menthol-based agents should contain a critical minimum structure. Therefore, we next investigated the separate moieties comprising menthol. From these studies we showed that isopropylcyclohexane is the core structure needed to completely inhibit ANO1 currents. Since the kinetics of current reduction by isopropylcyclohexane were slower than that for menthol, we focused on 4-isopropylcyclohexanol (4-iPr-CyH-OH), which has greater hydrophilicity, which could be valuable if the affinity site lies in the intracellular domain of the ion channel. According to our expectations, 4-iPr-CyH-OH showed rapid inhibition that was similar to that of menthol. Interestingly, 4-iPr-CyH-OH also inhibits TRPV1, TRPA1, TRPV4, and TRPM8 activity. Thus, 4-iPr-CyH-OH could have inhibitory effects on many different irritation pathways. The half inhibition concentration (IC_{50}) of 4-iPr-CyH-OH for mouse TRPA1, TRPV1, and ANO1 was 0.23, 0.73, and 1.09 mM, respectively (Fig. 3.2). IC_{50} of 4-iPr-CyH-OH in TRPV1 current induced by 100 nM capsaicin was lower than that of ANO1 current. However, the capsaicin at the concentration does not fully activate TRPV1, whereas 500 nM intracellular free calcium strongly activates ANO1 in our experiments. Three hundred micrometer allyl isothiocyanate (AITC) also induces the almost saturated TRPA1 activation. Thus, 4-iPr-CyH-OH could have a lower inhibitory effect toward TRPV1. Furthermore, we investigated the effects of 4-iPr-CyH-OH on capsaicin-evoked action potential in isolated small DRG neurons and capsaicin-induced pain-related behaviors in mice. In these experiments, 4-iPr-CyH-OH completely inhibited capsaicin-evoked action potentials with strong suppression of depolarization, and

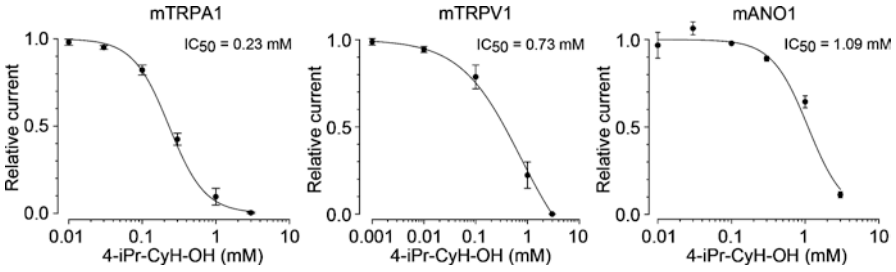

Fig. 3.2 Dose-response curves of 4-isopropylcyclohexanol (4-iPr-CyH-OH) at −60 mV. Mouse TRPA1, TRPV1, and ANO1 expressed in HEK293T cells were activated by 300 μM AITC, 100 nM capsaicin, and 500 nM free calcium, respectively

pain-related behaviors were significantly diminished with concomitant administration of 4-iPr-CyH-OH.

Although 4-iPr-CyH-OH is currently used as a food additive in Japan, the pharmacological understanding of its effects beyond those we found for pain sensation is limited [10, 19]. Thus, 4-iPr-CyH-OH could have potential as a basis for the development of novel drugs that target ion channels, particularly ANO1 and TRP channels.

3.5 Conclusion

TRP-ANO1 interactions are involved in several physiological mechanisms. For instance, TRPC2-ANO1 interaction could be involved in iodide homeostasis in thyroid cells and vomeronasal transduction [7, 33], and TRPC6-ANO1 interaction reportedly enhances vasoconstriction [35]. In addition, our findings indicated that ANO1 activation could generate sufficient depolarization to induce exocytosis in synapses between primary sensory neurons and secondary neurons in the spinal cord (Fig. 3.1). In fact, ANO1-dependent membrane potential changes could accelerate insulin secretion from pancreatic β-cells [4, 36]. Not only ANO1, targeting TRP-ANO interactions could be also a promising approach because ANOs are expressed in the whole body [8, 16, 25, 27], and ANOs have three functions, including chloride channel, scramblase, and internalization [24, 27]. Thus, additional physiological phenomena could be better explained by future investigations that focus on TRP-ANO interactions.

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Chapter 4
Roles of ASICs in Nociception and Proprioception

Cheng-Han Lee and Chih-Cheng Chen

Abstract Acid-sensing ion channels (ASICs) are a group of proton-gated ion channels belonging to the degenerin/epithelial sodium channel (DED/ENaC) family. There are at least six ASIC subtypes – ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4 – all expressed in somatosensory neurons. ASIC3 is the most abundant in dorsal root ganglia (DRG) and the most sensitive to extracellular acidification. ASICs were found as the major player involved in acid-induced pain in humans. Accumulating evidence has further shown ASIC3 as the molecular determinant involved in pain-associated tissue acidosis in rodent models. Besides having a role in nociception, members of the DEG/ENaC family have been demonstrated as essential mechanotransducers in the nematode *Caenorhabditis elegans* and fly *Drosophila melanogaster*. ASICs are mammalian homologues of DEG/ENaC and therefore may play a role in mechanotransduction. However, the role of ASICs in neurosensory mechanotransduction is disputed. Here we review recent studies to probe the roles of ASICs in acid nociception and neurosensory mechanotransduction. In reviewing genetic models and delicate electrophysiology approaches, we show ASIC3 as a dual-function protein for both acid-sensing and mechano-sensing in somatosensory nerves and therefore involved in regulating both nociception and proprioception.

Keywords ASIC3 · DRG · Mechanotransduction · Pain

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4.1 Introduction

Pain-associated tissue acidosis is common in humans and could occur during tissue injury (~pH 6.5), inflammation (pH 5.5–7.0), and ischemia (pH ≤7.1) [32, 46] and with tumors (pH 5.8–7.4) [63] and fatiguing exercise (pH 6.6–6.9) [52]. Somatosensory afferents projected from dorsal root ganglia (DRG), trigeminal ganglia (TG), nodose ganglia, and other ganglia are responsible for detecting such noxious acidosis and transduce the pain signal to the brain [6, 58]. Although acid (protons) can directly evoke pain in humans, the proton-sensing molecules that mediate the acid-induced pain are still unknown [34, 53, 59]. Proton-sensing ion channels and/or receptors expressed in somatosensory neurons include acid-sensing ion channels (ASICs), transient receptor potential (TRP) channels, two-pore potassium channels, and protein-sensing G-protein-coupled receptors. These acid-sensing molecules might be involved in different types of acid-induced nociception, as tested in rodent models.

With efforts in the development of pharmacological tools and genetic mouse models, ASICs and TRPs have drawn much attention for possible roles in acidosis-induced pain, although discrepant results have been reported from clinical studies. Of note, ischemic forms of acidosis lead to the accumulation of lactate, which enhances ASIC activity but inhibits TRP subfamily V1 members [11, 31]. Despite being important acid sensors in pain-associated tissue acidosis, ASICs are increasingly being found also involved in neurosensory mechanotransduction.

Here we review recent efforts from our work and that of others on the roles of ASICs in the somatosensory system, with a focus on nociception and proprioception.

4.2 Acid-Sensing Ion Channels

ASICs are amiloride-sensitive proton-sensing ion channels belonging to the degenerin/epithelial sodium channel (DEG/ENaC) family [61]. At least six ASIC subtypes have been identified in mammals: ASIC1a, ASIC1b (encoded by accn2), ASIC2a, ASIC2b (encoded by accn1), ASIC3 (encoded by accn3), and ASIC4 (encoded by accn4). A functional ASIC channel is composed of three subunits that could be an assembly of three identical ASIC subtypes (homomeric) or a combination of different ASIC subtypes (heteromeric). ASICs are known as proton-gated ion channels, because ASIC1a, ASIC1b, ASIC2a, ASIC3 or a combination of ASIC subtypes mediates a transient or biphasic (transient and sustained) current in response to external acidification in heterologous expression systems. The pH sensitivity among the ASIC subtypes is in the order of ASIC3 > ASIC1a > ASIC1b > ASIC2a [62]. ASIC3 is the most acid-sensitive subtype, being activated at ~pH 7.0, whereas activation of ASIC2a requires acidification decreased to ~pH 5.0. Neither ASIC2b nor ASIC4 is activated by acid, but they could form heteromeric channels with other ASIC subtypes and thus modulate their pH sensitivity and channel properties.

ASICs are widely expressed in the nervous system and many types of non-neuronal cells in the periphery. ASIC1a, ASIC2a, ASIC2b, and ASIC4 are predominantly expressed in the central nervous system. Especially, ASIC1a-containing channels are the major acid sensors in the brain and are involved in detecting tissue ischemia (e.g., stroke), neural inflammation, CO_2 inhalation, metabolic stress, and acidification of the synaptic cleft [19, 62]. In the peripheral nervous system, ASIC1a, ASIC1b, and ASIC3 are the major acid sensors, although ASIC2a and ASIC2b are also detectable. ASIC1b and ASIC3 are predominantly expressed in sensory neurons of dorsal root ganglia (DRG), nodose ganglia, and trigeminal ganglia and thus considered the major players in triggering pain-associated tissue acidosis.

4.3 ASIC3 and Pain

Several lines of evidence have suggested that ASIC3 might be the most important acid sensor involved in acidosis-induced pain. Studies of gene expression and electrophysiology of whole-cell patch clamp recordings have revealed that (1) ASIC3 is widely expressed in different types of nociceptors, (2) ASIC3 is the most abundant subtype expressed in the peripheral nervous system, (3) ASIC3 is the most sensitive ASIC subtype responding to external acidification, (4) ASIC3 is the only ASIC subtype mediating a non-desensitizing current in a mild acidification range, and (5) activation of ASIC3 can be potentiated by lactate [65]. The predominant expression of ASIC3 in nociceptors of deep tissues has provided hints of its involvement in muscle and joint pain, chest pain, and visceral pain [40, 65]. Genetic studies of ASIC3-knockout (*Asic3*$^{-/-}$) mice first proved a role for ASIC3 in pain associated with tissue acidosis in mice. In *Asic3*$^{-/-}$ mice, neurons expressing acid-induced currents were largely decreased in DRG nociceptors of muscle and cardiac afferents [26, 42]. In a mouse model of chronic widespread muscle pain (or fibromyalgia) induced by repeated intramuscular acid injections, long-lasting mechanical hyperalgesia developed in wild-type mice and *Asic1a*-knockout (*Asic1a*$^{-/-}$) mice but was totally abolished in *Asic3*$^{-/-}$ mice [54]. After inflammatory muscle injury, wild-type mice showed both primary muscle hyperalgesia and secondary hyperalgesia in the hind paw (distal to injured muscle). *Asic1a* knockout prevented the development of primary muscle hyperalgesia, whereas *Asic3*$^{-/-}$ prevented the development of secondary paw hyperalgesia [60]. Also, *Asic3*$^{-/-}$ mice failed to show secondary mechanical hyperalgesia induced by knee joint inflammation [30]. In a mouse model of chest pain, *Asic3*$^{-/-}$ mice were blunt to isoproterenol-induced cardiac ischemia and thus susceptible to ischemia-induced cardiac fibrosis [8]. The role of ASICs in visceral pain is, however, not clear, although *Asic3*$^{-/-}$ mice showed increased writhing responses to intraperitoneal acetic acid injection [5].

The identification of the ASIC3-selective antagonist APETx2 from sea anemone has resulted in an excellent tool for examining the role of ASIC3 in rodent models [16]. APETx2 is a 42-amino acid peptide that effectively inhibits the ASIC3-mediated current, with IC_{50} from 63 nM to 2 μM depending on the ASIC subtype

composition [13]. Accordingly, sub-micromolar amounts of APETx2 inhibited ASIC3-mediated currents in DRG nociceptors and the development of acidosis-induced pain in rodent models of inflammatory, postoperative, and muscle pain [65]. Also, APETx2 inhibited acid-induced hyperalgesic priming, the nociceptor plasticity required for pain chronicity, in muscle nociceptors [7]. Of note, the role of ASIC3 in acidosis-induced pain is closely related to acute pain induction, hyperalgesic priming, and/or chronic pain development but not chronic pain maintenance, because local blockade of the ASIC antagonist A-317567 in the muscle did not inhibit pain hypersensitivity in animals already showing chronic pain induced by repeated intramuscular acid injections [22]. However, this situation may not be the case in a model of rheumatoid arthritis: rheumatoid arthritis-induced mechanical hyperalgesia was long-lasting for more than 12 weeks in wild-type mice but was reduced in the late phase (after 6 weeks) in *Asic3*$^{-/-}$ mice [29].

With the development of pharmacological and genetic tools, accumulating evidence has shown ASIC3 as essential for the development of chronic pain associated with tissue acidosis occurring during inflammation and ischemia and with tumors (Table 4.1). That ASIC3 is largely expressed in a variety of nociceptors further emphasized the importance of its role in acidosis-induced pain. However, recent studies have revealed the expression of ASIC3 in resident macrophages in the muscle and its involvement in fatigue-induced hyperalgesia [24]. Surprisingly, removal of resident macrophages in muscle also prevented the development of chronic widespread pain induced by repeated acid injections [23]. Because ASIC3 is expressed in both muscle nociceptors and resident macrophages in muscle, further genetic studies are required to dissect the roles of ASIC3 in acidosis-induced pain.

Besides ASIC3, peripheral ASIC1a and ASIC1b are involved in pain associated with tissue acidosis in models of inflammatory pain [17, 60]. The ASIC1a/1b-selective antagonist mambalgin, a 57-amino acid peptide isolated from snake venom, was effective against inflammatory pain and neuropathic pain in rodents [15]. The roles of other ASIC subtypes in nociception remain to be addressed.

4.4 ASIC3 and Mechanotransduction

Besides having a role in acid-induced nociception, ASICs are proposed to be involved in neurosensory mechanotransduction, which was revealed in behavioral and/or in vivo physiological assays in mice lacking *Asic1a*, *Asic2*, and *Asic3* [6]. Especially, *Asic3*$^{-/-}$ mice showed many deficits in neurosensory mechanotransduction, such as acidosis-induced mechanical hyperalgesia [55], blood volume expansion-induced diuresis [37], pressure-induced vasodilation [20], and acoustic brainstem response [66]. However, *Asic3*$^{-/-}$ mice also showed enhanced mechanical sensitivity in response to cutaneous tactile stimuli [2, 5]. This unexpected enhanced mechanosensitivity of *Asic3*$^{-/-}$ mice might reflect in part the mixed effects of *Asic3* knockout on ex vivo recordings of skin-nerve preparations showing enhanced activity of rapidly adapting mechanoreceptors and reduced activity of A-fiber mechanoreceptors [47]. In comparison, *Asic1a*$^{-/-}$ mice showed no change of cutaneous

Table 4.1 Representative studies of research into pain-associated tissue acidosis in animal models

Sensor	Partners	Stimulation	Sites	Pain	Species	References
ASIC1	ASIC2	Carrageenan	Gastrocnemius muscle	Muscle pain	Mouse	[55]
ASIC3						[70]
						[60]
ASIC3		Occlusal interference	Masseter muscle	Muscle pain	Rat	[67]
TRPV1						
ASIC3		Muscle fatigue	Gastrocnemius muscle	Muscle pain	Mouse	[24]
ASIC3		Ischemia and reperfusion	Brachial artery	Ischemia myalgia	Mouse	[51]
ASIC3	P2X5	Acidic saline	Gastrocnemius muscle	Fibromyalgia	Mouse	[54]
TRPV1						[1]
						[7]
						[50]
ASIC3		Acidic saline	Knee joint	Joint pain	Mouse	[30]
		Carrageenan				[56]
ASIC3		Mono-iodoacetate	Knee joint	Osteoarthritis	Rat	[33]
ASIC3	TDAG8	CFA	Ankle joint	Rheumatoid arthritis	Mouse	[29]
	TRPV1					
ASIC3		Acidic saline	Gastrocnemius muscle	Inflammatory pain	Mouse	[12]
		CFA	Hindpaw		Rat	[70]
		Carrageenan				[38]
ASIC3	HTR3	Acidic saline	Hindpaw	Mirror-image pain	Mouse	[57]
ASIC3		Skin/muscle incision	Hindpaw	Postoperative pain	Rat	[14]
ASIC3	V-ATPase	Cancer cells	Tibia bone	Cancer pain	Mouse	[27]
					Rat	[73]
ASIC3	ASIC2a	Ischemia	Heart	Chest pain	Mouse	[26]
					Rat	[8]
						[71]
ASIC3		Nucleus pulposus	Dorsal root ganglion	Lumbar radiculopathy	Rat	[36]
ASIC3		Tooth movement	Teeth	Orofacial pain	Rat	[21]
ASIC3		Acidic saline	Ocular anterior surface	Ocular pain	Rat	[3]
TRPV1						
ASIC3		Acidic interstitial fluid	Cranial meninges	Migraine	Rat	[68]
						[69]

mechanoreceptor function [45], whereas *Asic2*$^{-/-}$ mice showed reduced activity of rapidly adapting and slow-adapting mechanoreceptors, with no change in A-fiber mechanoreceptors [48]. Unexpectedly, triple knockout of *Asic1a*, *Asic2*, and *Asic3* enhanced the activity of A-fiber mechanoreceptors but not other cutaneous mechanoreceptors [35]. Although issues of developmental compensation and composition of different ASIC subtypes in heteromeric ASIC channels may complicate the phenotypes of single or triple ASIC-subtype knockout, a direct role of ASICs in neurosensory mechanotransduction is thus questioned [44].

Members of the DEG/ENaC family have been found to be essential mechanical transducers in the nematode *Caenorhabditis elegans* and fly *Drosophila melanogaster* [4, 72]. However, the roles of ASICs, as the mammalian homologues of DEG/ENaC, in neurosensory mechanotransduction (especially as mechanical transducers) have been debated for more than 15 years [18, 44, 48]. One of the major issues is that neither *Asic2* nor *Asic3* knockout affects mechanically activated currents in isolated DRG neurons with use of the mechano-clamp approach [18]. This largely represents a technical problem in the field of mechanotransduction as discussed below.

Neurosensory mechanotransduction concerns the responses of the sensory nervous system to mechanical stimuli, which could be mediated by a variety of mechanically activated ion channels [49]. Our knowledge of the gating mechanisms of mechanically activated ion channels is limited. Two important gating models of mechanically activated ion channels have been proposed. In the bilayer model, channels are directly gated by tension in the cell membrane, and in the tether model, channels are opened by force transmitted from tethered elements of extracellular matrix proteins and/or intracellular cytoskeleton proteins [43]. However, approaches to measure the activity of mechanically activated ion channels are challenging and require sophisticated electrophysiology techniques of whole-cell patch clamp recordings [9, 25]. A whole-cell mechano-clamp technique has been developed to probe the mechanically activated currents mediated by the bilayer model, with a direct pipette indentation on the cell membrane used to induce the mechanically activated currents of cells [25]. The mechano-clamp approach has successfully led to the identification of Piezo proteins and tentonin 3 as mechanically activated ion channels [10, 28]. However, the mechano-clamp technique failed to demonstrate a role for ASICs in mechanotransduction, which is the putative mechanically activated ion channels of the tether model [4, 18].

A substrate deformation-driven neurite stretch (SDNS) approach was recently developed to probe mechanotransduction of the tether model [9, 41]. Different from the mechano-clamp, the SDNS approach can be used to stretch single neurites without contacting the cell membrane and thus could avoid changing the membrane tension and the induction of the mechanically activated current of the bilayer model. We combined the SDNS method with genetic models to show a role for ASIC3 in neurosensory mechanotransduction of proprioceptors [39]. In DRG proprioceptive neurons, SDNS induces an inward current that can be reversibly inhibited by the pan-ASIC blocker amiloride or the ASIC3-selective antagonist APETx2. Likewise, *Asic3* knockout abolishes the SDNS current in DRG proprioceptive neurons but has no effect on mechanically activated currents of the bilayer model. *Asic3*$^{-/-}$ mice also show behavioral deficits in proprioception, which further supports an important role

of ASIC3 in neurosensory mechanotransduction. With the evidence from SDNS studies, we have thus filled in the knowledge gap of in vitro evidence to demonstrate a role for ASIC3 in neurosensory mechanotransduction of the tether model. Moreover, *Asic3* knockout does not affect the mechanically activated currents of the bilayer model in DRG proprioceptive neurons, which can be abolished in mice lacking Piezo2 or tentonin 3 [28, 64].

4.5 Conclusion

The recent studies of ASICs have profoundly changed our knowledge of nociception and mechanotransduction. ASICs, especially ASIC3, contribute to chronic pain development associated with tissue acidosis and also modulate the neurosensory mechanotransduction of proprioceptors (Fig. 4.1). The development of the SDNS approach has been a milestone in probing the roles of each ASIC subtype in

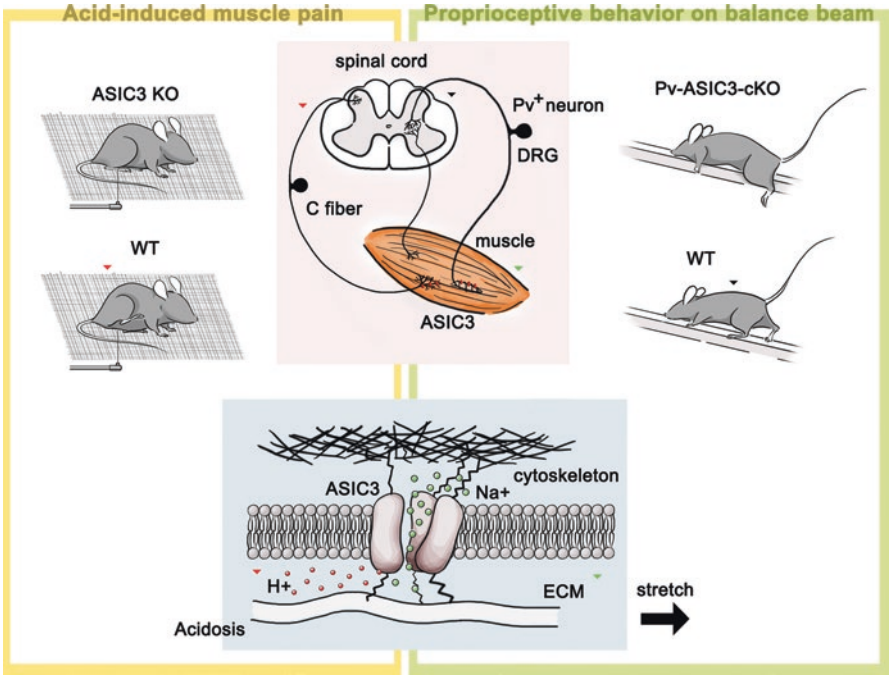

Fig. 4.1 The involvement of acid-sensing ion channel 3 (ASIC3) in acid-sensing of nociceptors and mechano-sensing of proprioceptors. ASIC3 is largely expressed in muscle afferents of nociceptors and proprioceptors. Tissue acidosis in muscle can activate nociceptor ASIC3 and induce muscle pain, which are abolished with ASIC3 knockout. In contrast, forced changes arising from muscle contraction can activate proprioceptor ASIC3 and modulate proprioceptive behaviors. Mice with conditional knockout of ASIC3 in proprioceptors perform poorly when they walk along a balance beam. *WT* wild type

mechanotransduction of the tether model. Moreover, studies of ASIC3 have brought new concepts in biology, including that (1) ASIC3 is a dual-function protein exerting both acid-sensing and mechano-sensing functions; (2) mechanotransducers of the bilayer and tether models can coexist in a single DRG neuron; and (3) DRG proprioceptors are mechanosensitive and also acid-sensitive. We thought we understood the role of ASICs, but actually we do not. Further research is definitely needed to determine how acid-sensing and mechano-sensing could work together in the somatosensory system.

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Chapter 5
Tackling Pain Associated with Rheumatoid Arthritis: Proton-Sensing Receptors

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Abstract Rheumatoid arthritis (RA), characterized by chronic inflammation of synovial joints, is often associated with ongoing pain and increased pain sensitivity. Chronic pain that comes with RA turns independent, essentially becoming its own disease. It could partly explain that a significant number (50%) of RA patients fail to respond to current RA therapies that focus mainly on suppression of joint inflammation. The acute phase of pain seems to associate with joint inflammation in early RA. In established RA, the chronic phase of pain could be linked to inflammatory components of neuron-immune interactions and noninflammatory components. Accumulating evidence suggests that the initial inflammation and autoimmunity in RA (preclinical RA) begin outside of the joint and may originate at mucosal sites and alterations in the composition of microbiota located at mucosal sites could be essential for mucosal inflammation, triggering joint inflammation. Fibroblast-like synoviocytes in the inflamed joint respond to cytokines to release acidic components, lowering pH in synovial fluid. Extracellular proton binds to proton-sensing ion channels, and G-protein-coupled receptors in joint nociceptive fibers may contribute to sensory transduction and release of neurotransmitters, leading to pain and hyperalgesia. Activation of peripheral sensory neurons or nociceptors further modulates inflammation, resulting in neuroinflammation or neurogenic inflammation. Peripheral and central nerves work with non-neuronal cells (such as immune cells, glial cells) in concert to contribute to the chronic phase of RA-associated pain. This review will discuss actions of proton-sensing receptors on neurons or non-neuronal cells that modulate RA pathology and associated chronic pain, and it will be beneficial for the development of future therapeutic treatments.

Keywords Rheumatoid arthritis · Chronic pain · Proton-sensing receptors · Neuron-immune interaction · Gut microbiota

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5.1 Introduction

Rheumatoid arthritis (RA), one of autoimmune diseases, is characterized by chronic joint inflammation leading to cartilage damage and ultimately total joint destruction. RA affects approximately 1% of the global population (the male/female ratio is 3–4:1) and induces significant morbidity and associated economic costs. The National Rheumatoid Arthritis Society estimates that about 9.4 million working days are lost because of RA. RA patients usually declare that pain is their greatest problem and highest priority [9]. Approximately 80% of RA patients rate pain as one of their top three priorities [37, 51]. Although RA drugs were the top 1 best-selling drugs in 2015 and 2016, 50% of RA patients still claimed ineffective treatments for current drugs.

Pain associated with RA is historically attributed to peripheral inflammation in the involved joints. Pro-inflammation mediators (such as TNFα, IL-1β, and IL-6) that play important roles in autoimmune diseases also contribute to the development of RA [30]. A number of agents that block TNFα, IL-1β, and IL-6 have been introduced into clinical trials or are currently marketed. Inflammatory pain symptoms can be partially relieved by nonsteroidal anti-inflammatory drugs (NSAIDs), biological or non-biological disease-modifying antirheumatic drugs (DMARDs), but many patients continue to suffer from moderate pain [96]. More than 10% of RA patients with inflammatory disease remission (disease activity score in 28 joints [DAS28] <2.6) have clinically significant pain (pain numeric rating score ≥4) [56]. There is a weak correlation between the intensity of pain and degree of peripheral inflammation [15, 88]. In early RA, functional impairment is believed to be mostly due to inflammatory process, but in established RA, disability may be due to joint damage [33]. Experimental animal models of inflammatory arthritis suggest that changes of neuronal sensitivity at both peripheral and central levels may be important for the chronic phase of RA pain [80]. In RA patients compared with controls, acute pain induction is associated with elevations in serum TNF-α levels that last for at least 1 h [27], and neutralization of TNF-α rapidly reduces nociceptive activity in central nerves [38]. These results indicate that the normal enhanced release of pro-inflammatory cytokines may contribute both inflammation and pain in early RA and may be the factor leading to development of central pain. However, in patients with established RA, chronic pain could not be only attributed to inflammatory process but also to other types of cells or to other comorbidities associated with RA. It may explain that biological therapies with cytokine inhibitors are effective in some RA patients but fail in others.

The occurrence of pain in conjunction with other centrally mediated symptoms (insomnia, fatigue, memory problems, mood, and sleep disturbances) is frequently observed in patients developing centralized pain [32]. It has been known that 25% of RA patients have a comorbid fibromyalgia (FM) and this rate is higher than the prevalence of FM in the general population (2%) [3]. RA patients with a comorbid FM have a higher disease activity and greater pain [3, 57]. It causes that DAS28 widely used in practice and clinical trials may be insufficient to evaluate real

inflammatory activity in patients with RA associated with chronic pain syndromes. Pain is indeed associated with disease activity [64]; disease activity, however, is not necessarily correlated with inflammatory components in established RA. Current treatments focusing on suppression of inflammation may produce an inadequate response in relieving chronic pain in many RA patients, especially in RA patients with other comorbidities.

Given the complexity of RA pathology, it is not surprising that pain associated with RA is difficult to treat. The causes of RA-associated pain could be different in the early and the late disease stages. The acute phase of pain could be associated with acute joint inflammation, but the chronic phase of pain could be linked to inflammatory components of neuron-immune interactions and noninflammatory components as central pain regulatory mechanisms. In the past decade, accumulating evidence reveals novel factors linked to RA and gains our understandings in RA pathology and associated pain. This chapter will discuss the roles of immune cells, glial cells, nerve fibers, and gut microbiota in RA and how proton-sensing receptors in these cells work in concert to contribute to the chronic phase of RA-associated pain.

5.2 Immune Cells

RA is characterized as a chronic inflammation in the synovial tissues, and such inflammation leads to progressive and erosive destruction of cartilage and bones. The main features of RA are the pannus formation and synovial hyperalgesia due to severe proliferation of macrophage-like and fibroblast-like synoviocytes (FLS) and infiltration of immune cells (such as neutrophils, macrophages, T lymphocytes, B lymphocytes, and dendritic cells) [30]. Macrophage-like and fibroblast-like synoviocytes in the intimal lining produce pro-inflammatory cytokines and degradative enzymes (as metalloproteinases, serine proteases, aggrecanases), which maintain inflammation, digest the extracellular matrix, promote osteoclast maturation and activation, and finally destroy the articular structure.

Neutrophils are the first effector cells at the site of inflammation and involved in the initiation of inflammatory responses. In the model of K/BxN serum-transfer arthritis, neutrophil depletion only affects the acute phase of inflammation rather than the chronic phase [100], supporting the role of neutrophils in the initiation of inflammation. The involvement of neutrophils in the acute inflammatory phase is to release high concentrations of cytokines, reactive oxygen species (ROS), granules containing enzymes, and so on, leading to inflammatory responses and joint destruction [68, 101]. Apoptotic neutrophils secrete mediators to attenuate inflammation, causing the resolution of inflammation. The impaired or defective clearance of dying neutrophils during inflammation can lead to sustained inflammation. Recent evidence reveals that neutrophils are also important in chronic inflammation and the function is linked to T-helper (Th) 17 cells [65, 85]. Neutrophils secrete chemokines, such as CCL20 and CCL2 to sustain Th17 cells for release of interleukin-17 (IL-17) that promotes disease progression and joint destruction [18]. Depletion of

neutrophils decreases IL-17 [65]. Having said that neutrophils have a dormant role in inflammatory responses in RA, neutrophils also contribute to inflammatory hyperalgesia [7, 58, 59]. Action of neutrophils on hyperalgesia could be due to downstream IL-17 production that directly acts on IL-17 receptors to sensitize joint nociceptors [24, 77]. Neutrophils also have anti-inflammatory and anti-nociceptive role by secreting opioid peptides [8].

In addition to FLS, synovial tissues also contain resident macrophages. When resident macrophages become activated in the inflamed joint, they along with infiltrated macrophages secrete pro-inflammatory cytokines (e.g., TNFα, IL-6), mediators, and enzymes to regulate synovial inflammation and joint destruction [49]. An increased number of macrophages are the early hallmark of active disease [92]. Activated macrophages also produce anti-inflammatory cytokines (e.g., IL-10) to promote the resolution of inflammation and tissue repair, ameliorating the disease. Two different macrophage phenotypes, M1 (classically activated) and M2 (alternatively activated), are responsible for the production of pro-inflammatory and anti-inflammatory cytokines, respectively [34]. The imbalance between pro- and anti-inflammatory cytokines (M1/M2) could be a key mechanism of rheumatic disease progression. Polarization of M1/M2 phenotypes depends on "flare" of rheumatic diseases and could contribute to distinct rheumatic diseases. RA patients display a more M1 macrophage profile than spondyloarthritis such as psoriatic arthritis (PsA) [94] or osteoarthritis (OA) [93]. Acute hypoxia environment favors M2 macrophage polarization, but chronic hypoxia condition results in high expression of an immunoregulatory receptor, triggering receptor expressed on myeloid cells (TREM)-1, inducing M1 polarization [75]. Thus, in the acute synovial inflammation, hypoxia environment induces macrophages to differentiate into M2 phenotype, preventing over-activation of immune cells. If inflammatory response progresses (chronic hypoxia), TREM-1 is expressed to trigger M1 polarization, promoting disease progression.

5.3 Autoimmunity and Gut Microbiota

Emerging data suggest that the initial inflammation and autoimmunity in RA (preclinical RA) begins outside of the joint and may originate at mucosal sites including the oral cavity, the lungs, and the gut [20]. It was found that aberrant immune response in RA may be associated with the dysbiosis of the oral or gut microbiota [11, 62, 106]. Specific alterations of gut bacteria can enhance or attenuate susceptibility to experimental arthritis [78, 102], recent-onset PsA [81], and new-onset and chronic RA patients [11, 106]. RA patients have different microbiota from FM patients, suggesting that the microbial composition could be related to autoimmunity.

Gut microbiota is associated with RA pathology via the binding of toll-like receptor 4 (TLR4), a lipopolysaccharide (LPS) receptor that regulates Th17 to produce IL-17, promoting arthritis. In TLR4-deficient mice, the severity of

arthritis is decreased [1]. The segmented filamentous bacteria, an unculturable gram-positive anaerobe in gut, are able to promote Th17 cells in the lamina propria at the small intestine and then trigger arthritis development [75, 102]. A recent study demonstrated that a 27-kD protein produced by *Prevotella copri* induces the immune response in RA patients, indicating that the *P. copri* is immune-relevant in RA pathogenesis [73].

Gut microbiota is also critical for the induction of pain. It was suggested that gut microbiota is related to chemotherapy-induced pain or visceral pain [63, 82] and pain can be reduced after treated with probiotics or antibiotics [70]. How mucosal inflammation triggers joint inflammation and pain remains unclear. It is likely that gut microbiota results in local mucosal inflammation by changing the mucosal permeability, increasing pro-inflammatory cytokines (e.g., IL-6, IL-17) and RA-related autoantibodies (e.g., autoantibodies to citrullinated protein antigens [ACAP]), which becomes systemic with circulating RA-related autoantibodies and pro-inflammatory mediators and finally leading to joint inflammation. IL-6 or IL-17 in joint tissues can activate nociceptors, triggering pain [23, 24]. ACAP can bind to $CD68^+$ osteoclasts in the bone marrow to induce a release of CXCL1/2 that activates nociceptors to cause pain independent of inflammation [97]. In addition, TLR4 activation by bacteria can produce pain hypersensitivity by functional coupling with transient receptor potential/vanilloid receptor subtype 1 (TRPV1) in nociceptors [21].

5.4 Neural Circuits and Neuron-Glial Interaction

Neural circuits balance the production and release of inflammatory mediators, playing important roles in promoting or resolving inflammation. Peripheral or central sensory neurons detect inflammatory signals and pass the information to the brainstem for the integration, and then the integrated signals are conveyed into the peripheral nerves to reduce inflammation, called the inflammatory reflex [86]. The vagal nerve is the longest cranial nerve and innervates visceral organs such as the liver, lung, and intestine. Vagal nerves not only detect mechanical and chemical stimuli, they also monitor peripheral inflammatory responses. Vagotomy disrupts the inflammatory reflex and reduces pro-resolving mediators, inhibiting resolution of inflammation [66]. Electrical stimulation of the vagal nerve reduces cytokine production and RA disease severity [55]. How inflammatory signals are transduced through the vagal nerve? It was suggested that intravenously applied IL-1ß increases the discharge activity in the gastric branch of the vagus nerve [26]. The studies in carotid body proposed that this organ responds to pH, cytokines, and LPS, relaying information through the carotid sinus nerve and the vagal nerve [83, 105]. Bacterial products could directly modulate neuronal excitability in certain subset of sensory neurons [14]. These lines of evidence suggested that the vagal afferent itself can directly sense cytokines or bacterial products produced by mucosal inflammation, but it cannot be excluded that inflammatory signals are detected indirectly through

other intermediate players. Once vagal afferents detect mucosal inflammatory signals, the information is passed to the brainstem and then via the vagal efferent to the celiac ganglion to activate norepinephrine (NE)-expressing neurons with fibers in splenic nerve (sympathetic). This leads to the NE release to activate a subset of T cells in spleen to produce acetylcholine, further inhibiting cytokine production and inflammation.

In contrast, activation of pain circuits or peripheral nerves regulates inflammation, called neurogenic neuroinflammation or neuroinflammation, respectively [46]. The hallmarks of neuroinflammation are infiltration of immune cells, activation of glial cells, and production of inflammatory cytokines. Denervation of peripheral nerves such as sciatic nerves [87] or removal of TRPV1-expressing peripheral nerves [10] by resiniferatoxin (RTX) increases synovial inflammation in the model of K/BxN serum-transfer arthritis. Collagen antibody-induced arthritis increases expression of spinal astrocytes and microglia, and inhibition of glial activity reverses mechanical hypersensitivity [4, 69], suggesting that spinal glial cells are also involved in the neuron-immune interaction.

5.5 Proton-Sensing Receptors

High hydrogen ion concentration [H^+] (acidosis) in synovial fluid is associated with disease activity of RA [28]. FLS responds to cytokines to release acidic components, lowering pH in synovial fluid [74]. Extracellular proton binds to proton-sensing ion channels, and G-protein-coupled receptors (GPCRs) in nociceptive fibers may contribute to sensory transduction and release of neurotransmitters, leading to pain and hyperalgesia [76, 90]. Proton-sensing GPCRs in synoviocytes was reported to link to the increase in intracellular calcium [17], which could have a contribution in the downstream inflammatory and cellular proliferative processes of synoviocytes. Extracellular proton, local production cytokines, and neurotransmitters released from joint fibers may work in concert to contribute to inflammatory responses and pain.

5.5.1 Transient Receptor Potential/Vanilloid Receptor Subtype 1 (TRPV1)

Transient receptor potential/vanilloid receptor subtype 1 (TRPV1) is a nonselective cation channel with a six-transmembrane domain. TRPV1 is activated by heat, vanilloid, capsaicin, and proton, and TRPV1 is involved in thermal and mechanical hyperalgesia [48].

In an arthritis model induced by complete Freund's adjuvant (CFA) injection in C57BL/6 mice, deletion of TRPV1 attenuates joint and paw swelling, synovial

inflammation, bone erosion, and cartilage damage in the early disease phase (≤5 weeks) [6, 29, 91]. In ICR mice with repeated intra-articular injection of CFA, deletion of TRPV1 gene not only attenuates synovial inflammation, bone erosion, and cartilage damage in the joint in the early disease phase but also at the later phase [39]. Given that removal of TRPV1-expressing peripheral nerves by RTX increases synovial inflammation in the model of K/BxN serum-transfer arthritis [10], reduction of synovial inflammation found in TRPV1-deficient mice is more likely due to the effects of TRPV1-expressed non-neuronal cells. TRPV1 is expressed in FLS [5, 25, 52], and stimulation of TRPV1 by capsaicin leads to increased IL-6 levels [25]. Thus, TRPV1 deletion may block the pro-inflammatory cytokine production, thereby reducing synovial inflammation. Protection of bone and cartilage destruction could be due to inhibiting synoviocyte invasion or synoviocyte-released signals of receptor activator of nuclear factor-kB ligand (RANKL) [30].

TRPV1 deficiency also attenuated RA-associated pain. As distinct models are used, whether TPRV1 acts on the early phase or the chronic phase of RA-associated pain remains arguable. In the model induced by CFA injection in C57BL/6 mice, TRPV1 deficiency reduces RA-associated hyperalgesia at the early phase (≤5 weeks) [29, 91]. However, in the model with repeated intra-articular injection of CFA, RA-induced mechanical hyperalgesia begins attenuated at the later phase (>8 weeks) in TRPV1-deficient mice [39]. Similarly, in the model of K/BxN serum-transfer arthritis, late mechanical hyperalgesia is reduced after removal of TRPV1-expressing nerves by RTX [10]. A previous model of muscle pain proposed that TRPV1 is involved in establishing hyperalgesic priming in the muscle pain model [12]; and the co-culture study of FLS and neurons demonstrated that synoviocytes from chronically, but not acutely, inflamed joints release inflammatory mediators to upregulate TRPV1 expression in DRG neurons [5], contributing to joint pain. These lines of evidence support that TRPV1 may not be essential for the initiation of arthritic pain but rather regulates the establishment of the chronic phase of arthritic pain. Although TRPV1 action on the RA-associated pain is mainly attributed to TRPV1-expressing nerves as removal of TRPV1-expressing nerves significantly attenuates mechanical hyperalgesia [10], the contribution of spinal glial cells cannot be excluded. TRPV1 is expressed in astrocytes and microglia [22, 50], and TRPV1 deficiency also reduces increased density of microglia and astrocytes in the spinal cord in adjuvant-induced arthritis [13].

5.5.2 Acid-Sensing Ion Channel 3 (ASIC3)

Acid-sensing ion channel 3 (ASIC3) belongs to the family of degenerin/epithelial amiloride-sensitive Na^+ channels and is activated by extracellular protons and plays an important role in pain [60].

Deletion of acid-sensing ion channel 3 (ASIC3) can reduce secondary mechanical hyperalgesia induced by carrageenan injection or anti-collagen antibody/LPS injection [42, 84]. Although ASIC3 deficiency can reduce arthritic pain, it

increases synovial inflammation [84]. Use of a selective ASIC3 blocker, APETx2, attenuates disease and pain progression of early-phase osteoarthritis (OA) in a rat model [45]. In ICR mice with repeated intra-articular injection of CFA [39], ASIC3 deficiency prevents synovial inflammation, bone erosion, and cartilage damage from the disease beginning (4 weeks), but ASIC3 deficiency affects RA-associated pain starting from the later phase (>6 weeks). Thus, ASIC3 is also involved in establishing hyperalgesic priming, as was suggested previously in a muscle model [12]. This observation was not reported before because most studies focused on the acute phase (<4 weeks) in different arthritis models [42, 45, 84]. ASIC3 is expressed in joint afferents [43] but also in synovial cells and cartilage [53], which could be one of the explanations for the different outcomes. ASIC3 could be similar to TRPV1 such that ASIC3-expressing neurons play a major role in regulating arthritis pain and ASIC3-expressing FLS or macrophages contribute significantly to synovial inflammation, but its effects are more complex than TRPV1.

Whether ASIC3 deficiency increases inflammation is arguable from previous studies using different arthritis models. In the collagen/LPS model, ASIC3 deficiency increases inflammation [84] but attenuates inflammation in the OA model [45]. In ICR mice with repeated intra-articular injection of CFA [39], ASIC3 deletion reduces pannus from weeks 4 to 8. At 12 weeks, ASIC3 deficiency seems to not fully suppress pannus, although it reduces arthritis scores, $CD68^+$ cells (FLS and macrophages) and $CD80^+$ cells (pro-inflammatory M1 macrophages) [39]. ASIC3 expressed in synoviocytes seems to mediate inflammatory cytokines or other substances to increase nerve innervation to synovium, thereby leading to pain and inflammation [53]. ASIC3 also mediates acid-induced endocytosis and maturation of macrophages to promote innate inflammation [54]. Macrophages have pro-inflammatory and anti-inflammatory phenotypes to balance synovial inflammation and joint destruction [49]. Although RA is considered to exhibit more inflammatory responses than OA, it still features anti-inflammatory responses, with macrophages playing a large part. Thus, deletion of ASIC3 reduced synovial inflammation from the disease beginning because of the inhibition of the FLS and pro-inflammatory macrophage function but with less reduction in the later phase of inflammation (inhibition of anti-inflammatory macrophage function).

Inhibition of FLS proliferation by ASIC3 deletion, it also explains the reduction of bone erosion and cartilage damage. Other lines of evidence support the observation that ASIC3 deficiency protects cartilage damage: (1) extracellular high $[H^+]$ concentration inhibits matrix synthesis by chondrocytes, affects biosynthetic ability of chondrocytes, and even induces chondrocyte apoptosis [41, 98, 103]; (2) inhibition of ASICs by amiloride prevents injury of chondrocytes induced by acid [41, 79, 104]; (3) ASIC3 acts as a pH sensor in chondrocytes to regulate hyaluronan expression [53]; (4) inhibition of ASIC3 by APETx2 protects against cartilage destruction in an OA model [45].

5.5.3 T-Cell Death-Associated Gene 8 (TDAG8)

T-cell death-associated gene 8 (TDAG8) is one of proton-sensing GPCRs and fully responsive to proton at pH 6.4–6.8, leading to accumulation of intracellular cAMP [40, 44, 95]. TDAG8 is suggested to be involved in bone cancer pain [35], acid-induced pain and itch [19, 61], and inflammatory agent-induced pain [19].

Deletion of TDAG8 increases the severity of collagen/LPS-induced arthritis, but arthritis-induced pain was not assessed in this study [71]. In ICR mice with repeated intra-articular injection of CFA [39], knockdown of TDAG8 expression in peripheral nerves reduces arthritis scores and joint swelling, but not synovial inflammation. TDAG8 activation in immune cells could inhibit inflammation as TDAG8 mediates acid-inhibited cytokine production in macrophages, T cells, neutrophils, and microglia [47, 67], while TDAG8 activation in FLS could facilitate FLS proliferation and downstream inflammatory responses. It may explain synovial inflammation is not attenuated when TDAG8 expression is knocked down in peripheral nerves [39]. Reduction of arthritis scores and joint swelling could be partly attributed to a decrease in the release of neurotransmitters as TDAG8 inhibition attenuates sensory transduction. In addition, TDAG8 is highly expressed in Th1-like effector/memory cells, and TDAG8-deficient mice reduce the number of Th17 cells and secretion of IL-17A [31]. It could be another reason for the reduction of arthritis scores in TDAG8-deficient mice. Recent studies found that vagal afferents also contain TDAG8 [99]. It remains unclear whether TDAG8 participates in the inflammatory reflex to regulate inflammation.

TDAG8 activation inhibits acidification-enhanced bone resorption [36], so TDAG8 inhibition should facilitate bone erosion. However, mice with TDAG8 knockdown show a significant chondroprotection effect, even though synovial inflammation is not inhibited. Which mechanism TDAG8 mediates to protect cartilage damage is unclear. TDAG8 likely responds to acid signals to mediate acid-induced injury of chondrocytes [41]. Suppression of TDAG8 prevents injury of chondrocytes.

TDAG8 was found involved in inflammatory pain and bone cancer pain [19, 35]. As well, TDAG8 participates in initiating hyperalgesia and establishing hyperalgesic priming in a dual-acid or CFA-induced inflammatory model [19, 89]. However, TDAG8 knockdown in peripheral nerves reduces hyperalgesia only in the acute phase (<4 weeks) but does not observe the priming effect of TDAG8 in the model with repeated CFA injection [39]. In contrast, using TDAG8 knockout mice, TDAG8 deficiency indeed attenuates chronic hyperalgesia (>6 weeks) in the same RA model [39], suggesting that TDAG8 is involved in the hyperalgesic priming in this RA model. The difference between TDAG8 knockdown and knockout mice is that TDAG8 deletion only occurs in the peripheral nerves in knockdown mice, while TDAG8 knockout mice have TDAG8 gene deletion in all cell types. It implies that the acute phase of RA pain is strongly linked to peripheral nerves, but the chronic phase could be also dependent on other cells such as immune cells, glial cells, synoviocytes, and central nervous system.

Genome-wide association studies (GWAS) demonstrated that genetic variants in the TDAG8 locus are associated with spondyloarthritis [16] and Th17 cells in spondyloarthritis patients have highly expressed TDAG8 gene [2]. Whether TDAG8 plays similar role in RA remains to be further characterized.

5.6 Conclusions

TRPV1, ASIC3, and TDAG8 are proton-sensing receptors involved in RA-associated chronic pain. These receptors are not only expressed on joint nociceptors and intestinal sensory neurons but also expressed on fibroblast-like synoviocytes, immune cells, and glial cells. These receptors are activated by extracellular proton, but acidic condition has distinct actions on nerves and immune cells. It is not surprising that TRPV1-, ASIC3-, and TDAG8-deficient mice have different influences on RA pathology and associated chronic pain. TDAG8 is essential for the acute RA pain and chronic RA pain, while both ASIC3 and TRPV1 are important for chronic RA pain. These three receptors may work in concert to regulate pain and inflammation. In the acute RA pain, the peripheral joint nerves responding to joint inflammation play a critical role, while in the chronic phase, non-neuronal cells and central nerves participate in the regulation of pain and hyperalgesia. Current therapies focus on inhibition of one receptor or single pathway, or deletion of a cell type may work on some patients but fail in others. More likely, different pharmaceutical strategies should be considered at different stages of RA pathology and pain and at different cell types.

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Chapter 6
Advances in Experimental Medicine and Biology: Intrafascicular Local Anesthetic Injection Damages Peripheral Nerve-Induced Neuropathic Pain

Kuang-Yi Tseng, Hung-Chen Wang, Lin-Li Chang, and Kuang-I Cheng

Abstract Peripheral nerve blockade (PNB) is advantageous for patients undergoing surgery to decrease the perioperative opioid consumptions and enhance recovery after surgery.

Inadvertent local anesthetic (LA) administration into nerve fiber intrafascicularly easily results in unrecognized nerve injury. Using nerve block guidance either by ultrasound, electrical nerve stimulator, or using pressure devices does not prevent nerve damage, even though most of the nerve injury is transiently. The incidence of neurologic symptoms or neuropathy is in the range of 0.02–2.2%, and no significant difference of postoperative neurologic symptoms is found as compared with using ultrasound or guided nerve stimulator technique. However, intrafascicular lidocaine brought about macrophage migration into the damaged fascicle, Schwann cell proliferation, increased intensity of myelin basic protein, and shorten withdrawal time to mechanical stimuli. In dorsal root ganglion (DRG), intrafascicular LA injection increased the activated transcriptional factor 3 (ATF-3) and downregulated Nav1.8 (Nav1.8). In spinal dorsal horn (SDH), the microglia and astrocytes located in SDH

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were activated and proliferated after intrafascicular LA injection and returned to baseline gradually at the end of the month. This is a kind of neuropathic pain, so low injection pressure should be maintained, the correct needle bevel used, nerve stimulator or ultrasound guidance applied, and careful and deliberately slow injection employed as important parts of the injection technique to prevent intrafascicular LA administration-induced neuropathic pain.

Keywords Intrafascicular · Intraneural · Peripheral nerve block · Local anesthetic

6.1 Introduction

Peripheral nerve blockade (PNB) is advantageous for patients undergoing surgery to reduce surgery-induced traumatize pain [11], to reduce intraoperatively consumptions of volatile agents [32, 33] or propofol [25], and to attenuate postoperative pain [23] and has been demonstrated to be one of the major components in postoperative multimodal pain regimens [7, 15, 28]. Appropriate nerve block is able to blunt surgery-induced stress responses as well as decrease blood loss. In addition, PNB presents its benefits by blocking a specific part of the body and limiting fluctuation of the hemodynamic responses [30, 39].

However, inadvertent local anesthetic (LA) administration into nerve fiber either via extrafascicular or intrafascicular pathways may easily result in unrecognized nerve injury [8, 18].

Using 22G insulated needles for axillary nerve approach under ultrasound guidance revealed 38.2% sensitivity to paresthesia during injection and presented a 75% motor response elicited by electrical nerve stimulation [36]. It indicated that nerve block guidance either by ultrasound, electrical nerve stimulator, or using pressure devices did not guarantee avoidance of damage, even though most of the nerve injury was transient [12, 17, 29]. Unfortunately, unintentionally administered LA into the intrafascicular space may lead to peripheral neuropathy producing axonal degeneration and inflammatory cell infiltrations [19]; therefore, we focused on analyzing information about intrafascicular injection in current clinical practice and the underlying mechanisms from animal models.

6.2 Pathophysiology Mechanisms of Peripheral Nerve Fibers Injury

Peripheral nerves may consist of sensory, motor, or mixed, carrying both sensory and motor nerve fibers. Dendrites receive information then passed to the neuron cells, and after processing or integration, the impulses transmit through the axons. Three essential connective tissue components include the epineurium, perineurium, and endoneurium covering the axons to prevent nerve fibers being easily damaged. The LAs administered near the nerve fibers need to traverse these three barriers (the

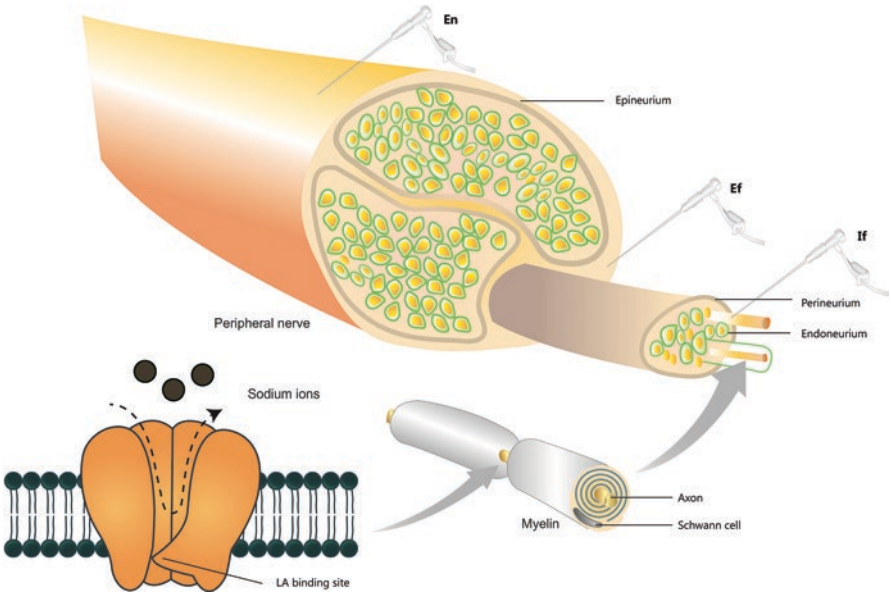

Fig. 6.1 The peripheral nerve is encapulated by the epineurium, a layer of collagen connective tissues. Extraneural injection (En) is administered local anesthetics (LAs) outside of the epineurium and perineurium that surrounds the nerve fascicle as a dense layer with active diffusion barrier characteristics. Extrafascicular injection (Ef) is defined as the LAs infiltrating between the epineurium and perineumium but without penetraring the perineurial barrier. If the needle tip pierces the perineurium and places the LAs in the endoneurial space, it is defined as the intrafascicular injection (If). As to the intrafascicular injection of LAs, the onset might be quicker than usual for mere diffusion through the lipid bilayer membrane to bind the inner receptor of the sodium channel and inhibit influx of sodium ions into the cytoplasm thereby impairing the generation of action potentials

epineurium, perineurium, endoneurium) and progress from the outside of the nerve fibers through the endoneurial space and then diffuse through double lipid layers of axonal cell membrane to interact with the inner surface of the sodium channel to reach its action site, thereby blocking the action potential transmission (Fig. 6.1). The epineurium enwraps a bunch of fascicles and is composed of collagenous extracellular matrix surrounding each fascicle [35]. The endoneurium has a nutritive and protective function containing fine bundles of fibrous connective tissue of collagen and is embedded in a matrix of ground substance to guarantee a constant environment for the nerve.

However, the perineurium consists of flat, squamous cells that are composed of one or more delicate, thin layers that form tight junctions preventing paracellular transportation [34]. The main barrier to delay LA acting on the inner receptor of the sodium channel is based on the thick basal membrane of the perineurium separating the endoneurium from extrafascicular tissues. Furthermore, in microscopic viewing of the ultrastructure of the perineurium, the mature perineural diffusion barrier is composed of strands with linear distribution of occludin, zonula occludens (ZO), and claudin tight junction protein labeling (that is continuous in adults) and matured

basement membrane components type IV collagen and laminin [35]. However, there is an active transcytotic transport through perineural cells showing that the perineurium constitutes a tight but selective barrier [46]. Therefore, unintentional needle tip penetration through the perineurium into the endoneurial space when administering LAs might produce significant shortening onset time compared with extraneural LA administration [4].

6.3 Intraneural LA Administration

Although PNB is a commonly executed procedure perioperatively, patients might present peripheral neuropathy associated with extraneural or intraneural LA administration under ultrasound or nerve stimulator guidance [3, 16, 31, 41, 42]. Tracing back the last 10 years of PNB-related information, the incidence of neurologic symptoms or neuropathy is in the range of 0.02–2.2%, and no significant difference of postoperative neurologic symptoms is found as compared using ultrasound with guided nerve stimulator technique [3, 24, 45]. During PNB procedure guidance by ultrasound, unintentional intraneural injection occurred in 53 out of 325 patients with subgluteal sciatic nerve block, but no clinical evidence of nerve injury in the postoperative follow-up was found [14]. According to scholars [4], intraneural LA injection compared with extraneural LA administration induced nerve swelling and increased diameters found with fascicle dissection. Patients in either intraneural or extraneural groups all showed similar sequelae with subclinical, significant reduction in amplitude of the action potentials in the 5th week, but no single state of any clinical neurological complications after 6 months was found [4]. Although transient neurologic deficits occur frequently with an incidence of 8.2–14% [9, 22], there are still some case reports where patients receiving PNB suffered from sensory/motor neurologic deficit over 6 months [9, 26, 31, 41, 42].

PNB induced long-term neurologic deficit that might be closely associated with intrafascicular LA injection. Regarding the intrafascicular LA injection, if the bevel of the needle tip completely penetrates into the perineural sheath, it might produce high-pressure injection within the fascicle resulting in a fascicle of nerve fiber presenting as a wedge-shaped demyelination along with focal axonal loss accompanied with bordering normal myelinated axons but with endoneurial edematous change [48].

6.4 Intrafascicular Lidocaine Administration

However, intrafascicular high LA injection pressure gives rise to nerve fiber structure and functional changes. Intrafascicular lidocaine brought about macrophage migration into the damaged fascicle, Schwann cells proliferation, and increased intensity of myelin basic protein (MBP) [6]. Although high intrafascicular pressure injection induced rapidly increased interleukin-1 beta (IL-1ß), IL-6, and tumor

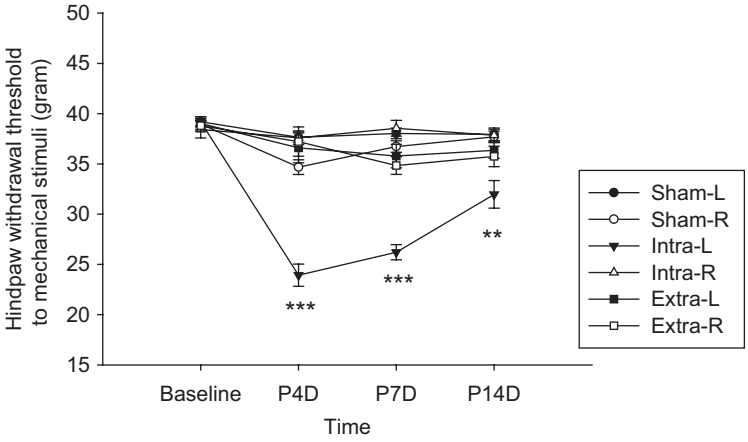

Figure Behavioral responses to mechanical stimuli on hind paws after intrafascicular or extraneural lidocaine injection into the left sciatic nerve. Mann-Whitney U test, **P < 0.01, ***P < 0.001.

Fig. 6.2 Intrafascicular lidocaine injection-induced hind paw mechanical allodynia. One hundred microliter of lidocaine was intrafascicularly administered into rat left sciatic nerve resulting in increased hind paw withdrawal responses to mechanical stimuli revealing significant difference in postinjection 4 days (P4D), P7D, and P14D, respectively. *Sham-L* sham, nerve left; *Sham-R* sham, nerve right; *Intra-L* intrafascicular LA, nerve left; *Intra-R* intrafascicular LA, nerve right; *Extra-L* extrafascicular LA, nerve left; *Extra-R* extrafascicular LA, nerve right. ***, $P < 0.001$; **, $P < 0.01$

necrosis factor alpha (TNF-α) cytokines expression within 12 h, the uprising expression of macrophages, Schwann cells, and MBP in damaged nerve segments might persist for 14 days or more. In addition, as shown in Fig. 6.2, a rat model demonstrated that intrafascicular lidocaine 100 microliter into the sciatic nerve increased hindpaw withdrawal responses to mechanical stimuli (mechanical allodynia) at least post-injection 14 days. The induced neuropathic pain is mainly based on the functional changes of the dorsal root ganglion (DRG) and spinal dorsal horn (SDH). After intrafascicular LA injection, it exhibited increased activated transcriptional factor 3 (ATF-3) and downregulated Nav1.8 (Nav1.8) in involved DRGs (Fig. 6.3). A significantly increased ATF-3 protein expression in the DRG may persist over 3 weeks.

Although there is limited information of the correlation between ATF-3 and intrafascicular LA injection, the ATF-3 protein expression increased in peripheral nerve axotomy [47] and herpes zoster infection-induced neuropathy [10]. The ATF-3 mRNA may increase shortly (usually within 2 h) when exposed to stress signals and might reach peak more than 8 h after exposure [13]. Nevertheless, mere chronic inflammation generated by intra-plantar completion of Freund's adjuvant did not increase ATF-3 protein levels [1].

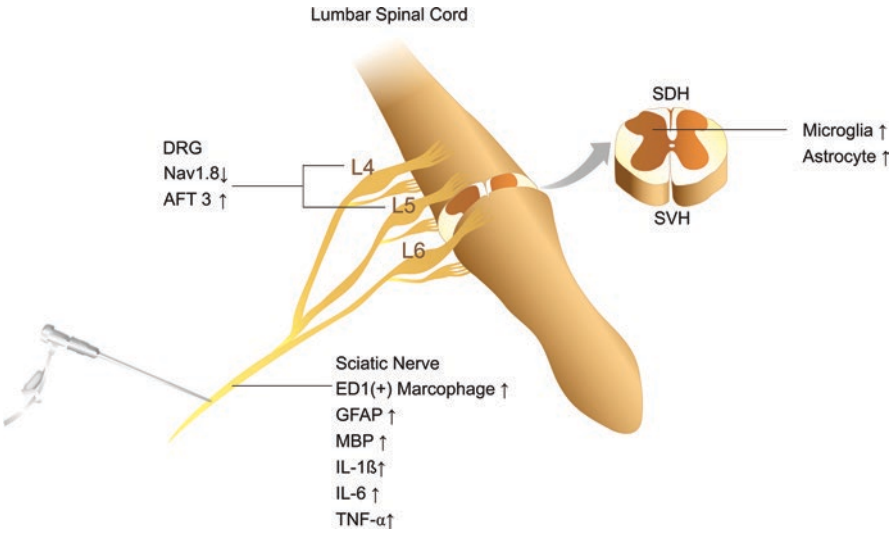

Fig. 6.3 Schematic diagram illustrates pathophysiology mechanisms of nerve damage induced by lidocaine intrafascicular administration into the sciatic nerve. With macrophage migration into the damaged fascicle, increased interleukin-1 beta (IL-1ß), IL-6, tumor necrosis factor alpha (TNF-α) cytokines expression, glial fibrillary acidic protein (GFAP)-positive Schwann cell proliferation, and increased intensity of myelin basic protein (MBP) were found within the sciatic nerve. Downregulation of sodium channel 1.8 (Nav1.8) and increased activated transcriptional factor 3 (ATF-3) were presented in L4 and L5 dorsal root ganglions (DRGs). As viewing from the transverse section of the spinal cord, glia cells were activated in spinal dorsal horn (SDH). Data are obtained from our previous report [6]. Some of the data from our unpublished results include cytokines expression in sciatic nerve and Nav1.8 expression in DRG that were measured by ELISA and Western blotting, respectively. Lumbar spinal cord, LSC; SVH, spinal ventral horn

Persistent downregulation of Nav1.8 in the damaged DRG is demonstrated in rats submitted to intrafascicular lidocaine injection. Downregulated Nav1.8 expression in injured neuron cells has been demonstrated in peripheral nerve axotomy and diabetic rats [5]. It is a necessary mediator of nerve growth factor-induced thermal hyperalgesia [20]. Therefore, intrafascicular LA injection might disturb microtubule functions and interrupt nerve growth factor retrograde transmission to the involved neurons. In the spinal cord, the microglia and astrocytes located in SDH were activated and proliferated after intrafascicular lidocaine injection and returned to baseline gradually at the end of the month [5]. From measurement of pain behavior responses and assessment of the nerve damage sites along with the DRG and spinal cord, the intrafascicular LA-induced pain is a kind of neuropathic pain.

6.5 High-Pressure Intrafascicular Injection

In measurement of the mean application pressure during intrafascicular injection, its average pressure was 1485 ± 390 mmHg and the extrafascicular injection was 400 ± 134 mmHg by using Bio Bench software [27]. From measurement of the intrafascicular canine sciatic nerve, high injection pressure (>25 PSI (pound per square inch), 1293 mmHg) might result in nerve fiber axonolysis, demyelination, and macrophage infiltration but not in low injection pressure (<4 PSI, 207 mmHg) [12]. However, injection-producing pressures measured in the injection line are not equal to pressures at the needle tip excepting no flow through the needle. Using a porcine model for extraneural injections, pressures at the needle tip and at the injection line varied substantially as 114.8 and 222 mmHg, respectively, at 0.5 ml injectate volume up to the 203.3 and 515.3 mmHg, respectively, at the end of 2 ml saline infusion at a rate of 10 ml/min (0.16 ml/s) [37]. In human cadavers, 1 mL of 0.9% sodium chloride was injected over 10 s, and the average pressure for intraneural injections was 24.1 ± 5.7 PSI (1246 ± 295 mmHg) and 6.1 ± 2.1 PSI (315 ± 109 mmHg) for perineural injections [21].

6.6 Needle Characteristics

There is no significant difference on intentional nerve puncture injury between tapered-tip needles of pencil-point and Tuohy-tip needles. However, both needle types injured nerves similarly in an examination of the occurrence of posttraumatic regional inflammation, myelin damage, and intraneural hematoma [44]. Compared with long-bevel needles, short-bevel needles are commonly used in PNB when difficulty in penetrating the perineurium is encountered and there is low incidence of related neural injury. If the short-bevel needle unintentionally impales the perineurium, the lesion might get worse and take longer to repair than for long-beveled needles [38]; however, regardless of the needle type, the needle gauge is proportional to the extent of the nerve damage as demonstrated by 22-G needles (3%) and 17- to 18-G needles (40%) [43].

6.7 Practical Advisory Recommendation to Prevent Intrafascicular Injection

In use of peripheral nerve stimulation, the presence of an evoked motor response at a current of <0.5 mA with duration 0.1 ms indicates intimate needle-nerve relationship and needle-nerve contact or an intraneural needle placement, and the absence

of a motor response at current of up to 1.8 mA does not exclude needle-nerve contact or intraneural needle placement [29]. In using injection pressure monitoring, presently, animal data presents high-pressure intrafascicular injection-induced nerve injury, but there is no human data confirming or refuting the effectiveness of injection pressure monitoring. In addition, the common practice of subjectively assessing injection pressure by "hand feel" is inaccurate [29]. In using ultrasound guidance for nerve blockage, ultrasound can detect intraneural injection, but the technique is not easy to discern between interfascicular and intrafascicular injections. In addition, images of needle-nerve interface are not consistently obtained by all operators and in all patients [29]; therefore, ultrasound combined with pressure monitoring or with peripheral nerve stimulation should be recommended to prevent unintentional intrafascicular nerve injection.

6.8 Treatment of Intrafascicular Injection-Induced Neuropathic Deficit or Pain

Intraneural needle tip placement was indicated with any one of the following presenting conditions such as pain or paresthesia, motor responses with a minimal stimulating current <0.4 mA, needle tip observed inside the nerve guidance by ultrasound, nerve swelling following local anesthetic injection, a >4.3% increase in electrical impedance [2], or increased injection pressure PSI. Once intrafascicular injection occurs, treatments are suggested as below.

1. Intrafascicular high injection pressure-induced neuropathic pain is reasonably treated pharmacologically based on antidepressants or anticonvulsants [40].
2. Functional deficits from neurological injuries should be rehabilitated in concert with rehabilitation specialists [29].
3. Corticosteroids may have a beneficial effect for trauma possibly resulting from interventional procedures. However, balance should be maintained between the positive effects and the risk of steroid-induced hyperglycemia in patients [29].
4. Pretreatment and post-nerve injury minocycline administration is effective in alleviating mechanical behaviors, mitigating macrophage recruitment into the sciatic nerve, and suppressing activated microglial cells in the spinal cord [6].
5. Injection of ATP into the damaged peripheral nerve might improve nerve regeneration [49].

6.9 Conclusion

Intraneural LA injection shows no obvious nerve damage unless the needle tip has penetrated into the perineurium resulting in intrafascicular LA high-pressure injection where the structures and functions of nerve fibers have been altered.

Intrafascicular LA injection increased the sensitive dynamic mechanical responses in rat hind paw models resulting in peripheral nerve macrophage invasion, Schwann cell proliferation, ATF3 increased expression in DRG, and glia cell proliferation in SDH. This is a kind of nerve injury-induced neuropathic pain. Though high-pressure injection monitoring, ultrasound guidance, or using peripheral nerve stimulation to avoid nerve injury during LA administration are administered, unintentional intrafascicular injection might still occur. Therefore, careful and deliberately slow injection under ultrasound guidance or using nerve stimulation is the most important part of the injection technique to prevent intrafascicular LA administration-induced neuropathic pain.

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Chapter 7
Microglia in the CNS and Neuropathic Pain

Makoto Tsuda

Abstract Neuropathic pain occurring after peripheral nerve injury is not simply a consequence of temporal continuity of acute nociceptive signals, but rather of maladaptive nervous system function. Over the past decades, a body of literature has provided evidence for the necessity and sufficiency of microglia, the tissue-resident macrophages of the central nervous system, for nerve injury-induced alterations in synaptic function. Recent studies have also revealed active roles for microglia in brain regions important for emotion and memory. In this chapter, I highlight recent advances in our understanding of the mechanisms that underlie the role of spinal and brain microglia in neuropathic pain, with a focus on how microglia are activated and alter synaptic function. I also discuss the therapeutic potential of microglia from recent advances in the development of new drugs targeting microglia, which may facilitate translation from the bench to bedside.

Keywords Microglia · Neuropathic pain · Spinal cord · Brain

7.1 Introduction

Injury to the nervous system as a consequence of cancer, diabetes, infection, autoimmune disease, chemotherapy, and trauma often causes debilitating chronic pain syndrome (neuropathic pain). Its symptoms include spontaneous pain, hyperalgesia (increased pain by a stimulus that normally provokes pain), and allodynia (pain due to a stimulus that does not normally provoke pain). Neuropathic pain does not resolve even after the overt tissue damage has already healed and can persist for long periods of time, indicating that the pain is not simply a temporal continuum of acute nociceptive pain, but rather due to pathologically altered nervous system function [5, 58, 79, 105]. Such pathological alterations have been extensively studied

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using rodent models of neuropathic pain, for example, models developed by peripheral nerve injury (PNI). Accumulating evidence indicates that PNI causes a variety of plastic modifications in neuronal synapses, connections, and networks at the molecular and cellular levels. These modifications shift the balance between synaptic excitation and inhibition in lamina I projection neurons toward excitation, which may account for development and maintenance of pain hypersensitivity [5, 58, 79, 105]. These alterations were long thought to be a consequence simply of changes in neurons, but mounting evidence indicates the important role of non-neuronal cells of the nervous system, including monocytes, macrophages, T cells, and glial cells [43, 45]. Microglial cells, which are known as the tissue-resident macrophages of the central nervous system (CNS) and constitute 5–10% of total cells in the adult CNS, have received much attention. In the late 1970s, it was found that non-neuronal cells (which were later identified as microglia) are increased in the spinal dorsal horn (SDH) after PNI [27, 28]. About 30 years later, a causal role of spinal microglia in neuropathic pain was first reported [46, 97]. Currently, numerous microglia-selective molecules (approximately 40) implicated in PNI-induced pain have been identified, providing compelling evidence that microglia are the key cell type for pathogenesis of neuropathic pain. In this chapter, we highlight recent advances in understanding of the role of CNS microglia in neuropathic pain.

7.2 Microglia

Microglia were originally described by Pio del Rio-Hortega in 1919 [19] and proposed to have a mesodermal origin [51]. In fate mapping studies enabling cell marking and gene regulation at the developmental stage, prenatal hematopoietic precursor cells were identified as the origin of microglia [29, 30, 52]. Microglia arise from yolk sac precursors genetically labelled as runt-related transcription factor 1 (Runx1)-expressing cells. Erythromyeloid progenitors in the yolk sac develop into microglia progenitors via an immature and more mature stage. The progenitors then leave the yolk sac, migrate to the brain through blood vessels, appear in the neuroepithelium with an amoeboid morphology, and finally take on a ramified. The development of microglia is independent of transcription factors required for development of other myeloid cell populations [52, 83]. As microglia have a unique molecular signature compared with other myeloid and immune cells [7, 25, 31, 37], this indicates a distinct developmental program of microglia from other myeloid cell types. The microglial development program is regulated by interleukin-34 (IL-34) signaling via CSF1R [29, 103]. Promoting terminal differentiation and acquiring adult microglia properties require TGF-β1 as a key factor [7]. In the healthy adult CNS, microglia remain throughout life and are maintained by self-renewal [88] with little contribution from bone marrow-derived circulating monocytes [2]. For maintaining microglia in adults, CSF1R signaling might have an ongoing role since pharmacological inhibition of CSF1R eliminates microglia in the adult brain [21]. In adults, microglia represent a morphologically unique type of cell, which, under normal conditions, has a small

soma bearing thin and branched processes. Two photon in vivo imaging studies have revealed that microglia processes are highly dynamic [17, 18, 73]. The processes of microglia rapidly move toward the site of injury [18, 34]. Furthermore, microglia directly appose synaptic regions (presynaptic terminals and dendritic spines) and, in response to neuronal activity, steer their processes toward active synapses, which facilitates contact with highly active neurons [102]. Now, microglia in the CNS are increasingly recognized as being crucial for sculpting the structure of the CNS, refining neuronal circuitry and network connectivity, and contributing to plasticity.

7.3 Microgliosis After PNI

As seen in the initial reports in the late 1970s [27, 28], PNI increases the number of microglia in the SDH. Such microgliosis is considered to occur through two mechanisms. First is proliferation of resident microglia because SDH microglia are immunohistochemically labelled by proliferation markers [26, 42]. Second is infiltration of bone marrow-derived circulating monocytes into SDH, which differentiate into microglia-like cells [106]. However, the latter was only observed in bone marrow chimeric mice receiving a high dose of irradiation [87], a treatment that can produce toxic effects including disruption of the blood-brain/spinal cord barrier [59]. Recent studies demonstrated no contribution of circulating monocytes to the PNI-induced microgliosis in the SDH, using parabiosis mice (a model in which two mice are surgically joined and share circulating blood in order to generate a chimera without irradiation and transplantation) [87] and transgenic mice enabling distinct visualization of resident microglia and circulating monocytes [32]. Therefore, local expansion of resident microglia by proliferation is the primary cellular mechanism for SDH microgliosis after PNI [32, 87]. Nonetheless, it should be noted that monocyte infiltration might be dependent on the neuropathic pain model. For example, in experimental autoimmune encephalomyelitis (a model of multiple sclerosis, with chronic pain being a common symptom), massive monocyte infiltration is observed in the spinal cord with demyelinating lesions [1]. However, these monocytes do not permanently contribute to the resident microglia pool.

SDH microgliosis seems to be a crucial step in neuropathic pain because interrupting this process suppresses PNI-induced pain hypersensitivity [32]. What triggers microgliosis? There are currently many reports showing that gene knockout reduces PNI-induced microgliosis [43]. Among them, neuregulin-1 might be one candidate. This is expressed in dorsal root ganglion (DRG) neurons, and its receptor ErbB2 is activated in spinal microglia after PNI [8]. Inhibition of neuregulin-1/ErbB2 signaling suppresses the PNI-induced microgliosis. Another potential candidate factor recently identified is colony-stimulating factor 1 (CSF1). CSF1 is rapidly induced in injured DRG neurons [33, 77] presumably by IL-1β signaling from surrounding satellite glia [61]. By contrast, IL-34 expression was not changed in DRG neurons [77]. The PNI-induced microglial proliferation and mechanical hypersensitivity were reduced by conditional knockout of CSF1 in DRG neurons

[33] and intrathecal administration of a CSF1R inhibitor [77]. Conversely, intrathecal CSF1 administration to normal mice induced proliferation and pain [33]. These findings suggest that CSF1 in injured DRG neurons activates CSF1R in microglia and induces proliferation. DNAX-activation protein 12 (DAP12) is a putative molecule downstream of CSF1R signaling, but the PNI-induced microglial proliferation might underlie a DAP12-independent mechanism because DAP12-deficient mice had no effect on the proliferation [33]. However, DAP12-deficient mice do not show PNI-induced pain [33, 55] or increased microglial number [55]. Thus, it is conceivable that DAP12-dependent signaling might presumably be involved in microglial migration from surrounding areas or changes in survival [55]. In addition, it should be noted that the upregulation of CSF1 and CSF1R persists until a few weeks after PNI [33, 77], when microglial proliferation has already terminated [32], suggesting a distinct role for CSF1-CSF1R signaling at this later phase, such as the control of the expression of microglial genes.

7.4 Molecularly Activated Microglia After PNI

SDH microglia are in an activated state following PNI through a change in their gene expression. For this process, one of the key regulators is interferon regulatory factor 8 (IRF8), a member of the IRF family [85]. Within the SDH, IRF8 is upregulated exclusively in microglia after PNI [66]. IRF8 regulates microglial genes including cell surface responses such as purinergic P2 receptors (P2X4R and P2Y12R), toll-like receptor 2 (TLR2), and C-X3-C motif chemokine receptor 1 (CX3CR1) and diffusible factors (IL-1β, cathepsin S (CatS), and brain-derived neurotrophic factor (BDNF)). The mechanism underlying IRF8 expression remains to be determined, but microglial IRF8 in the SDH has been shown to be upregulated by intrathecal administration of CSF1 or an activator of triggering receptor expressed on myeloid cells 2 (Trem2) [33, 55]. IRF8 also directly regulates transcription of IRF1 and IRF5 [63, 64]. It was found that IRF5 binds to the P2X4R promoter and induces its expression [64]. Loss of IRF5 suppresses the PNI-induced spinal P2X4R upregulation and pain hypersensitivity. Thus, the IRF8–IRF5 transcription cascade would be a core mechanism for producing P2X4R-expressing microglia after PNI and neuropathic pain. Microglial P2X4R upregulation also involves factors released from damaged DRG neurons such as CSF1 [33] and cysteine-cysteine chemokine ligand 21 (CCL21) [3] and by other extra- and intracellular factors [95, 96, 98, 99]. Pharmacological blockade and genetic knockout of P2X4R suppress the PNI-induced mechanical hypersensitivity [94, 97, 100]. Intrathecal administration of P2X4R-stimulated cultured microglia to normal rats induces allodynia, indicating that P2X4R-expressing microglia are not only necessary but sufficient to produce pain hypersensitivity [92, 97]. For activating P2X4Rs, extracellular ATP is required. ATP is known to be released from primary afferents [71], SDH neurons [47], and glia [6, 22, 41], but it was recently found that SDH neurons that express vesicular nucleotide transporter (VNUT [82], also known as SLC17A9; a secretory vesicle

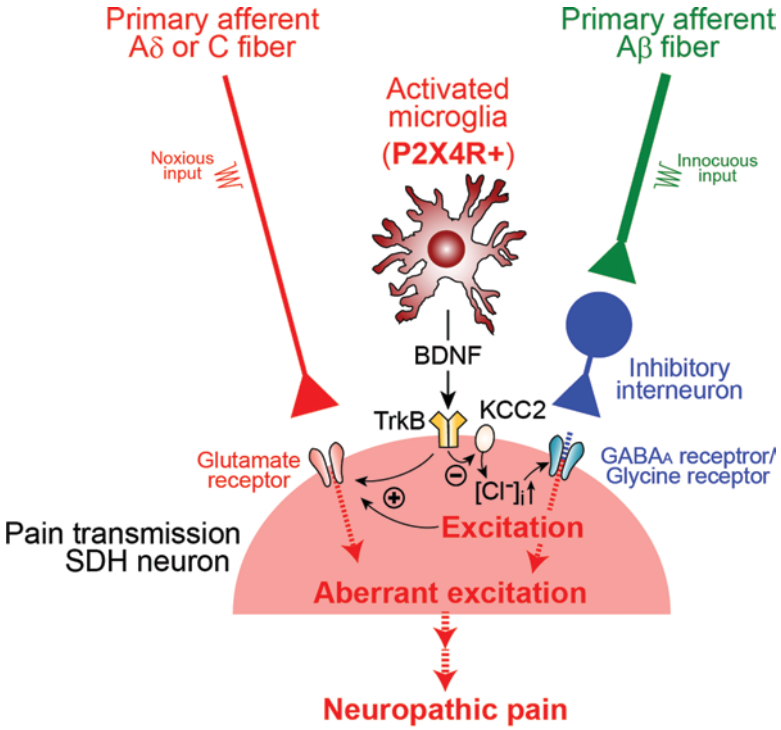

Fig. 7.1 Role of P2X4R-expressing spinal microglia in neuropathic pain. After PNI, microglia in the SDH become activated. The activated microglia upregulate P2X4R expression. P2X4R-stimulated microglia releases the signaling molecules BDNF. BDNF downregulates KCC2 in SDH pain transmission neurons, via TrkB, which causes an increase in intracellular Cl^- and leads to a depolarizing shift in the anion reversal potential. Under these conditions, GABA or glycine released as a result of innocuous stimulation induces neuronal depolarization. TrkB signaling also potentiates glutamatergic excitation via glutamate receptors. The resulting hyperexcitability of pain transmission in neurons contributes to neuropathic pain

protein responsible for storage and release of ATP) are a crucial source of the ATP that causes pain hypersensitivity [65]. Following stimulation of P2X4R, microglia release BDNF [16, 91]. BDNF activates tyrosine receptor kinase B (TrkB), in lamina I neurons, and induces an altered transmembrane anion gradient by downregulating KCC2, which caused changes in GABA- and glycine-evoked responses from inhibitory to excitatory and mechanical hypersensitivity [16] (Fig. 7.1). This change also potentiates their glutamatergic excitation via N-methyl-D-aspartate receptors (NMDAR) [38]. The crucial role of microglial BDNF was demonstrated by the finding that microglia-selective BDNF deficiency reduces PNI-induced pain [84]. By contrast, the conditional knockout of BDNF in primary afferent neurons has no effect [107]. These studies identifying the microglial P2X4–BDNF–KCC2 pathway provide evidence for the causal role of microglia-to-SDH neuronal signaling in neuropathic pain (Fig. 7.1).

Another microglial signaling to SDH neurons for neuropathic pain involves inflammatory factors. In particular, IL-1β and tumor necrosis factor-α (TNFα) have been extensively studied [43]. Important microglial receptors for producing and releasing these proinflammatory cytokines might be P2X7R and TLRs [10, 53, 54, 86]. In the SDH, P2X7R is required for ATP-induced IL-1β release from TLR4-primed microglia [13]. PNI-induced IL-1β transcription in the spinal cord involves TLR2 [53] and TLR4 [86]. At a posttranscription level, the Nod-like receptor family, pyrin domain containing-3 protein (NLRP3) inflammasomes activate pro-caspase-1, which promotes pro-IL-1β processing and secretion of mature IL-1β [35]. P2X7R is one of the most potent activators of the NLRP3 inflammasome [20]. IL-1β has been shown to phosphorylate NMDARs [101] and to enhance excitatory synaptic transmission [11, 50, 80]. IL-1β also decreases GABA- and glycine-mediated synaptic inhibition [50]. In addition, microglial IL-18, which can also be produced via NLRP3 inflammasomes, signals to astrocytes and contributes to neuropathic pain [69]. SDH astrocytes also become activated after PNI and contribute to maintenance of pain hypersensitivity [56, 93, 110], suggesting a crucial role of microglia-astrocyte signaling in chronicity of neuropathic pain.

TNFα is also a potent neuromodulator contributing to neuropathic pain. Expression of this cytokine in the SDH is exclusively increased in microglia after PNI via p38 mitogen-activated protein kinase (p38MAPK) [48]. TNF receptors (TNFR) in the SDH are found in multiple cell types [48]. In SDH neurons, TNFα rapidly increases excitatory responses evoked by activation of NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) in SDH neurons [50]. TNFα has recently been shown to contribute to a form of synaptic plasticity for pain amplification in the SDH [57]. TNFR expressed at presynaptic terminals of primary afferents modulates glutamate release [78]. Furthermore, microglia, astrocytes, and endothelial cells in the SDH also express TNFR [48]. Microglial TNFR activation increases expression of BDNF, which leads to an increase in dendritic structural remodeling and synaptic connectivity strength in lamina I SDH neurons [62]. TNFα acts on astrocytes and enhances expression of chemokines, which rapidly increase excitatory synaptic transmission [9, 24]. In endothelial cells, TNFR upregulates cyclooxygenease-2 (COX-2) and prostaglandin I2 synthase (PGIS) [48]. Pharmacological inhibition of COX-2 and prostaglandin I2 (IP) receptors reduces pain hypersensitivity. Since IP receptors are localized in SDH neurons [48] and primary afferents [76], microglial TNFα can activate neurovascular communication and produce pain [48]. Collectively, TNFα modulates synaptic structure and strength in SDH neurons by multiple mechanisms involving direct and indirect effects.

CatS is a lysosomal cysteine protease that is also a crucial microglial molecule for a communication to SDH neurons and for neuropathic pain [14]. CatS expression is upregulated in microglia in the SDH after PNI. Microglial CatS is released in response to P2X7R activation via p38MAPK and then cleaves membrane-bound fractalkine expressed on SDH neurons and astrocytes [12]. The cleaved fractalkine is considered to act on microglia again because the fractalkine receptor CX3CR1 is found exclusively in microglia [32, 109]. Activation of the P2X7R–p38MAPK–

CatS–fractalkine–CX3CR1 pathway leads to IL-1β secretion from microglia [11], which in turn modulates synaptic excitation and inhibition, as described above.

7.5 Brain Microglia and Neuropathic Pain

Recent studies have shown that PNI also activates microglia in several brain regions. These include the thalamus, amygdala, ventral tegmental area (VTA), nucleus accumbens (NAc), ACC, bed nucleus of stria terminalis, hippocampus, and periaqueductal gray [62, 68, 72, 89, 90]. Although the mechanism underlying microglia activation in the brain after PNI remains unknown, the role of brain microglia in neuropathic pain has recently been shown. It was found that inhibition of VTA microglia activation suppresses the PNI-induced reduction of dopamine release in the NAc and altered reward behavior [89], suggesting that activated microglia contribute to impairment of the VTA–NAc mesolimbic dopamine system after PNI. In the hippocampal CA1 region, dendritic structural complexity (including spine density), functional synaptic connectivity and BDNF levels were all reduced in PNI mice [62]. Microglial ablation and TNFR deficiency also prevented pain hypersensitivity and memory deficits after PNI. These findings provide evidence indicating that PNI activates brain microglia, which contributes to structural and functional synaptic alterations and pain hypersensitivity, as well as reward and memory deficits of PNI. It was also found that PNI also causes infiltration of circulating monocytes selectively in the central nucleus of the amygdala about 1 month later [81]. The infiltrated cells expressed IL-1β, and blocking the IL-1β signal reversed anxiety but not mechanical hypersensitivity. Because information about the aversive nature of the pain experience is thought to be processed in the central nucleus of the amygdala [4], ongoing signaling derived from infiltrated monocytes might also be crucial for the emotional component of neuropathic pain.

7.6 Therapeutic Implications

The mounting findings from studies using preclinical models described above provide much interest in microglia as a promising target for treating neuropathic pain. There are so far no clinically approved drugs that selectively target microglial molecules, but drug discovery efforts are currently in progress. A recent study identified NP-1815-PX as a novel P2X4R antagonist with a potent inhibition to rodent and human P2X4Rs [67]. Intrathecal administration of this compound to pathological pain models produces an anti-allodynic effect. Unfortunately, NP-1815-PX had poor CNS penetration, but the pharmaceutical company Nippon Chemiphar successfully developed a more potent and specific P2X4R antagonist with CNS-penetrating properties (NC-2600), which has been tested in phase I trials in Japan. Furthermore, the first-generation bisphosphonate clodronate was identified as a

potent and selective allosteric inhibitor for VNUT. Clodronate has shown to impair vesicular ATP release from neurons and to attenuate neuropathic pain [49]. Thus, these compounds can inhibit the activation of the P2X4R–BDNF–TrkB–KCC2 signaling pathway. P2X7R antagonists [44] and CatS inhibitor [36] could target the P2X7–CatS–fractalkine–CX3CR1–p38 MAPK–IL-1β pathway.

An alternative therapeutic potential of microglia for treating pain might be to increase the usefulness of opioids. Recent studies have revealed a crucial role of spinal and brain microglia in these side effects of opioids. Chronic morphine treatment activates microglia in the SDH and some brain regions [40]. Analgesic tolerance to opioids is suppressed by depleting spinal microglia [60] and by inhibiting microglial molecules [39, 60, 104, 108]. However, spinal microglia have little role in already established tolerance [23], suggesting that spinal microglia contribute to the development, but not maintenance, of morphine analgesic tolerance. Furthermore, morphine is known to produce a paradoxical increase in pain sensitivity. This side effect seems to be dependent on microglial P2X4R signaling in the SDH [23]. Moreover, it was also recently found that spinal microglia depletion also attenuates the behavioral sequela of withdrawal from chronic morphine [6]. Microglia activated by chronic morphine treatment release ATP via pannexin 1 that has interacted with P2X7R, and inhibition of microglial ATP release attenuates withdrawal behavior and long-term synaptic facilitation [6]. These findings suggest that targeting spinal microglia might selectively prevent the undesirable side effects caused by chronic opioid use without reducing their pain-relieving effect. However, whether opioids act directly on μ-opioid receptors (MOR) expressed by microglia remains controversial. Some studies showed that opioids upregulate microglial molecules (like P2X4R, P2X7R, and pannexin 1) in cultured microglial cells in vitro via microglial MOR, but a recent study reported that MOR is undetectable in spinal microglia isolated from adult mice. The latter study also showed that a conditional loss of MOR in primary afferent nociceptors eliminates morphine-induced tolerance and hyperalgesia without suppressing activation of spinal microglia [15]. Further investigation is needed to clarify this issue.

Several studies have recently established methods for generating human microglia through the differentiation of induced pluripotent stem (iPS) cells to erythromyeloid progenitor-like cells [70], which may provide a major step forward to understanding an alteration in microglial functions in neuropathic pain patients. If circulating monocytes recruited to the brain also contribute to neuropathic pain [81], a technique for developing induced microglia-like (iMG) cells from human blood monocytes [75] would be useful. It was recently found that iMG cells of fibromyalgia patients display a TNFα-releasing inflammatory phenotype, and interestingly the ability of iMG cells to release this cytokine correlates with the pain severity of patients [74]. Thus, it is possible that iMG cells may be used to study the mechanisms of neuropathic pain and also as biomarkers for diagnosis and therapeutics. However, it should be noted that there are dramatic differences between cultured microglia and microglia in vivo [7], and thus further studies are needed to examine whether human microglia derived from iPS cells and human iMG derived from monocytes are indeed useful for translation.

7.7 Conclusions

An accumulating body of literature has not only provided compelling evidence for the necessity and sufficiency of microglia in neuropathic pain but also greatly advanced our understanding of the molecular and cellular mechanisms of this contribution. The recent identification of microglia-selective genes [7, 25, 31, 37] will accelerate investigations. Furthermore, recent work has revealed a crucial role for brain microglia in sensory and/or emotional aspects of neuropathic pain, although the underlying mechanism(s) remain unknown. Because pharmacological, molecular, and genetic manipulations of the function or expression of microglial molecules substantially influence chronic pain behaviors and have no effect on acute physiological pain under normal conditions, glial cells and their expressing molecules might be good targets for treating chronic pain. Indeed, potent and selective antagonists and/or inhibitors targeting microglial molecules have been developed and exhibit therapeutic effects on neuropathic pain hypersensitivity in preclinical models. Structure-based drug discovery together with technological advances in establishing human microglia from iPS cells and iMG from circulating monocytes from patients will help us to establish a strategy to effectively suppress activated microglia and to diagnose neuropathic pain.

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Chapter 8
Descending Noradrenergic Inhibition: An Important Mechanism of Gabapentin Analgesia in Neuropathic Pain

Ken-ichiro Hayashida and James C. Eisenach

Abstract Gabapentinoids are effective in a wide range of animal pain models and in patients with neuropathic pain and has become one of first-line treatments for neuropathic pain. Because spinal plasticity and sensitization have been intensely studied in neuropathic pain, most laboratory studies have focused on actions of gabapentinoids in the spinal cord, where they reduce primary afferent traffic and excitation of spinal nociceptive neurons, via interaction with α2δ subunits of voltage-gated Ca^{2+} channels. However, a recent clinical study questioned the relevance of this in vitro and in vivo rodent studies by demonstrating a complete lack of clinical efficacy of intrathecal gabapentin in patients with chronic pain. Curiously, preclinical studies continue to focus on spinal cord actions of gabapentinoids despite this lack of translation to humans.

We and others demonstrated that gabapentin inhibits presynaptic GABA release and induces glutamate release from astrocytes in the locus coeruleus (LC), thereby increasing LC neuron activity and spinal noradrenaline release, and that gabapentin relies on this action in the LC for its analgesia. We also recently discovered that, with prolonged time after neuropathic injury, noradrenergic neurons in the LC become less responsive to gabapentin, leading to impaired gabapentin analgesia, and that astroglial glutamate dysregulation is critical to this impaired LC response. The clinically available drug valproate increases glutamate transporter-1 (GLT-1) expression in the LC to restore this impaired gabapentin analgesia.

Keywords Neuropathic pain · Gabapentin · Locus coeruleus · Astrocyte · Glutamate transporter

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8.1 Introduction

Peripheral nerve injury can result in neuropathic pain, hyperalgesia, and allodynia which respond poorly to nonsteroidal anti-inflammatory drugs. Although opioids are effective acutely [15], their chronic use is complicated by tolerance and therapy-limiting side effects, and hence alternatives to opioids have been sought for decades. Only a few of these have shown efficacy in the clinic.

Gabapentin was licensed as an antiepileptic drug in 1993 and was rapidly recognized to be effective to reduce pain in patients with neuropathic pain. This clinical experience is paralleled in the laboratory, where gabapentin has been shown to possess analgesic properties in a wide range of neuropathic pain models [7, 9, 13, 19]. Gabapentin interacts with the α2δ subunit of voltage-gated calcium channels, which modulate the release of excitatory amino acids at the level of the spinal dorsal horn [3]. α2δ subunits are upregulated in the spinal cord and primary sensory afferents after nerve injury in rodents [11], and gabapentin blocks voltage-gated calcium channels in transgenic mice with upregulated α2δ-1 subunits but not in normal mice [10], suggesting efficacy of gabapentin depends on this subunit. For this reason, most laboratory studies have focused on the theory that gabapentin primarily acts on spinal pain mechanisms. However, a recent clinical study questioned this theory by demonstrating a complete lack of clinical efficacy of intrathecal gabapentin in patients with noncancer pain [16], despite the known efficacy of oral gabapentin in this patient population.

Tanabe et al. first reported the involvement of the locus coeruleus (LC)-spinal descending noradrenergic pathway in gabapentin analgesia by demonstrating that depletion or blockade of spinal noradrenergic signaling abolished the anti-hypersensitivity effect of systemically administered gabapentin in mice after peripheral nerve injury [19]. Similar behavioral results have also been reported in various rodent models of neuropathic and postoperative pain after systemic, intracerebroventricular, or intra-LC administration of gabapentin [6, 7, 9, 13]. Consistent with these behavioral observations, we demonstrated that systemically administered gabapentin increases phosphorylated cAMP response element-binding protein expression, as a measure of neuronal activation, in LC neurons and subsequently induces spinal noradrenaline release in rats 2–3 weeks after spinal nerve ligation (SNL) [7]. Gabapentin likely acts similarly in humans, since its oral administration, in a dose that produces postoperative analgesia, increases noradrenaline concentration in cerebrospinal fluid from patients scheduled for orthopedic surgery for painful degenerative joint disease (Fig. 8.1) [6]. Together, these laboratory and clinical observations suggest that gabapentin recruits descending inhibition and that this inhibition plays a key role in gabapentin-induced analgesia.

This mini-review discusses the mechanisms by which gabapentin activates the LC for analgesia, how gabapentin loses its efficacy during chronification of neuropathic pain, and pharmacologic approaches to restore or enhance gabapentin analgesia in chronic neuropathic pain.

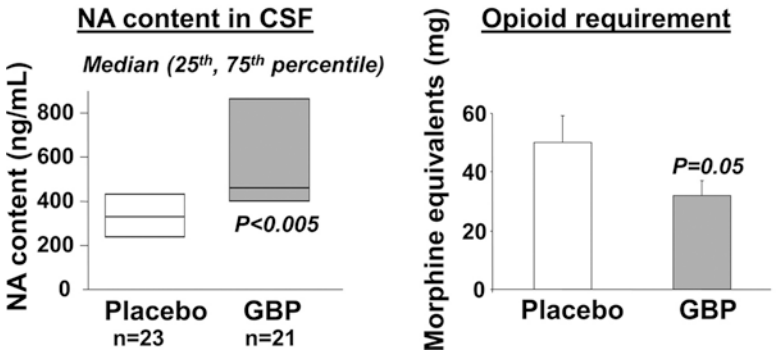

Fig. 8.1 On the day of orthopedic surgery, patients with chronic, painful degenerative joint disease were randomly assigned to receive oral 1200 mg gabapentin (GBP) or placebo approximately 90 min before collecting cerebrospinal fluid (CSF) samples for noradrenaline (NA) measurement (left panel) and the amounts of opioid (right panel) administered in the first 24 h. (Adapted from Hayashida et al. [6])

8.2 Mechanisms of LC Activation by Gabapentin

Despite its name and structural similarity to GABA, gabapentin does not have direct effects on GABA receptors. However, analgesic effects of gabapentin may reflect modulation of GABA release. Gabapentin's actions on GABA release are controversial and likely depend on the site in the central nervous system. For example, gabapentin does not affect GABA release from spinal dorsal horn synaptosomes in rats [23]. Some studies show an increase in GABA release in rat and human brain from gabapentin [4, 14, 21], whereas other studies show a direct reduction in GABA release when rat cortical synaptosomes are exposed to gabapentin [1]. In the LC, gabapentin and other α2δ ligands reduce presynaptic GABA release and thereby disinhibit noradrenergic neurons in rodents [18, 23], suggesting that gabapentin reduces the influence of GABA in the LC as one of the mechanisms by which it activates descending inhibition.

In rats 2–3 weeks after SNL surgery, gabapentin-induced analgesia and activation of LC neurons were blocked by local application of a AMPA glutamate receptor antagonist [7]. This suggests that, other than reducing the influence of GABA, gabapentin also relies on glutamate-dependent mechanisms in the LC to activate descending inhibition. In vivo microdialysis, systemic or local perfusion of gabapentin in rats consistently increases extracellular glutamate concentrations in the LC but not in the spinal cord [17]. In addition to its direct inhibition on LC neurons via postsynaptic GABAA receptors, GABA is known to inhibit glutamate release via presynaptic GABAB receptors [5, 20]. However, blockade of GABAB receptors in the LC affects neither basal glutamate levels nor the gabapentin-induced glutamate increase in rats [17], suggesting that tonic influence of GABA on glutamatergic terminals via presynaptic GABAB receptors is either minor or absent in the LC and that gabapentin's effect on the glutamate levels is independent of GABA-mediated mechanisms.

We proposed that gabapentin increases extracellular glutamate in the LC via actions on the glutamate transporter-1 (GLT-1) in astroglia based on our observations that gabapentin and its related α2δ ligand pregabalin increase co-transport of Na^+ ions and glutamate via glutamate transporters and enhance glutamate-induced intracellular Ca^{2+} response via the reverse mode of Na^+-Ca^{2+} exchange and by these mechanisms facilitate glutamate release in cultured astrocytes [22]. We also showed the in vivo relevance of gabapentin's action in astrocytes, by demonstrating in rats 2–3 weeks after SNL that selective blockade or knockdown of GLT-1 abolished gabapentin's effects on glutamate increase in the LC and on gabapentin-induced anti-hypersensitivity [8, 17]. Since there is no evidence for the presence of α2δ subunits in astrocytes, α2δ interactions probably are not involved in the effect of gabapentin on astrocytes. This is further supported by our observation that pregabalin increases extracellular glutamate concentrations in the LC, whereas 3-exo-aminobicyclo[2.2.1]heptane-2-exo-carboxylic acid does not, despite its high binding affinity to α2δ subunits [12, 17].

Taken together, these results suggest that gabapentin inhibits presynaptic GABA release and induces glutamate release from astrocytes in the LC, thereby increasing LC neuronal activity and spinal noradrenaline release, and that gabapentin relies on this action in the LC for its analgesia, at least during the early phase (2–3 weeks after nerve injury) of neuropathic pain (Fig. 8.2).

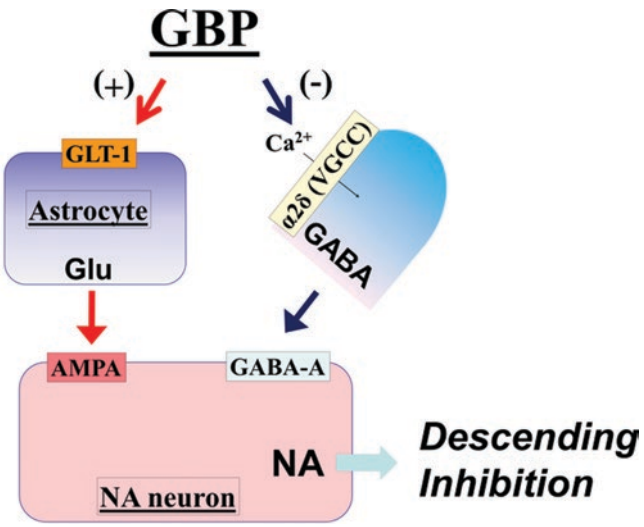

Fig. 8.2 Proposed mechanisms of gabapentin (GBP) action in the locus coeruleus (LC). GBP interacts with the α2δ subunit of voltage-gated calcium channels (VGCC) to reduce presynaptic GABA release and activates glutamate transferase-1 (GLT-1)-dependent mechanisms to induce glutamate (Glu) release from astrocytes in the LC, thereby increasing LC neuronal activity and spinal noradrenaline (NA) release to recruit descending inhibition

8.3 Impaired Gabapentin Analgesia in Chronic Neuropathic Pain

In various neuropathic pain models in rodents, gabapentin is remarkably and uniformly effective [7, 9, 11, 19]. However, gabapentin often fails to provide sufficient analgesia in patients with neuropathic pain [2]. In the past two decades, pain research has primarily focused on the mechanisms of clinical efficacy of gabapentin, but less attention has paid to understand why all rodents, but only some patients respond to gabapentin. From a public health perspective, understanding mechanisms of this heterogeneity in response and how efficacy might be improved is arguably as important as understanding therapeutic mechanisms.

We demonstrated that the early uniform anti-hypersensitivity effect of gabapentin in rats after SNL is lost as time progresses after surgery (Fig. 8.3) [8]. For a large effect size to reduce hypersensitivity (>30 g), this is uniformly lost around 5 weeks after injury, and for smaller effect sizes (>10 g), it is lost in nearly half of the animals 10 weeks after injury. This questions the relevance of much of the previous

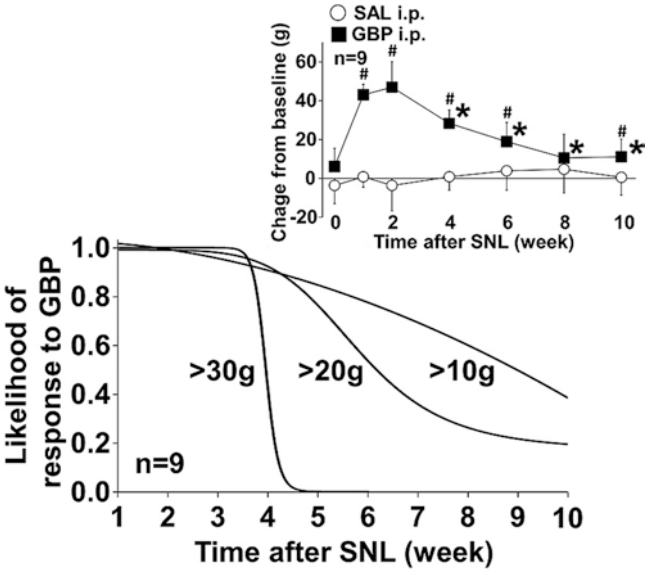

Fig. 8.3 Upper panel: withdrawal thresholds in the ipsilateral hindpaw to spinal nerve ligation (SNL) were measured 1 h after an intraperitoneal injection of saline (SAL) or gabapentin (GBP, 100 mg/kg) in rats 0–10 weeks after SNL(upper right graph). Data (mean ± SD) are presented as change from baseline.*$P < 0.05$ vs. 2 weeks after SNL. #$P < 0.05$ vs. SAL. Lower panel: the likelihood, assessed by logistic regression, that gabapentin (GBP) will produce an increase in mechanical withdrawal threshold of three levels from baseline hypersensitive level (30 g = strong effect, 20 g = moderate effect, or 10 g = weak effect) as a function of time after spinal nerve ligation (SNL) injury. (Adapted from Kimura et al. [8])

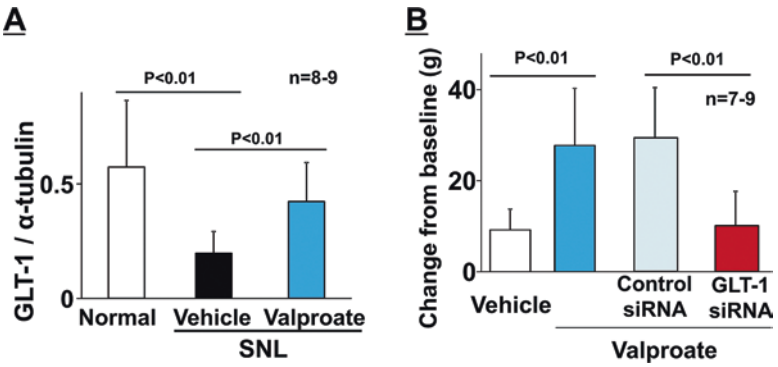

Fig. 8.4 (**a**) Representative Western blotting images and quantification of GLT-1 in the LC from normal and SNL rats treated with oral vehicle or valproate (200 mg/5 mL/kg/day) for 14 days starting from 6 weeks after SNL surgery. Data (mean + SD) are presented as ratio to α-tubulin. (**b**) Vehicle or valproate-treated animals with or without repeated intra-LC injections of the control (8.3 pmol/0.5 μl) or GLT-1 (8.3 pmol/ 0.5 μl) siRNA for the last 5 days received an intraperitoneal gabapentin (100 mg/kg). Withdrawal thresholds in the hindpaw ipsilateral to SNL were measured 1 h after gabapentin administration. Data (mean + SD) are presented as change from baseline. (Adapted from Kimura et al. [8])

laboratory studies that were performed within the first 2–4 weeks of surgical injury, to clinical use of gabapentin in long-standing pain, and suggests that future studies aimed to better understand individual lack of response to gabapentin should focus on times more remote than 2–4 weeks after injury.

Given that expression of GLT-1 in the LC is essential to gabapentin's analgesia and that GLT-1 is downregulated in the LC 6 weeks after SNL [8, 17], we recently hypothesized that downregulation of GLT-1 results in reduced gabapentin efficacy over time after nerve injury. Consistent with this hypothesis, inhibition of histone deacetylase (HDAC) by the clinically available drug valproate restores downregulated GLT-1 expression in the LC 8 weeks after SNL (Fig. 8.4a) and also restores the anti-hypersensitivity effect of gabapentin (Fig. 8.4b). This effect of valproate is reversed by the knockdown of GLT-1 in the LC. These results strongly suggest not only a causal relationship between GLT-1 expression in the LC and the anti-hypersensitivity effect of gabapentin but also a possible treatment strategy to restore impaired gabapentin analgesia using HDAC inhibitors in chronic neuropathic pain.

8.4 Conclusion

Although interactions with α2δ subunits of voltage-gated calcium channels are shown to be essential for gabapentin's effects in rodents [10, 11], the lack of efficacy of intrathecal gabapentin in patients [16] questions whether gabapentin relies exclusively on spinal actions for its analgesic efficacy. We demonstrated that gabapentin inhibits presynaptic GABA release and induces glutamate release from

astrocytes in the LC, thereby increasing LC neuron activity and spinal noradrenaline release, and that gabapentin relies on this action in the LC for its analgesia, at least during the early period after neuropathic injury. However, when neuropathic pain becomes chronic, noradrenergic neurons in the LC become less responsive to gabapentin, leading to impaired gabapentin analgesia. Downregulation of astroglial GLT-1 in the LC is critical to this impaired response of the LC to gabapentin. Oral valproate increases GLT-1 expression in the LC, possibly via HDAC inhibition, to restore the impaired anti-hypersensitivity effect of gabapentin. Given clinical availability and established safety profiles, valproate should be tested to rescue gabapentin efficacy in those who initially fail to respond to gabapentin.

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Chapter 9
Chronic Neuropathic Pain Protects the Heart from Ischemia-Reperfusion Injury

Yi-Fen Cheng and Chien-Chang Chen

Abstract The prevalence of chronic pain increases with age. The pain occurrence in the elderly ranges from 25% to 80% in different countries. Ischemic heart disease is also prevailing in the aged people. Restored blood flow quickly rescues myocardium but also causes ischemia-reperfusion (IR) injury. Brief episodes of ischemia at a distant organ could reduce the myocardial reperfusion injury. This is called remote ischemic preconditioning (RIPC) cardioprotection. Several circulating factors and neurogenic signals contribute to the cardioprotection by RIPC. Preinfarction angina, a form of chest pain, is associated with significant cardioprotection in myocardial infarction patients. Activation of peripheral nociception also induces cardioprotection against IR injury via neurogenic pathway. It is possible that angina also induces nociceptive signal pathway to provide cardioprotection. It is unclear whether preexisting chronic pain will also have a cardioprotection effect. We recently reported chronic neuropathic pain attenuates cardiac IR injury in mice. ERK activation in anterior nucleus of paraventricular thalamus (PVA) is required for this remote cardioprotection. Direct activation of PVA neurons also provides cardioprotection against cardiac IR injury. Chronic neuropathic pain-induced cardioprotection requires activation of parasympathetic nerves. This review summarizes the potential interaction of chronic pain and cardiac IR injury.

Keywords Cardiac ischemia-reperfusion injury · Chronic neuropathic pain · Anterior nucleus of paraventricular thalamus (PVA) · ERK

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9.1 Introduction

Cardiovascular diseases (CVDs), including heart failure, hypertension, stroke, and coronary artery disease, are the leading cause of death in the world [2]. The number of people died from CVDs estimated 17.5 million and will increase to 23.3 million by 2030 [1, 56]. Smoking, eating an unhealthy diet, obesity, diabetes, and low physical activity are risk factors for CVDs [2]. Ischemic heart disease (IHD) accounts for 12.7% of total global mortality. It is responsible for sudden death due to insufficient blood supply to the heart [29, 61]. Even though mortality rate is getting lower in high-income countries, it remains high in developing countries. Therefore, innovative and better treatments for CVDs are still needed.

Most IHD is caused by atherosclerosis. The major interventions of IHD are timely thrombolysis or percutaneous coronary intervention to restore blood supply to reduce ultimate infarct size [11]. Paradoxically, rapidly reperfusion of the infarcted myocardium induces the death of cardiomyocytes and exacerbates the extent of heart injury which is called myocardial ischemia-reperfusion (IR) injury. The pathogenesis processes of IR injury include calcium overload, increased reactive oxygen species (ROS), and pH paradoxes. These three events give rise to mitochondrial dysfunction and membrane rupture due to opening of the mitochondrial permeability transition pore (mPTP). Occlusion of coronary arteries leads to an imbalance between the production and consumption of energy. During ischemia, cardiomyocytes turn to anaerobic metabolism which leads to accumulation of lactic acid and proton which decrease intracellular pH to less than 7.0. When blood flow of coronary artery promptly rebuilds in the infarcted myocardium, rapid exchange of proton restores the pH by Na^+-H^+ exchange pathway. The pH shift permits mPTP opening and cardiomyocyte hypercontracture [35, 48]. Rapidly restoration of pH also leads to accelerated Ca^{2+} entry through Na^+-Ca^{2+} exchanger resulting in calcium overload [69]. Massive ROS are generated due to reactivation of the electron transport chain in mitochondria during reperfusion. ROS is also a chemoattractant which mediates leukocyte infiltration [35]. ROS would also cause mPTP opening and induce cardiomyocyte death. There are four recognized forms of myocardial IR injury such as arrhythmia, myocardial stunning, microvascular obstruction, and lethal reperfusion [35, 70]. Lethal reperfusion injury accounts for up to 50% of the final size of myocardial infarction, and there is no effective treatment available to reduce lethal myocardial reperfusion injury [35, 39].

Conditioning the heart could reduce IR injury with brief periods of ischemia followed by reperfusion before or after restoration blood supply in the infarcted region. This phenomenon is called ischemic conditioning [44]. Cardioprotection can also be induced by nonischemic stimulation. For example, peripheral nociception induced by skin incisions on the abdomen provided cardioprotection and called remote preconditioning of trauma (RPCT) in rodent [41]. Furthermore, patients with angina, a form of chest pain, have better prognosis and less reperfusion injury compared to those without chest pain [59]. It is possible that angina also induces nociceptive signal pathway to provide cardioprotection. It is unclear whether preexisting chronic pain will also have a cardioprotection effect.

9.2 Ischemia Preconditioning in Remote Sites Prevent Myocardial Ischemia-Reperfusion Injury

Brief episodes of ischemia-reperfusion before prolonged occlusion could protect the myocardium against reperfusion injury. Murry et al. first demonstrated that brief episodes of ischemia-reperfusion decreased the rate of ATP depletion and washed out the catabolites that stored during ischemia. They applied four cycles of coronary artery occlusions for 5 min followed by reperfusion for 5 min in canine before beginning a sustained occlusion of the coronary artery for 40 min. Their results showed ischemic preconditioning (IPC) in the heart could decrease the subsequent infarction size after 4 days of reperfusion compared to control [62]. IPC has two windows of protection. It provides an acute time windows last between 4 and 6 h and the second window 24–72 h after preconditioning [87]. The detailed signaling mechanism of IPC has been well-studied. IPC initiates several autacoids including adenosine, opioids, and bradykinin binding to their membrane receptors on cardiomyocytes. In the acute phase of IPC protection, the stimulation of these triggers conducts signaling through ERK, Akt, and eNOS to produce nitric oxide (NO). NO stimulates mitochondria to generate mild ROS which induces protein kinase C (PKC) to open mitochondrial K_{ATP} channel. NO also inhibits mPTP opening. In the second window time point, IPC activates transcription factors, including AP-1, hypoxic-inducible factor 1 (HIF-1α), and signal transducer and activator of transcription (STAT). These transcription factors contribute to synthesize mediators including heat shock proteins and inducible nitric oxide synthase [36]. However, IPC needs to be applied prior to the known ischemic event and requires to be invasively applied to the heart; it may not be feasible in clinical practice [51].

Intermittent occlusions in remote vascular bed also minimize infarct size in myocardium subjected to sustain ischemia. Przyklenk et al. used four cycles, 5 min of ischemia followed by 5 min of reperfusion in the circumflex coronary artery in the canine model [71]. Their results showed this kind of preconditioning could also reduce the infracted size in the territory which occluded by ligating the left anterior descending artery. This is called remote ischemic preconditioning (RIPC) [71]. Followed studies suggested RIPC-induced cardioprotection could be elicited by applying to an organ or tissue remote from the heart [13, 31, 34, 42]. RIPC is more feasible in clinical settings and provides a similar protective effect. Clinical trials have been conducted to investigate remote limb ischemic preconditioning by blood pressure cuff when patients undergo acute myocardial infarction or before receiving angioplasty or coronary artery bypass graft (CABG) surgery [14, 79]. Candilio et al. found significant reduction in the troponin T level and less incidence of postoperative atrial fibrillation 72 h after surgery in patients receiving RIPC compared to those in control group [14]. Botker et al. showed a randomized clinical trial of 333 patients with first acute myocardial infarction received RIPC by repeated ischemia-reperfusion cycles in an upper limb by a blood pressure cuff during ambulance transferring to the hospital. They found patients with RIPC had a greater myocardial salvage assessed by single-photon emission CT (SPECT) after angioplasty, but left

ventricular ejection fraction was not significantly different 30 days after angioplasty between the preconditioned and control group [6, 83]. These studies indicated a successful translation of remote ischemic preconditioning, and it is a noninvasive, simple, safe, and cheap intervention clinically. However, two multicenter randomized trials, the Remote Ischemic Preconditioning for Heart Surgery (RIPHeart) study by Meybohm et al. [58] and the effect of remote ischemic preconditioning on clinical outcomes in patients undergoing coronary artery bypass graft surgery (ERICCA) trial by Hausenloy et al. [33], suggest no clinical improvement between RIPC and sham-RIPC groups. The negative results of these two trials have been attributed to the use of hypnotic drug propofol [46]. Propofol is known to mask the effect of ischemia-induced cardioprotection [45]. Future studies of RIPC avoiding the use of propofol are required to confirm the effect of RIPC clinically.

Several studies pointed out remote organs would produce endogenous factors such as bradykinin, opioids, adenosine, nitrite, and microRNAs to trigger protective effect [9, 34, 49, 72]. RIPC also activated similar kinases in IPC in myocytes and eventually targeted to mitochondria. Ligands bind to the cell surface and then activate intracellular kinases such as protein kinase C (PKC) and signaling components such as nitric oxide and ROS. The end effectors of RIPC lead to the opening of mitochondrial K_{ATP} channel, inhibiting mPTP formation or opening [34, 36]. RIPC and IPC have similar intracellular signal pathways but how the signal transmits from the remote site to the heart is currently controversial. Daily limb ischemic preconditioning for 15 min, 24 h, or 9 days in mice provides similar cardioprotection in a global IR injury model using isolated hearts by enhancing autophagy signaling [73]. This study suggests that cardioprotection could be achieved by chronic ischemic preconditioning. Long-term remote ischemic preconditioning is associated with downregulation of mTOR and enhanced autophagy signaling [73].

The remote cardioprotective signal from a distant tissue or organ to the heart needs a humoral and/or neuronal transmission. Remote ischemic preconditioning at distant sites releases molecules such as nitrite, bradykinin, and adenosine as humoral factors which bind to several Gi-coupled receptors on cardiomyocytes [36]. Previous studies show intravenous pretreatment of adenosine, bradykinin, or opioid receptor antagonists could inhibit RIPC [50, 53, 76, 78]. Acetylcholine (Ach) could promote vascular relaxation and if treated prior to ischemia could reduce reperfusion injury in the heart [20].

Other studies used the ganglionic blockers such as hexamethonium to show that RIPC derived protective effect via the autonomic nervous system [31, 76, 86]. Gho et al. demonstrated brief occlusion of anterior mesenteric artery reduced myocardial IR injury, whereas hexamethonium applied 15 min prior to ischemia stimulus blocked the protective effect [31]. This study suggested remote ischemic preconditioning delivered protective signals via a neurogenic pathway. Mastitskaya et al. established an important role for parasympathetic outflow against myocardial reperfusion injury using an optogenetic approach directly stimulating the dorsal vagal motor nucleus (DVMN), and inhibition of DVMN by chemogenetics could abolish cardioprotection from RIPC [55]. The cardioprotective signal from the preconditioned remote site could be attenuated by atropine pretreatment or vagotomy [5, 26].

These studies provide strong evidence that cardioprotection provided by RIPC requires parasympathetic nervous system.

Lim et al. showed that resection of femoral nerve and sciatic nerve or occlusion of femoral vein before RIPC could abolish cardioprotection [52]. Pickard et al. showed that RIPC signal releasing is dependent on prior activation of the vagus nerve [68]. They performed bilateral cervical vagotomy before RIPC in rats and transferred the dialyzed venous blood plasma from the RIPC rat to naïve-isolated rat hearts before IR injury. The naive recipient hearts perfused with RIPC-treated plasma showed reduced infarct size after reperfusion injury; but the RIPC-treated plasma from bilateral cervical vagotomy did not provide protection in naïve rat hearts [68]. Therefore, the intact neural pathway of RIPC to the heart is required for the cardioprotection.

Patients with acute myocardial infarction accompany with preinfarction angina have better prognosis and smaller infarct size [28]. Preinfarction angina results from ischemia that excites chemosensitive and mechanoreceptive neuron in the heart. The cardiac ischemic episodes release factors such as adenosine and bradykinin that activate the receptors of the sympathetic and vagal afferent pathways [30]. The beneficial effect of angina against reperfusion injury may depend on the neurogenic pathway, but the mechanism is still unclear.

9.3 Peripheral Nociceptive Stimuli Elicited Protective Effect Against Myocardial Reperfusion Injury

In 2009, Jones et al. showed an incision in the abdomen provided cardioprotection and named this phenomenon remote preconditioning of trauma (RPCT). Application of capsaicin which caused acute pain in the abdomen in mice also elicited cardioprotection [41]. They found RPCT-induced protection went through the spinal cord, activated the sympathetic nerve, and induced translocation of PKCε to the membrane fraction in cardiomyocytes [41]. This is the first study that showed a beneficial effect of acute pain to cardiac IR injury. Stimulating the sensory neurons by capsaicin injection into the hind paw elicits cardioprotection in rats only when applied before myocardial ischemia [5]. These results suggest that activation of remote sensory neurons could reduce myocardial IR injury. However, whether chronic nonischemic preconditioning could provide cardioprotection is unclear.

Pain is not only an unpleasant feeling with actual or potential tissue damage but also a complex sensory modality. The feeling of pain is essential for survival to avoid tissue damage. Acute pain serves as a protective mechanism for avoiding a damage or threat to the body. People who could not sense pain congenitally are easily injured, and most of them die at an early age. Acute pain usually disappears less than 30 days when the underlying cause of pain has been treated or diminished. Chronic pain persists even the injury is healed, and pain signals last longer in the nervous system. About 25% of the population suffers from chronic pain and espe-

cially in the aged. A systematic review shows that 6.9–10% of adults have chronic pain with neuropathic characteristic [66]. Neuropathic pain is a complex, chronic pain state and often results from alcoholism, chemotherapy, amputation, or diabetes. The prevalence of chronic neuropathic pain is about 7–8% of the population and lack of effective therapy [7, 21, 80].

Stimulus-evoked pain in rodents is measured by the response of latency and thresholds to tactile, chemical, or thermal stimuli. Spared nerve injury (SNI) model mimics human chronic neuropathic pain [8]. The common peroneal, tibia, and sural nerves are the distal branches of the sciatic nerve. Lacerating the common peroneal and sural nerves and keeping the tibia nerve intact produced robust mechanical pain response in the SNI model. Contralateral side can be used as a control. SNI in mouse provides an easy and reproducible mechanical neuropathic pain response [77].

Pain transmission and modulation is a complex and dynamic interaction including intrinsic spinal interneurons, glia. Noxious stimuli transmitting from painful initiation sites to the cortex allow for conscious awareness, producing an emotionally unpleasant pain, learning, and memory of noxious stimuli, removing of stimuli, and ultimately returning to the non-active state [82]. Activation of peripheral sensory fibers or injury to nerve fibers induces pain response. An endogenous top-down inhibitory descending modulatory circuit modulates pain transmission. Descending pain modulatory circuit receives inputs from hypothalamus and amygdala; rostral anterior cingulate cortex suppresses ascending pain signals and output signals through periaqueductal gray (PAG) to the medulla [65]. The inhibitory impulses from neurons within the nucleus raphe magnus and nucleus reticularis gigantocellularis within the rostral ventromedial medulla (RVM) descend to the medullary dorsal horn or the spinal cord and form connection to activate modulatory neurons and then attribute to the motor function [65, 82]. Stimulation of the PAG or RVM could induce the release of serotonin in the spinal cord and increase norepinephrine level in the cerebrospinal fluid to elicit anticonception. Moreover, brief shock of the forepaws causes stress-induced analgesia and was abolished by systematic application of naloxone or microinjection of μ-opioid receptor antagonists into the PAG or RVM, indicating the involvement of endogenous opioidergic pain inhibitory system [27, 65].

A particular set of central nervous system (CNS) structures including somatosensory cortices, anterior cingulate cortex (ACC), thalamus, prefrontal cortices (PFC), and insula respond to painful stimuli. These specific regions in response to pain have displayed a pain matrix from human brain imaging studies. Activation of this so-called pain matrix signature has been related to perceived pain intensity [75]. It also has been showed alterations in the connectivity in the CNS for acute pain in healthy subjects and chronic pain patients have distinctive features [3]. A study using functional magnetic resonance (fMRI) showed activity within the brainstem, and the anterior thalami remained increased during central sensitization in comparison with normal state in human [47]. Animal studies using different approaches showed the importance of CNS in mediating hyperalgesia. Carrasquillo et al. illustrated the activation of ERK in central nucleus of amygdala (CeA) altered the formalin modulating mechanical hyperalgesia in mice [15].

We have shown that the $Ca_v3.2$ T-type Ca^{2+} channels in the anterior nucleus of the paraventricular thalamus (PVA), a brain region located at the rostral portion of paraventricular thalamus (PVA), are required for the development of chronic muscle pain induced by repeated acid injection into lateral gastrocnemius muscle [18]. PVA is known to modulate circadian rhythm by receiving the projections from suprachiasmatic nucleus (SCN). Increases of nociception correlated neuronal activity markers, c-fos and pERK, have been observed in PVA in rodents [10, 19, 24, 63]. Inhibiting ERK activity in PVA prevented the development of chronic mechanical hyperalgesia in mice [18]. We also showed that activation of ERK in PVA plays a crucial role in the modulation of mechanical hyperalgesia in chronic neuropathic pain [17]. Previous tracing studies demonstrate that PVA sends projections to many brain regions, including amygdala and the bed nucleus of the stria terminalis (BNST) in rats and monkeys [38, 60, 84]. We also observe similar projections from PVA to BNST and amygdala in mice (unpublished data). BNST projects to the nucleus tactus solitarii (NTS) and nucleus ambiguus and modulates baroreflex response via parasympathetic nerve system [22, 32, 37]. It is possible PVA neurons affect autonomic nervous system via the BNST/NTS/nucleus ambiguus connection.

9.4 Potential Link in Chronic Pain and Myocardial Ischemia-Reperfusion Injury

Acute pain and capsaicin application in the abdomen could elicit remote cardioprotection [41]. However, it is unclear whether chronic pain could have any effect on myocardial IR injury. People worldwide suffer from chronic pain especially in the elderly. Cardiovascular heart disease is the leading cause of death in people older than 65 years [16]. A prospective study showed patients had experience angina before first-time acute myocardial infarction (AMI) associated with decreased infarct size and with better protection of global and regional left ventricular contractility [59]. Moreover, remote acute nociception by trauma or directly activating C sensory fibers in the skin also provides cardioprotection through spinal nerve and sympathetic nervous system [41]. Pain may alter autonomic nervous system and further regulate myocardial injury. Direct stimulation in DVMN also elicited cardioprotection against IR injury [55]. A number of studies showed that electrical stimulation in vagal neuron in canine, cats, and rats reduced myocardial IR injury and the risk of subsequent heart failure. Vagal stimulation-induced cardioprotection may be mediated through cholinergic anti-inflammatory pathway, independent of the bradycardic effect [4, 88, 89]. However, the results of clinical trials for vagal stimulation remained controversial, and further investigation is required [12].

Jin et al. investigated whether neuropathic pain affects autonomic nervous activities by measuring heart rate and arterial blood pressure and plasma level of norepinephrine in rats after chronic constriction injury (CCI)-induced neuropathic pain

[40]. Their results demonstrated that the cardiovascular function was increased through sympathetic and non-sympathetic activity for 2 weeks after CCI, followed by a predominance of parasympathetic tone [40]. Dean et al. showed anandamide content was lower and the catabolic enzyme fatty acid amide hydrolase was higher in the dorsal part of PAG (dPAG) in chronic neuropathic pain rats [81]. Their results suggest that maladaptive endocannabinoid signaling in the dPAG could contribute to chronic hyperalgesia and reduced heart rate in neuropathic pain model. On the contrary, a meta-analysis showed the reduction of high-frequency heart rate variability in chronic pain studies that implicated a dysregulation in parasympathetic nervous system. However, these effects were heavily influenced by fibromyalgia studies [81]. The effect of chronic pain on autonomic nervous activities might depend on the types of chronic pain and when the autonomic nerve activity is measured. It is possible that a dynamic change of autonomic nerve activity occurs after chronic pain.

To investigate whether chronic pain affects cardiac IR injury, we performed left anterior descending coronary artery ligation for 45 min followed by 24 h reperfusion 5 days after SNI-induced chronic mechanical hyperalgesia in mice. Our study showed that infarct size was smaller, cardiac fibrosis was reduced, and cardiac function was improved in SNI but not sham-operated group [19]. We also demonstrated that ERK activity in PVA is involved in the SNI-induced cardioprotection by showing the abolishment of cardioprotection by inhibition of PVA ERK activity. We also demonstrated that activation of PVA neurons using pharmacological or optogenetic stimulation is sufficient to induce cardioprotection against IR injury. Silencing PVA neuronal activity with Gi-coupled DREADDs efficiently attenuated SNI-induced cardioprotection. This PVA-dependent cardioprotection was abolished by hexamethonium and glycopyrrolate but not propranolol, which suggests the involvement of parasympathetic nervous system. Heart rates were significantly reduced in conscious mice subjected to SNI or optogenetic stimulation. These results suggest that the parasympathetic nerve is responsible for this unexpected cardioprotective effect of chronic neuropathic pain in mice [19]. Vagus nerve stimulation ameliorated cardiac function in animal ischemia-reperfusion model and in patient with heart failure [25, 43]. This effect has been attributed primarily to muscarinic acetylcholine receptors [57]. Moreover, intracardiac acetylcholine has been proven to have an important role in ischemic preconditioning [67]. Chronic neuropathic pain may activate parasympathetic nervous system via PVA neurons in the brain to provide cardioprotection against ischemia-reperfusion injury.

9.5 Conclusion

Chronic pain is associated with physiological and psychological modifications including autonomic dysregulation but what influences the heart disease is unclear. Many studies discussed alterations in the autonomic nervous system function in patients with chronic pain [23, 54]. Decreased vagal activity may result in greater

somatic and visceral input which reduced pain threshold and enhance pain sensitivity in those patients with chronic widespread pain. Most of the studies applied heart rate variability (HRV) analysis to define autonomous nerve function. However, the meaning of parameters in HRV analysis is more complex and hard to explain. Our study showed that SNI neuropathic pain induces cardioprotection against IR injury in mice. However, some case-control retrospect studies showed a positive correlation between chronic pain and cardiovascular risk in human [64, 74, 85]. There could be several reasons for the discrepancy. We studied the effect of SNI-induced neuropathic pain on cardioprotection against IR injury. Most of the retrospective analyses include a wide spectrum of cardiovascular disease, ranging from hypertension, coronary heart disease, pulmonary heart disease and diseases of pulmonary circulation, other forms of heart disease, cerebrovascular disease, and diseases of arteries to diseases of veins. Potential confounding factors such as physical activities, metabolic diseases, and vitamin D deficiency in different types of chronic pain patients could be associated with CVD risks. It is hard to confirm the causal relationship in the chronic pain and CVD risks in these retrospective case-control studies. More prospective cohort studies will be helpful to elucidate the interaction between chronic pain and cardiovascular diseases.

Angina was found to be associated with better prognosis in patients with acute myocardial infarction after thrombolysis. It is possible that angina induces cardioprotection via a nociceptive signal pathway. Our study supports the idea that chronic neuropathic pain activates PVA neurons which then increase the vagus nerve activity, release of ACh, and activation of PKCε and thus protect the cardiac myocytes from IR injury (Fig. 9.1).

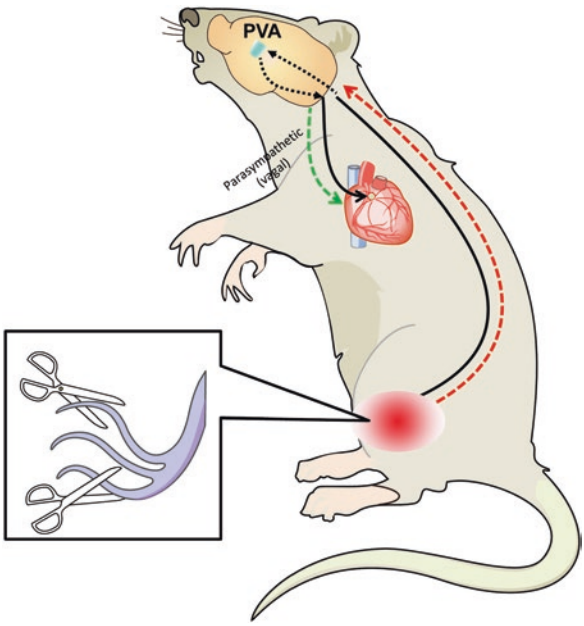

Fig. 9.1 Proposed model for SNI-induced cardioprotection. Spared nerve injury activates the afferent nociceptive signal pathway via the spinal cord to PVA neurons in the brain. Activation of PVA neurons leads to cardioprotection against ischemia-reperfusion injury in a parasympathetic (vagal) nerve-dependent manner

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Chapter 10
Knowing the Neuronal Mechanism of Spontaneous Pain to Treat Chronic Pain in the Future

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Abstract Spontaneous pain is the major complain for the patients to see a doctor. Human imaging studies presented that spontaneous pain is mainly associated with activity changes in medial pain pathway, while broader brain regions were activated by allodynia pain. On behavioral level, temporally disassociation between the evoked pain and spontaneous pain was observed; these data gave a hint that the spontaneous pain and evoked pain may be mediated by different neuronal mechanisms. And more attentions should be paid to the spontaneous pain to treat the chronic pain in the future.

Keywords Neuropathic pain · Spontaneous pain · Evoked pain · Central sensitization

10.1 Introduction

Pain refers to "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (IASP, https://www.iasp-pain.org). Subjects feel physiological pain when tissue is damaged; although the feeling is not good, it provides warning signals and protects subjects to avoid further damage; the causation of physiological pain is known and it will only last for several weeks. About 30% people in our society are also suffering from chronic pain, which will last for several months, and the causation usually is unknown. Major pathological phenomena of chronic pain include allodynia ("pain due to a stimulus that does not normally provoke pain"), hyperalgesia ("increased pain from a stimulus that normally provokes pain") (IASP, https://www.iasp-pain.org), and spontaneous pain (pain felt without apparent external stimulus). Based on the taxonomy, allodynia and hyperalgesia are evoked by external physical

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stimulations that may be mechanical or thermal stimulations. Conversely, the spontaneous pain is not dependent on the external stimulus and unavoidable. Some intrinsic mechanical forces come from the blood pressure or muscle contraction may also induce pain; under this situation, the spontaneous pain also has sensory components [1].

In clinical research, by using neuropathic pain questionnaires and neuropathic pain scale, spontaneous pain was found on about 96% of neuropathic pain patients, while stimulus-evoked pain was only presented on less than 25% patients [2]. Results from noninvasive brain imaging also showed that brain regions related to spontaneous pain were different from evoked pain; on the behavioral level, it was showed that the spontaneous pain was temporally dissociated with evoked pain. These observations shed a light that evoked pain and spontaneous pain may be mediated by different neuronal mechanism. However, to what degree the neuronal mechanism was different should be paid attention; since most of chronic pain patients have spontaneous pain, this knowledge will help for translational research in chronic pain managements.

10.2 Measurements of Spontaneous Pain

For chronic pain patients, the spontaneous pain can be evaluated by questionnaires and neuropathic pain scale. In preclinical research, it is easy to judge the sensory-discriminative components by withdrawal- or reflex-based measurements. Spontaneous pain is evaluated by indirect behavioral readout, different behavioral paradigms have been developed to measure spontaneous pain, including facial expression, evaluation of quality of life of mice by daily locomotion, feeding, drinking, and sleeping, and conditioning behavioral paradigm, like conditioned place preference, conditioned place aversion and drug self-administration, more information have been reviewed in [3].

Static and dynamic weight-bearing has been used in diverse pain models. Relative unbalance of weight-bearing and guarding index, which measure body weight distribution between the monoarthritic and control paw, are useful and can be partially blocked by relevant analgesics [4]. Home-cage behaviors such as grooming, rearing, climbing, moving, distance, drinking, and eating may constitute indicators for daily well-being of the animals. By monitoring home-cage behaviors, Rochelle Urban et al. found home-cage behavior was altered only early after CCI, and behavioral indices of ongoing pain were largely unchanged in male mice with tissue or nerve injury-induced mechanical hypersensitivity [5]. RA Whitehead et al. employed acute wheel-running exercise with limited repeated exposures which offers a behavioral assay complementary to stimulus-induced measures of neuropathic pain without altering allodynia [6]. However, spontaneous neuropathic pain in mice cannot be assessed by using locomotion and gait analysis [7].

For infants and people with cognitive impairments, pain facial expression is a reliable measurement method. Nonhuman animals also have facial expression, so

Langford DJ et al. developed the mouse grimace scale (MGS) [8] to evaluate the emotion stage. But this assay needs highly trained personnel spending a lot of time to score. With the development of artificial intelligence, Tuttle, Alexander H et al. developed an automated mouse grimace scale by a deep neural network which provided an objective and rapid way to quantify spontaneous pain and pain relief in mice [9].

The results of using ultrasound vocalizations to measured spontaneous pain [10] are controversial. Jourdan et al. thought ultrasonic vocalization could not be used to evaluate chronic pain in rats at the beginning [7]. However, Martina Kurejova improved behavioral assay and proved that ultrasound vocalizations was a liable indicator of chronic cancer pain and neuropathic pain [11]. Conditioned place preference (CPP) test [12], combined with pain and reward system to measure ongoing pain by King et al. [13], was widely employed to neuropathic pain and inflammatory pain models in rats and mice [3]. This test has also been used to analyze the abirritation of particular brain region or to test the efficiency of drugs [14, 15]. But it has limitations: first, the animals must have normal memory to remember different contextual and non-contextual cues-paired chambers; second, if the targeted chemical or manipulated brain region is associated with memory or reward function, it might affect CPP results.

10.3 Brain Regions Related to Regulation of Spontaneous Pain

The noninvasive brain imaging approaches were applied to investigate the brain regions that are involved in the regulation of pain under normal condition or activity change under neuropathic pain condition. Three major imaging modalities including metabolic, functional, and anatomical were mainly used [16]. Metabolic imaging such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance spectroscopy (MRS) were mainly used in evaluating changes in brain chemistry; fMRI was mainly used to observe the task-related brain activities or functional connectivity based on the change in blood-oxygenation-level dependent (BOLD) signal; anatomical MRI techniques were used to investigate the structural changes under chronic pain condition [17].

10.3.1 Brain Regions Related to Evoked Pain

The brain regions related to evoked pain were investigated on pain induced on healthy subjects or neuropathic pain patients. For example, topical capsaicin induced significant mechanical allodynia or hyperalgesia. After topical capsaicin application, mechanical allodynia combined significantly increased BOLD signals in contralateral S1, PA, and IFC and bilateral S2/insula [18]. Thermal hyperalgesia

increased activation of bilateral anterior insular cortices, MFC, GC, and contralateral SFC and IFC, while the mechanical hyperalgesia led to activations of primary and secondary somatosensory cortices (S1 and S2), associative-somatosensory cortices, and insula and superior and inferior frontal cortices (SFC, IFC), and activations of GC, contralateral MFC, and anterior insula were related to the stimulus-related unpleasantness [19]. In the menthol-induced allodynia model, significantly increased activations were found in bilateral dorsolateral prefrontal cortices (DLPFC) and the brainstem (ipsilateral parabrachial nucleus) during cold allodynia [20].

In patients with complex regional pain syndromes (CRPSs), allodynia increased activities in contralateral S1 and motor cortex (M1), parietal association cortices (PA), bilateral S2, insula, frontal cortices, and both anterior and posterior parts of cingulate cortex (aACC and pACC) [21]. In neuropathic pain patients, it was found that the intensity of allodynic pain was correlated with magnitude of activation in the caudal anterior insula (cAI), independent of the level of ongoing pain [22].

In the neuropathic pain animal models, acetone application induced widespread and functionally diverse changes in somatosensory, cingulate cortices and subcortical areas such as the thalamus and the periaqueductal gray in SNI rats [23], while higher BOLD signals in the anterior cingulate area (ACA) and thalamus were reported in the mice with injury at the L4 spinal nerve root [24].

10.3.2 Brain Regions Related to Spontaneous Pain

Modern brain imaging techniques also improved our understanding of spontaneous pain perception in brain. It was found that the spontaneous pain was chieflly associated with medial pain system, including medial prefrontal cortex and thalamus. The author concluded that the change of thalamic activity and the medial pain system is mainly associated with spontaneous neuropathic pain, suggesting that the spontaneous pain may more related to the emotional dimension of pain [25]. Another article summarized 40 representative articles found that spontaneous neuropathic pain is associated with changes in thalamic activity [26].

Later on, by using serial fMRI, Apkarian group found that CBP spontaneous pain was mainly associated with medial prefrontal cortex [27] with increased high-frequency BOLD oscillations (0.12–0.20 Hz) [28]; the involvement of mPFC to regulation of spontaneous pain was also found in the patients with OA [29]. However, broader brain regions related to spontaneous pain were reported under postherpetic neuralgia (PHN), both affective and sensory-discriminative areas: the thalamus, primary and secondary somatosensory, insula, and anterior cingulate cortices were involved in the regulation of spontaneous pain [30]. Furthermore, decreased resting-state functional connectivity between the thalamus and the cortex was observed in diabetic neuropathic pain [31].

Therefore, based on the brain imaging results from human, different brain regions were involved in the evoked pain and spontaneous pain, more broader brain regions were activated by allodynia pain, while the spontaneous pain were mainly associated with the affective and emotion areas, including the prefrontal cortex and medial thalamus.

10.4 Time Courses of Evoked Pain and Spontaneous Pain

Time course of spontaneous pain was important to understand the neuronal mechanisms of chronic pain. The temporal characteristics of spontaneous pain and evoked pain have been paid much attention. Scratching behavior, such as a vibratory-like shaking of the hind paw in the air, was treated as a sign of spontaneous pain after loose ligation on common sciatic nerve; it was found that the thermal allodynia lasted for 4 weeks, and increased scratching behavior was still observed at 10 weeks after operation [32]. Later studies on rats with chronic constriction injury (CCI) of the sciatic nerve showed dissociation of time course of evoked pain and spontaneous pain; in one study, spontaneous pain evaluated by the resting paw posture of hind paw and persistent for 12 weeks was reported, while the mechanical allodynia only lasted for 6 weeks on the ligated side; interestingly, thermal allodynia only developed 4 weeks after surgery [33]. In another study, weight-bearing deficit was used as an index of spontaneous pain, and it was found that spontaneous pain reached to peak at a week post-operation and gradually recovered for over 7 weeks. Longer-lasting evoked mechanical allodynia was reported [34]. On the mice with spared nerve injury, stable paw withdrawal threshold but temporally changed paw print intensity, stance phase duration, paw print area, paw placement ratio (ipsilateral/contralateral), and regularity index were observed after surgery, suggesting the dissociation of time course of mechanical allodynia and dynamic weight-bearing (gait) changes [7]. Wang et al. examined the analgesic effects of clonidine on the mice with ligation of common peroneal nerve (CPN), a neuropathic pain mice model with very little impairments on motor system [35], and found that the mechanical allodynia could lasted for at least 14 days, which could be alleviated by the application of clonidine, while clonidine induced place preference was only observed at day 7, but not at day 14 after nerve injury [36].

These studies suggest that the evoked pain and spontaneous pain are temporally disassociated on rodents' model. These observations not just indicate the difference between spontaneous pain and evoked pain, but also showed the temporal change of neuronal mechanism.

10.5 The Neuronal Mechanism for Spontaneous Pain: Peripheral Sensitization

Spontaneous pain was persistent and unavoidable, which needed the appropriate way to be evaluated. Despite the methodologies associated with spontaneous pain have been gradually developed, the underlying neuronal mechanism mediated spontaneous pain was still poorly understood. Recent studies showed that evoked pain and spontaneous pain may be mediated by peripheral sensitization. For example, increased membrane-bound PKC in the lumbar spinal cord was involved in the development of both thermal allodynia and spontaneous pain [37]. In nerve-injured rodents, increased spontaneous activity (>0.05 imp/s in 3 min) originating within or

close to the DRG [38] or abnormal ectopic discharges were observed [39]; these abnormal discharges were thought to contribute to low back pain, sciatica, hyperalgesia, and tactile allodynia [39]. In adjuvant inflamed rodents, long-lasting increased nerve growth factor (NGF) [40] and sodium channel [41] led to spontaneous activities in DRG neurons; this change persisted in small neurons for at least 2 months and roughly paralleled the time course of behaviorally measured changes in pain threshold [41]. On the basis of a model of advanced osteoarthritis, high dose of intra-articular monosodium iodoacetate (MIA) induced ongoing pain was dependent on afferent fiber activity but apparently independent of TRPV1 or TRPA1 activation [42]. Therefore, the lasting molecular changes in DRG neurons were associated with both evoked pain and spontaneous pain.

10.6 The Neuronal Mechanism for Spontaneous Pain: Central Sensitization

Given reliable increased activities were observed within extensive brain regions after nerve injury, the increased neuronal excitability would be accomnied with evoked pain and spontaneous pain. In rats with sciatic nerve ligation, reliable increases in 2-DG metabolic activity were observed within extensive brain regions that have been implicated in nociceptive processing as compared to sham-operated rats [43]; similarly, detectable bilateral increases in regional cerebral blood flow (rCBF) were detected within multiple forebrain structures, like the hindlimb region of somatosensory cortex, multiple thalamic nuclei, limbic system, cingulate cortex (18%), retrosplenial cortex (30%), and habenular complex (53%), which suggest that the neural mechanisms of the ongoing pain produced by a unilateral injury involved bilateral somatosensory and limbic forebrain structures [44].

The anterior cingulate cortex (ACC) has critical role in the regulation of spontaneous pain [45]. Lesion of the rACC blocked conditioned place preference elicited by RVM lidocaine but did not alter acute stimulus-evoked hypersensitivity [46]. In the CPN ligation neuropathic pain mice model, significantly increased excitatory synaptic transmission was observed in the anterior cingulate cortex, which was mediated by both presynaptic glutamate release and postsynaptic AMPA receptors phosphorylation [47]. The increased activities of PKM zeta (**PKMζ**) were involved in the maintenance of neuropathic pain, while local application of a selective inhibitor zeta-interacting protein (ZIP) of **PKMζ** erased synaptic potentiation and alleviated both mechanical allodynia and spontaneous pain induced by CPN ligation [48]; the similar analgesic effects of ZIP were observed in the spinal cord [49, 50]. Recently, it was found that the expression of cingulate adrenergic receptors α2A subunits was changed temporally, presented as upregulation at day 7, but downregulation at day 14 after CPN ligation, which was paralleled with the analgesic effects of clonidine on spontaneous pain, evaluated with conditioned place preference [36]; the activities of α2A further increased the pair-pulse ratio of AMPA receptor-

mediated currents (unpublished data), suggesting the presynaptic regulation of α2A receptors. And nerve injury also upregulated BDNF-tropomyosin receptor kinase B (TrkB) activities and NR2B receptors activities, which further led to developments of spontaneous pain [51]. Although current data did not cover all the brain regions that were involved in the regulation of spontaneous pain, one point can be concluded that the increased excitatory ability is involved in the regulation of spontaneous pain.

10.7 Conclusion: Challenges and Opportunities

Given the spontaneous pain and evoked pain were mediated by both peripheral centralization and central sensitization, what's the difference for the neuronal mechanisms of spontaneous pain and evoked pain? One point may be the neuronal circuit differences. The spontaneous pain was mainly associated with the activity changes in the prefrontal cortex and thalamus, while the evoked pain was associated with activity change in more broader brain regions. Except for the long-range neuronal circuits, the difference may also happen on the local neuronal circuits; compared to the somatosensory cortex, no layer IV in the ACC and prefrontal cortex, the information processing flow may be different. Another point may be on the molecular level, different molecular pathways were involved in the regulation of cellular excitability, in addition, current omics approaches have been applied to investigate the possible molecular changes related to chronic pain, which may provide a more broader view for molecular mechanisms of spontaneous pain.

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Chapter 11
Role of Neuroinflammation in Opioid Tolerance: Translational Evidence from Human-to-Rodent Studies

Chih-Peng Lin and Dai-Hua Lu

Abstract Opioid analgesics remain the most effective and widely used analgesics for the management of moderate to severe pain, including cancer pain and chronic non-cancer pain. However, the efficacy of long-term opioid analgesics is attenuated by tolerance and/or hyperalgesia after long-term use, preventing adequate pain relief under stable opioid dosages for chronic pain patients. Classical neuron-centered concepts about tolerance, such as internalization of opioid receptors, upregulation of N-methyl-D-aspartate receptor function, or downregulation of glutamate transporter activity, can only partially explain the phenomenon of tolerance. Recent evidence revealing glial activation and upregulation of inflammatory mediators in the rodent central nervous system has confirmed the pivotal role of neuroinflammation in neuropathic pain or opioid tolerance, or both. However, human evidence is still sparse.

Based on our clinical practice, we conducted translational research by investigating the cerebrospinal fluid (CSF) cytokine and chemokine profiles of opioid-tolerant patients after research ethic committee approval. CSF samples from opioid-tolerant patients and opioid-naive subjects were compared. We found CXCL1, CXCL12, and leukemia inhibitory factor (LIF) were significantly upregulated among the opioid-tolerant patients and positively correlated with the opioid dosage.

We translated these findings back to lab animal experiment; after induction of tolerance by morphine infusion, the spinal cord expression of CXCL1, CXCL12, and LIF were all upregulated. Although CXCL1 and CXCL12 infusion alone did not affect baseline tail-flick latency, morphine analgesic efficacy dropped significantly after intrathecal infusion of CXCL1 and CXCL12. After establishing tolerance by intrathecal continuous infusion of morphine, tolerance development was accelerated by co-administration of CXCL1 and CXCL12. In parallel, the effect was attenuated by co-administration of CXCL1- or CXCL12-neutralizing antibody or concordant receptor antagonists.

On the contrary, although chronic morphine administration still induced LIF upregulation in rat spinal cords, intrathecal injection of LIF potentiated the analgesic

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action of morphine and delayed the development of morphine tolerance. Upregulation of endogenously released LIF by long-term use of opioids might counterbalance the tolerance induction effects of other pro-inflammatory cytokines.

CXCL1, CXCL12, and LIF are upregulated in both opioid-tolerant patients and rodents. The onset and extent of opioid tolerance were affected by modulating the intrathecal CXCL1/CXCR2, CXCL12/CXCR4, and LIF signaling and could be novel drug targets for the treatment of opioid tolerance.

Keywords Opioid tolerance · Neuroinflammation · Chemokine · Cytokine · Translational research

11.1 Introduction

Access to proper pain treatment has been widely accepted as a human right. Thus, adequate pain management is an essential part of modern medicine that improves patients' quality of life and maintains their psychosocial function. Insufficiencies in pain management have many causes, including cultural, societal, religious, and political attitudes.

Opioids are the most effective and widely used analgesics for the management of moderate to severe pain, including cancer pain and chronic non-cancer pain. In fact, the World Health Organization has accepted that opioid analgesics are an essential medicine for pain management. Although the use of opioids to treat chronic non-cancer pain remains controversial in terms of both its efficacy and its adverse physical and psychological effects [48], the practice has escalated in recent years, making opioids one of the most commonly prescribed medications [5, 33]. Clinical use of opioid analgesics is usually limited by opioid-related side effects that include respiratory depression, constipation, nausea, and vomiting. The potential for fear of addiction also complicates the use of opioids to treat chronic non-cancer pain. Repetitive administration of opioids over time can lead to tolerance, so that a higher dose of opioids is needed to achieve the same level of pain relief. This might lead to further serious side effects, as well as to physical dependence.

11.2 Opioid-Related Signaling

Opioid systems modulate antinociception and pain behavior. Endogenous opioid peptides and their receptors are expressed throughout the nociceptive neural circuitry, as well as in critical regions of the central nervous system (CNS) involved in reward- and emotion-related brain structures. Similar to endogenous opiates such as endorphins, enkephalins, and dynorphins, opioid analgesics exert their pharmacological action by binding to G-protein-coupled opioid receptors, which can be categorized into four subtypes: μ-opioid receptors (MORs), δ-opioid receptors (DORs),

κ-opioid receptor (KORs), as well as the recently discovered nociceptin receptor. All these receptors are distributed throughout the CNS and also occur in the peripheral processes of sensory neurons [49]. Moreover, opioid receptors are expressed in sympathetic neurons and in the immune system, but their function there is yet to be elucidated [34, 60]. Most of the analgesic effects of both endogenous opiates and opioid medications are derived from the activation of MORs, while DORs also mediate some of the analgesic, antidepressant, and physical dependence effects. Finally, KORs function in pain relief, sedation, dysphoria, and pupil constriction. Activation of MORs is also linked with respiratory depression and supraspinal analgesia [1]. In general, all four opioid receptor subtypes are G-protein-coupled receptors that share common signaling pathways. Selective ligands bind to each opioid receptor and can induce one or more of these downstream pathways [1]. Activation of the opioid receptor causes inhibition of adenylyl cyclase, leading to a reduction in intracellular cyclic adenosine monophosphate (cAMP) levels and activation of protein kinase A (PKA).

11.3 Traditional Viewpoint of Opioid Tolerance

Although the activation of opioid receptors leads to antinociceptive effects, it can also cause undesirable effects, such as tolerance and hyperalgesia [24]. Agonist binding to the opioid receptor triggers a series of downstream molecular events, including receptor phosphorylation by G-protein receptor kinase and beta-arresting binding that is saturated within minutes. Rapid desensitization and re-sensitization reaches equilibrium within minutes, while short-term tolerance, as manifested by receptor endocytosis, happens within 1 day. In contrast, long-term tolerance involves multiple regulatory processes that cannot be fully explained in terms of molecular events. Neuron-centered concepts such as the internalization of opioid receptors, upregulation of N-methyl-D-aspartate (NMDA) receptor function, or downregulation of glutamate transporter activity can only partially explain the phenomenon of tolerance.

In the CNS, opioids reportedly lead to NMDA receptor upregulation. Indeed, in one study, MK-801—an NMDA receptor antagonist—attenuated the development of morphine tolerance and dependence, suggesting that NMDA receptor activation is involved in pharmacological opioid tolerance [29, 51]. Accordingly, the opioid increase pain sensitivity was thought to be caused by MOR activation and NMDA receptor utilization. However, an unremitting hyperalgesia was found in opioid receptor knockout mice after continuous infusion of morphine [23], indicating that opioid-induced hyperalgesia is independent of MORs and that non-neuronal cells are involved. For now, it is widely accepted that the neuronal activity of opioids cannot fully explain the initiation and maintenance of opioid tolerance and hyperalgesia.

11.4 Interplay Between Neuroinflammation and Opioids

Non-neuronal immunocompetent cells in the CNS, including astrocytes, microglia, and endothelial cells, have been recognized as important modulators of pain and opioid pharmacodynamics [19]. Neuroinflammation manifesting as morphological glial cell proliferation and hypertrophy, with pro-inflammatory cytokine/chemokine overproduction, has been recognized as a key contributor to multiple CNS diseases, including pathological and chronic pain [31].

Several studies have revealed that astrocytes play a critical role in the analgesic effects of morphine and in complications associated with opioids. Opioids were first reported to affect non-neuronal cells when Beitner-Johnson, Guitart, and Nestler [2] found that chronic morphine treatment increased levels of glial fibrillary acidic protein (GFAP) immunoactivity in the rat ventral tegmental area. Another report linking glial activation to opioid tolerance demonstrated that chronic systemic morphine use increases astroglial activation, as indicated by increased GFAP immunostaining in the spinal cord [32]. Song and Zhao also showed a circumstantial association between astrocytes and morphine tolerance by revealing that intrathecal delivery of the astrocyte inhibitor fluorocitrate (a glial metabolic inhibitor) eliminated morphine tolerance and morphine-induced elevation of GFAP expression [46]. Astrocyte activation in the spinal cord has also been identified in cases of chronic morphine treatment. The neuroimmune mechanism of opioid-induce hyperalgesia was confirmed in another study using either inhibitors of glial activation or pro-inflammatory antagonists [19].

Conversely, several studies have shown that, by suppressing astroglial activation, both neuropathic pain and morphine tolerance are attenuated [14, 25]. This provides evidence that chronic morphine administration increases astrocyte activation and upregulates IL-1β and matrix metalloprotease-9 (MMP9) in the dorsal root ganglion [3]. Furthermore, knockdown of IL-1β in the dorsal root ganglion can prolong morphine analgesia.

On the other hand, microglial activation is another important part of the neuroimmune response to morphine-mediated analgesia. Microglia are derived from the bone marrow during the perinatal period and participate in neuropathic and postoperative pain, as well as in opioid tolerance [53]. Microglial activation in response to chronic morphine administration was examined through immunostaining with the microglial marker CD11b. It was found that chronic codeine exposure resulted in CD11b augmentation in the rodent spinal cord [21]. In addition, cell surface receptors such as P2X4 and P2X7 were upregulated in spinal microglia, suggesting that morphine enhances microglial activation through P2X4 receptor signaling [16]. Similarly, inhibition of microglial P2X4 receptors attenuated morphine tolerance [21]. With this in mind, several studies have delivered a selective microglial inhibitor—minocycline—intrathecally to antagonize morphine tolerance through inhibition of p38 in spinal microglia [8, 18].

These non-neuronal CNS cells are thought to boost nociceptive transmission by producing immune signaling molecules such as pro-inflammatory cytokines and chemokines that enhance neuropathic pain behavior.

Several studies have emphasized the role of cytokines and their receptors under neuropathic conditions. Microglia and astrocytes are considered the principal source of cytokines contributing to opioid tolerance in the CNS. Upon stimulation, activated glial cells release pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which act as critical communication molecules with opioid receptors. Several preclinical studies have demonstrated that the orchestrated action of different cytokines (TNF-α, IL-1β, and IL-6), chemokines (CCL2, CCL21, CX3CL1, CXCL1, and CXCL12), and other neuromodulators (growth factors, neurotransmitters, and proteases) powerfully modulates synaptic transmission, leading to central sensitization and enhanced chronic pain states [22, 36, 50].

Recent studies have revealed that cytokines and their receptors are upregulated by neuronal and non-neuronal cells in response to chronic morphine treatment [22, 54]. In addition, intrathecal injection of cytokines leads to the activation of astrocytes and microglia; it also causes hyperalgesia [7]. It follows that opioid-induced tolerance can be reduced by directly blocking the action of pro-inflammatory cytokines.

Many studies have demonstrated that morphine tolerance can be slowed or reversed by inhibition of spinal pro-inflammatory cytokines and by knockout of IL-1β signaling [22, 36, 37, 41]. For example, several investigations have revealed that the expression of TNF-α is increased in patients with morphine tolerance. During neuroinflammation, both astrocytes and microglia can release TNF-α, which exerts neuroprotective or neurotoxic effects. In a rat model of chronic morphine exposure, a TNF-α receptor inhibitor reversed morphine tolerance by inhibiting the pro-inflammatory cytokine [42].

Following receptor binding, TNF-α induces rapid expression of CCL2 (monocyte chemoattractant protein [MCP]-1), CXCL10 (interferon-γ inducible protein [IP]-10), and CXCL1 (growth-related oncogene [GRO]-α) in primary astroglial cell cultures [13]. This mechanism may contribute to morphine tolerance. CCL2, which is mainly produced by astrocytes and microglia, was also released in rat models of injury [11] and ischemia [6]. It has an important role in the development of neuropathic pain [55, 56]. However, little information is available regarding its role in morphine tolerance [59]. Relatedly, in another study, chronic morphine treatment led to an increase in spinal CCL2 immunoactivity, and a CCL2-neutralizing antibody significantly reduced the incidence of morphine tolerance [59]. Likewise, intrathecal administration of CX3CL1-neutralizing antibody enhanced acute morphine analgesia and slowed the development of tolerance [22].

11.5 Evidence of Chemokine/Cytokine Among Patients with Opioid Tolerance

We recently analyzed cerebrospinal fluid (CSF) from patients with opioid tolerance who had regularly taken strong opioids to manage their pain for more than 1 month. Opioid tolerance was identified when patients were prescribed a daily dose of intravenous morphine greater than 100 mg or a dosage of other strong opioids delivered at an equipotent dosage by other routes of administration (e.g., transdermal fentanyl, oral, or intraspinal opioids). Patients with evidence of CNS involvement were excluded from this study. CSF samples were collected immediately after intrathecal catheterization or immediately before a scheduled refilling of the implanted intrathecal pump. Patients who had not taken opioids within 3 months and who had no chronic or ongoing acute pain were used as controls.

The concentrations of TNF-α, IL-6, leukemia inhibitory factor (LIF), CXCL1, CXCL10, CCL2, and CX3CL1 (fractalkine) in patients with opioid tolerance were also examined by Lin et al. [26]. Unlike some other studies [43], we did not find a difference in the CSF concentrations of TNF-α or fractalkine between opioid-naive and opioid-tolerant subjects (Fig. 11.1a). However, the concentrations of CXCL1, CXCL12, and LIF were markedly greater in the CSF of patients with opioid tolerance than in age-matched opioid-naive patients (Fig. 11.1b) [27, 52]. Additionally, the CXCL1 and LIF concentrations in CSF were positively correlated with daily morphine equivalent dose in our human study (Fig. 11.1c). These findings suggest that the upregulation of CXCL1, CXCL12, and LIF occurs when opioid tolerance is already in the well-established stage rather than in the initial stages.

11.6 Physiological Role of CXCL1/CXCR2, CXCL12/CXCR4, and LIF Signaling

CXCL1 has been detected in humans in a variety of neurological diseases. For example, it is markedly upregulated in bacterial meningitis, but not in aseptic meningitis or healthy controls [61]; it is upregulated in neuroinflammatory diseases such as multiple sclerosis, acute disseminated encephalomyelitis [12], and opsoclonus myoclonus syndrome [35]. Moreover, CXCL1 has been demonstrated in rats with brain injury [20]. The involvement of CXCL1 in neuroinflammatory pain has been demonstrated in a rat model of spinal nerve ligation. In this model, upregulation of CXCL1 occurred primarily in reactive astrocytes and paralleled neuropathic pain behaviors such as mechanical allodynia and heat hyperalgesia [58]. Moreover, knockdown of CXCL1 persistently attenuated hypersensitivity to spinal nerve ligation-induced pain.

CXCL12 is also known as stromal cell-derived factor 1 (SDF-1). CXCL12/CXCR4 signaling may mediate morphine-induced tactile allodynia and offset the analgesic potency of morphine in rodent models; it may also be involved in the

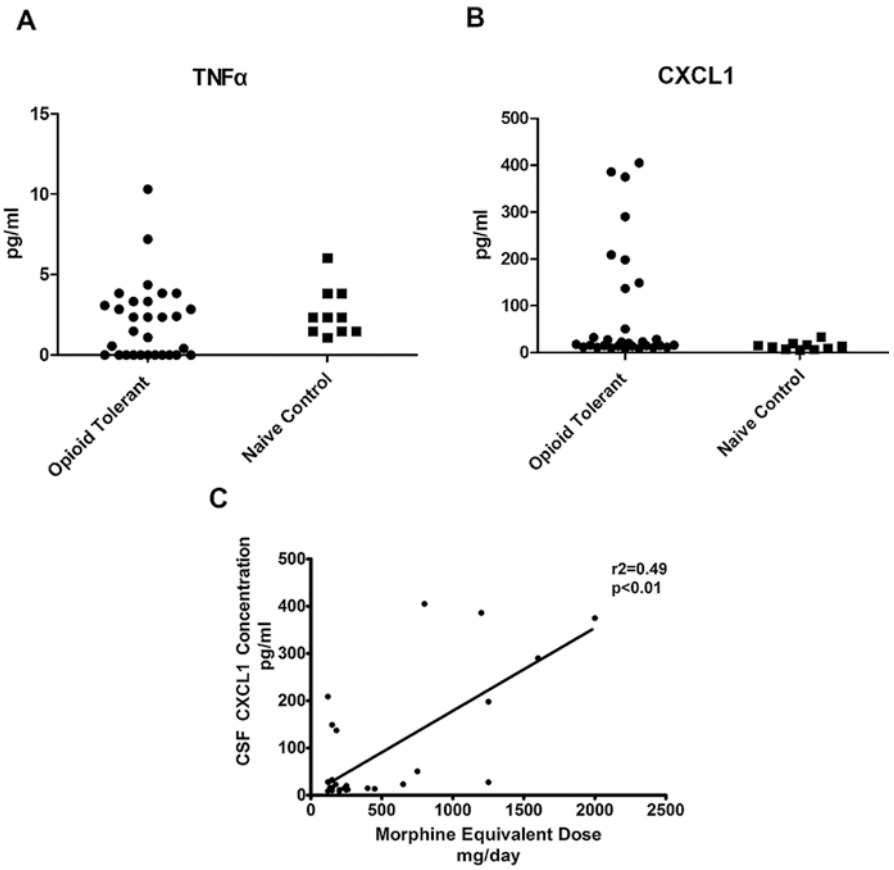

Fig. 11.1 Comparison of TNF-α and CXCL1 levels in the cerebrospinal fluid of patients with opioid tolerance and in opioid-naive patients. (**a**) There were no significant differences in CSF concentrations of TNF-α between the two groups. (**b**) CXCL1 levels were significantly greater in the CSF of morphine-tolerant patients than in naive controls. (**c**) The concentration of CXCL1 in CSF was positively correlated with daily morphine equivalent dose [26]

pathogenesis of opioid-induced hyperalgesia [40, 57]. CXCL12/CXCR4 is widely distributed in the CNS [38, 39] and is involved in the development and maintenance of pathological pain. It has been extensively studied in different animal models, including chronic constriction injury of the sciatic nerve [9], partial sciatic nerve ligation [28], HIV-associated sensory neuropathy [4], diabetic neuropathy [30], and bone cancer [17, 44].

Like CXCL1 and CXCL12, LIF is also involved in inflammation [15], pain [47], and neurogenesis [45]. Furthermore, recent studies have indicated that LIF is a key mediator in sensory neurons [10].

Because CXCL1/CXCR2, CXCL12/CXCR4, and LIF signaling are involved in neuroinflammation, it is likely that modulation of these signaling molecules would be a promising therapeutic approach to attenuate opioid tolerance.

11.7 Targeting CXCL1/CXCR2, CXCL12/CXCR4, and LIF Signaling as Potential Drug Target to Treat Opioid Tolerance

In light of our clinical findings, we performed translational studies using clinically relevant animal models to confirm that CXCL1/CXCR2, CXCL12/CXCR4, and LIF are related to morphine tolerance.

We first tested the commonly used regimen by injecting 10 mg/kg of morphine daily via the intraperitoneal route. In addition, CXCL1 or CXCL12 were infused intrathecally using an osmotic pump (1.2 ng/h) 24 h before morphine injection. To mimic intrathecal morphine infusion therapy, we carried out continuous intrathecal infusion of morphine (15 μg/h) using osmotic minipumps. After tolerance had been established using this method, neutralizing antibodies or receptor blockers were applied to evaluate the role of CXCL1/CXCR2 and CXCL12/CXCR4 signaling in opioid tolerance.

Different from the CXCL1 and CXCL12, the effect of LIF on acute or chronic morphine-induced analgesia was evaluated by subcutaneous injection (2 mg/kg in the acute analgesia paradigm; 10 mg/kg/day in the morphine tolerance paradigm). The exogenous LIF or neutralizing anti-LIF antibody were delivered via intrathecal infusion 30 min before morphine injection.

11.7.1 CXCL1/CXCR2 Signaling

Parallel to the evidence in humans, CXCL1 mRNA expression in the rat spinal cord was significantly increased after continuous intrathecal morphine infusion (Fig. 11.2). Moreover, exogenous CXCL1 significantly decreased the antinociceptive efficacy of morphine in daily morphine intraperitoneal injection paradigm. Conversely, by blocking CXCL1/CXCR2 signaling using a co-infused CXCL1-neutralizing antibody or receptor antagonist, the analgesic efficacy of morphine could be partially preserved (Figs. 11.3a, b). These results are consistent with the hypothesis that CXCL1 plays a role in the development of opioid tolerance.

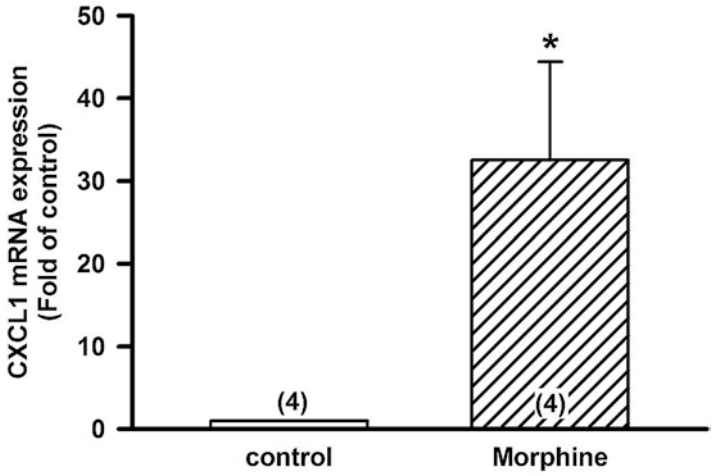

Fig. 11.2 Intrathecal morphine infusion increases the expression of CXCL1 in the rat. Morphine was administered via osmotic pump at an infusion rate of 15 μg/h. The CXCL1 expression levels in the spinal cord were significantly increased after morphine administration [26]

11.7.2 CXCL12/CXCR4

A similar hypothesis was used to evaluate the possible role of CXCL12/CXCR4 in morphine tolerance. After intrathecal infusion of morphine for as little as 2 days, CXCL12 mRNA was significantly upregulated in the dorsal horn tissue of the spinal cord when opioid tolerance was well established. The acute antinociceptive effect of morphine was not affected by exogenous CXCL12. However, tolerance development was accelerated by very low doses of exogenous CXCL12. Conversely, blocking CXCL12/CXCR4 signaling can persistently suppress opioid tolerance (Fig. 11.3c).

11.7.3 LIF Signaling

Chronic morphine administration increased the expression of endogenous LIF in the rat spinal cord in a dose-dependent manner. Compellingly, the development of tolerance was markedly suppressed by exogenous LIF, whereas neutralizing endogenously released LIF using anti-LIF antibodies accelerated tolerance induction (Fig. 11.3d).

In our study, we revealed that CSF levels of the chemokines CXCL1/CXCR2, CXCL12/CXCR4, and LIF were significantly upregulated in both patients and rodents with opioid tolerance [26]. We also showed that morphine tolerance was

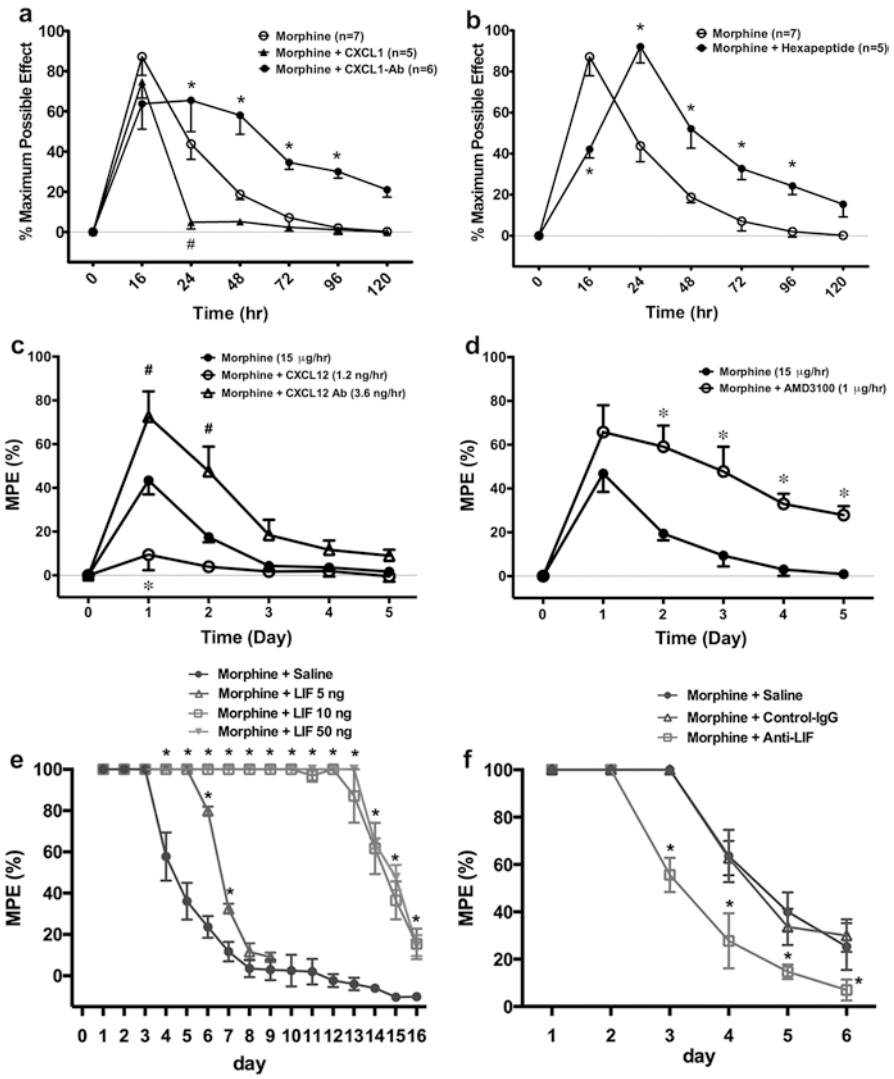

Fig. 11.3 The effects of inhibition of CXCL1/CXCR2, CXCL12/CXCR4, and LIF signaling on morphine tolerance. Intrathecal administration of exogenous CXCL1 or CXCL12 using an osmotic pump significantly accelerated the development of morphine tolerance (**a**–**c**), whereas CXCL1 or CXCL12 neutralizing antibodies (**a**–**c**) or receptor blockers (**b**–**d**) inhibited the induction of morphine tolerance and partially restored the analgesic efficacy of morphine [26, 27]. (**e**) Exogenous LIF was administered intrathecally at different doses 30 min before subcutaneously morphine injection (S.C). Morphine tolerance was suppressed in a dose-dependent manner. (**f**) Morphine tolerance was accelerated by intrathecal administration of an anti-LIF antibody (5 μg) 30 min before daily morphine treatment [52]

accelerated by co-administration of CXCL1/CXCL12 and attenuated by co-administration of CXCL1/CXCL12-neutralizing antibody or receptor blocker. On the other hand, we discovered that LIF is upregulated among patients and animals with opioid tolerance. Moreover, in translational lab animal research, we found that exogenous LIF can potentiate the acute antinociceptive effects of morphine and attenuate the development of tolerance [52].

11.8 Conclusion

Chronic unremitting pain is a debilitating disease that is a major socioeconomic burden to our society. Opioid therapy remains the most effective and widely accepted treatment strategy for moderate to severe pain, especially in patients with cancer. The present review discussed the involvement of neuroinflammation in opioid tolerance, focusing on clinical studies and translational rat experiments.

The review mentioned recent insights into the general principles underlying opioid tolerance. Specifically, several studies have suggested that immunoregulatory products ameliorate both acute and chronic opioid-induced analgesia and that a number of cytokines or chemokines may be drug targets for the prevention of opioid tolerance.

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Chapter 12
Neural Mechanisms of Offset Analgesia

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Abstract One feels a disproportionately large decrease of pain sensation on a slight decrease of thermal pain stimulus. Such phenomenon is termed offset analgesia and considered mediated by endogenous analgesic mechanisms. Offset analgesia was found attenuated in patients with neuropathic pain. We further found that such attenuation occurred in a more heterogeneous population of patients with chronic pain. By functional magnetic resonance imaging, we also found negative blood oxygenation level-dependent signals at those areas concerned with descending pain modulatory and reward systems during offset analgesia in the same cohort of patients. We propose that dysfunction of those systems, as revealed by attenuation of offset analgesia, might well be part of neural mechanisms of pain chronification.

Keywords Offset analgesia · Chronic pain · Functional magnetic resonance imaging · Descending pain modulatory system · Reward system

12.1 Introduction: History and Methodology of Offset Analgesia

One feels suffering on increase of pain and relief on decrease of pain. Such nociceptive intensity-dependent dynamics of pain perception are considered physiologically normal responses in terms of strategy to estimate nociceptive stimulus in a timely manner to protect oneself from injury. One feels motivated to escape from nociceptive stimulus on its increase and relieved or even rewarded after successful avoidance or decrease of pain.

Being compatible with such basic mechanisms of pain perception, offset analgesia (OA) was first described by Grill and Coghill in 2002 as a disproportionately

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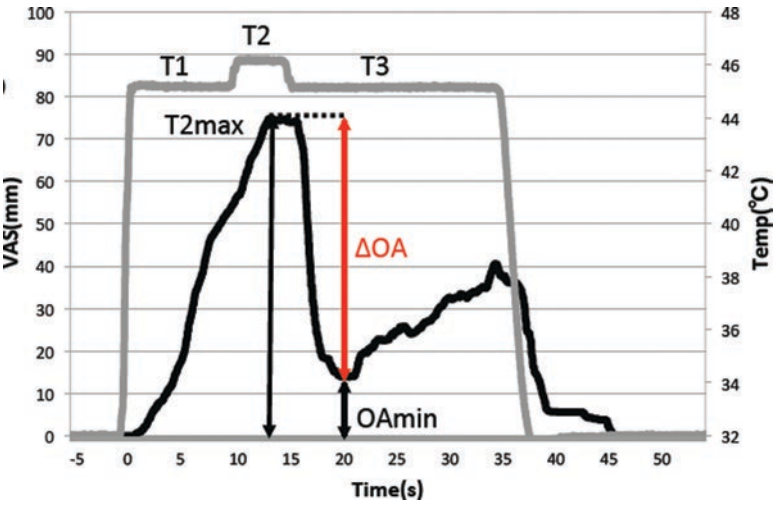

Fig. 12.1 A typical time course of offset analgesia. A Peltier-type thermode was used to give pain stimulation (gray line) to the left volar forearm of a healthy volunteer subject and a continuous pain rating response was obtained with an analogue-to-digital visual analogue scale (VAS) recorder (black line). T1, the first plateau at 45°C for 10 s; T2, the second plateau at 46°C for 5 s; T3, the third plateau at 45°C for 20 s; T2max, maximum VAS response during T2; OAmin, minimum VAS during T3; ΔOA, magnitude of offset analgesia

large decrease of pain perception following a tiny decrease, usually by 1°C, of thermal nociception on the skin in humans (Fig. 12.1) [1]. Experiments of OA have usually been performed using a Peltier-type thermal stimulator capable of changing the temperature of thermal stimulation at rapid ramp rates. A typical three-temperature stimulation paradigm of OA consists of the first hot painful stimulus at 48°C for 5 s (T1), followed by a 49°C stimulus for 5 s (T2) and a 48°C stimulus for 20 s (T3). A continuous monitoring of pain intensity in visual analogue scale indicates abrupt decrease of pain sensation from 60 mm to a trough of 20 mm, followed by a gradual return of pain sensation toward usual pain intensity during a 48°C stimulus (Fig. 12.1). Magnitude of OA (ΔOA) is usually obtained by the difference between the maximal pain intensity during T2 and the minimum pain intensity at the trough during T3.

OA is considered a physiological phenomenon that mediates relief and reward after a decrease of pain. It works as a temporal sharpening mechanism of pain perception, which might facilitate one's behavioral adaptation in response to rapid changes in nociceptive stimulus.

12.2 Neuroimaging Studies on the Mechanisms of Offset Analgesia

What are the neural mechanisms of OA? Although peripheral mechanisms have also been implicated [2], OA is considered mediated primarily by the central mechanisms, i.e., the descending pain modulatory system, which is one of the endogenous pain modulatory systems. Earlier neuroimaging studies revealed possible involvement of periaqueductal gray, a relay center for descending pain modulation, in mediating OA [3]. Yelle et al. used functional magnetic resonance imaging (fMRI) and found that the periaqueductal gray (PAG) showed a significant increase of blood oxygenation level-dependent (BOLD) signal intensity during offset analgesia. PAG is considered a brainstem relay center for descending modulation of pain, mediating the top-down signals toward the dorsal horn of the spinal cord, where nociceptive information from the primary afferent neurons is blocked.

12.3 Offset Analgesia in Patients with Chronic Pain

Niesters et al. examined OA in ten patients with neuropathic pain and showed significantly attenuated OA in those patients in comparison with healthy controls [4]. Their results implied possible involvement of dysfunctional endogenous analgesia in pathophysiology of chronic pain disorders.

In a more general, heterogeneous population of patients with chronic pain (n = 12) and sex-, age-matched controls (n = 12), we performed OA experiments and tested a hypothesis that OA might be attenuated in patients with OA in comparison with controls depending on various disease profiles, such as severity and duration of chronic pain [5]. We used a Peltier-type, thermal pain stimulator (PATHWAY, Medoc, Israel) to give hot pain stimulation on the left volar forearm and simultaneously recorded pain sensation using a continuous analogue-to-digital converter of visual analogue scale (0–100 mm, 0 = no pain, 100 = the most intense pain imaginable; CoVAS, Medoc, Israel). We further examined whether different durations of T2 (5, 10, and 15 s) might influence the extent of OA, with T1 and T3 set at 10 s and 20 s, respectively [5]. We used two kinds of thermal pain stimulation blocks including OA and constant paradigms, the latter serving as control for OA.

Magnitude of OA was significantly smaller in patients than in controls (35.3% vs. 61.8%, $p < 0.001$), but the difference grew smaller as T2 was increased to 10 or 15 s. That is, longer duration of pain sensitization resulted in more enhanced OA in both patients and controls. We further found that latency of maximum pain intensity in the constant paradigm (45 °C for 35 s) was longer in patients than in controls (16.4 s vs. 11.5 s, p = 0.004). This fact implied that patients with chronic pain showed a slower speed of pain perception. Furthermore, ΔOA grew smaller as pain duration increased. This fact implied that longer-lasting pain might be associated

with more severe deterioration of descending modulation of pain as revealed by attenuated OA.

From all those findings, we devised an "OA index" as ΔOA divided by latency to reach maximum pain intensity during a constant paradigm. We found that the OA index successfully distinguished patients with chronic pain from healthy controls with the area under the receiver operating characteristic (ROC) curve at 0.897. Attenuation of OA, in conjunction with deceleration of pain perception, has thus been found to be sensitive in diagnosing chronic pain [5].

12.4 Functional Neuroimaging of OA in Patients with Chronic Pain

What would be the neuropathological mechanisms underlying attenuation of OA in patients with chronic pain? We further attempted to answer this question using functional neuroimaging. In the same cohort of subjects, we performed simultaneous fMRI and OA stimulation using a 3-Tesla MRI scanner (SIGNA, GE Healthcare, USA) and an MRI-compatible, Peltier-type thermal pain stimulator (PATHWAY, Medoc, Israel) and a VAS recorder (CoVAS, Medoc, Israel) [6]. In 17 patients with chronic pain and 17 sex- and age-matched controls, we gave a pseudorandomized mixture of 3 different stimulation paradigms (OA, constant, and short) while obtaining a whole-brain, gradient-echo echo-planar imaging.

We found a typical pattern of pain-related cerebral activation including deactivation of the default mode network. Furthermore, after a focused whole-brain comparison of OA-related BOLD signals during the middle 10 s of T3 between OA and contrast paradigms, we found a significant difference in activation at specific areas that belonged to the descending pain modulatory (anterior cingulate, dorsolateral prefrontal cortices, and brainstem) and reward systems (putamen, medial prefrontal cortex, and nucleus accumbens). Although those areas showed positive activation in healthy controls, they showed contrasting negative activation in patients with chronic pain ($p < 0.05$, uncorrected) (Fig. 12.2).

From the above findings, we inferred that OA was specifically mediated by activation of both the descending pain modulatory and reward systems [6]. Attenuation of OA in patients with chronic pain was associated with inactivity of those areas as revealed by contrasting deactivation of BOLD signals. The present study proved, for the first time, that chronification of pain involved dysfunction of both the descending pain modulatory and reward systems.

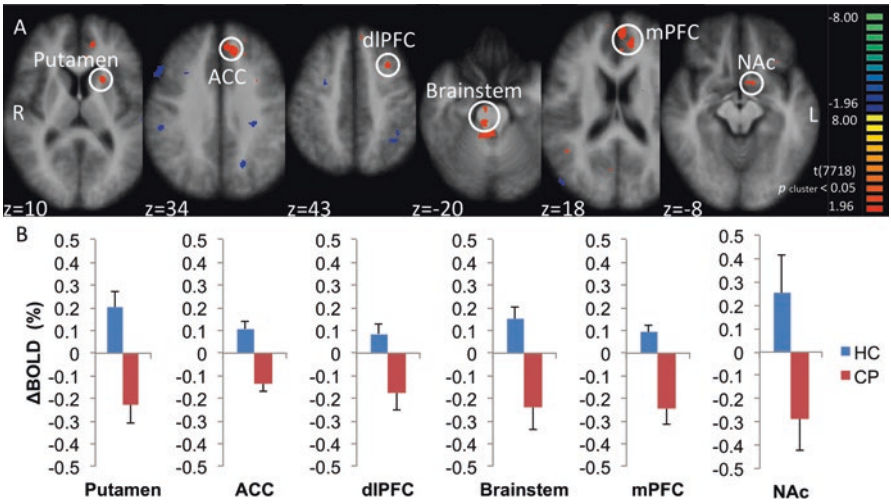

Fig. 12.2 Brain areas that showed larger offset analgesia-related blood oxygenation level-dependent (BOLD) signals in healthy controls than in patients with chronic pain. (**A**) A between-group whole-brain subtraction analysis revealed significant clusters ($p < 0.05$, uncorrected) associated with offset analgesia. *ACC* anterior cingulate cortex, *dlPFC* dorsolateral prefrontal cortex, *mPFC* medial prefrontal cortex, *NAc* nucleus accumbens. Color bars indicate *t* values. Z values indicate axial z-coordinates in Talairach stereotaxic space. (**B**) A BOLD signal time-course analysis revealed contrasting activities at the above regions of interest between the two groups. *HC* healthy controls, *CP* patients with chronic pain. (Adapted from Zhang et al. [6])

12.5 Cerebral Mechanisms of Pain Chronification

The current series of psychophysical and neuroimaging studies of OA successfully provided significant insights into the cerebral mechanisms of pain chronification. First, patients with chronic pain might have obtundation in temporal enhancement mechanisms of nociceptive perception. In contrast to healthy controls showing large OA and fast nociceptive responses, patients with chronic pain required longer duration of nociception to trigger OA and adaptation. Although it remains undetermined whether such obtundation is caused by central, peripheral mechanisms, or both, pathophysiological alteration of cerebral modulatory mechanisms should probably play a major role.

Second, altered behavioral responses to nociception have been confirmed to be associated with cerebral dysfunction of descending pain modulatory and reward systems. Earlier separate reports already showed evidence for plastic deterioration of descending modulatory centers, i.e., gray matter density reduction at the dorsolateral prefrontal cortex [7], and failed reward responses on pain offset, i.e., diminution of nucleus accumbens activity [8]. Our current fMRI study has successfully shown novel evidence for simultaneous inactivity of both systems during dynamic perception of pain in patients with chronic pain.

In future studies, we will need to explore causal relationships between persistent pain and cerebral plastic changes. Longitudinal behavioral and neuroimaging studies of patients with chronic pain, along the course of treatment, might potentially give clues to such questions.

12.6 Conclusion

Patients with chronic pain showed attenuation of OA and obtundation in nociceptive perception in comparison with healthy controls. Attenuation of OA was associated with inactivity of principal brain areas for descending pain modulation and reward in patients with chronic pain. Those findings supported significant pathophysiological roles for both of those systems in pain chronification.

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Chapter 13
Cortical LTP: A Synaptic Model for Chronic Pain

Min Zhuo

Abstract Cumulative evidence indicates that cortical synapses not only play important roles in pain perception and related emotional functions but also undergo long-term potentiation (LTP) and contribute to chronic pain. LTP is found at two key cortical regions such as the anterior cingulate cortex (ACC) and insular cortex (IC), and inhibition of cortical LTP produces analgesic effects as well as anxiolytic effects. In this chapter, I will summarize our work on ACC and IC and provide evidence for calcium-stimulated AC1 as a key molecule for cortical LTP and chronic pain.

Keywords Anterior cingulate cortex · Insular cortex · Long-term potentiation · NMDA · AC1

13.1 Introduction

Chronic pain is a major health issue all over the world and is caused by tissue or nerve injury under different disease conditions. Due to poor understanding of the molecular mechanisms of chronic pain, especially at central regions, current pain medicines are ineffective. Consequently, patients mostly depend on the use of opioids to control pain. While opioids fail to erase chronic pain, they only produce analgesic effects by nonselectively inhibiting synaptic transmission. The long-term use of opioids has caused widespread drug abuse problems. In this chapter, I will discuss previous and recent discoveries related to the cortical mechanism of chronic pain and propose LTP as a key synaptic mechanism for chronic pain.

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13.2 ACC and IC

Human and animal studies have consistently demonstrated that neurons in the anterior cingulate cortex (ACC) and insular cortex (IC) are two key cortical regions for pain perception and chronic pain [1, 2, 13, 14, 24, 26, 28, 29, 30]. Activation of the ACC and IC has been reported to be caused by various noxious heat, cold, and chemical stimuli. In animal models of chronic pain, activity-dependent immediate early genes have been reported to be activated in these two areas after peripheral inflammation or nerve injury. In genetic knockout mice in which chronic pain has been significantly reduced, these immediate early genes are also reduced in the ACC and IC [18, 20, 25]. Electrophysiological experiments have provided direct evidence that neurons in the ACC and IC are activated by noxious stimuli and most of these cells are likely excitatory pyramidal neurons [21, 25]. Moreover, activation of the ACC causes fearful memory, supporting the roles of ACC in the unpleasantness of pain [16]. In human brain imaging experiments, it has also been found that ACC and/or IC regions are triggered by psychological pain and social exclusion, providing further evidence for their importance in the process of pain [2]. Furthermore, biochemical and anatomic studies have indicated that plasticity-related signaling pathways are activated in the ACC and IC after peripheral injuries. It is believed that cortical synapses undergo long-term plastic changes after peripheral injuries.

Recently, activation of the ACC induced by peripheral nerve injury increases the turnover of specific synaptic proteins in a persistent manner. Ko et al. [5] demonstrate that neural cell adhesion molecule 1 (NCAM1) is one of the molecules involved and show that it mediates spine reorganization and contributes to the behavioral sensitization.

13.3 Long-Term Potentiation (LTP) in the ACC and IC

LTP can be readily induced using a variety of experimental methods, including field excitatory postsynaptic potential (EPSP) recordings, whole-cell patch-clamp recordings, and multielectrode array (MEA) recordings. Field recordings in slices from adult mice have shown that glutamatergic synapses in the ACC exhibit LTP lasting many hours in response to theta burst stimulation (TBS). The activation of NMDA receptors and L-type voltage-gated calcium channels (L-VGCCs) are required for ACC LTP [25]. ACC LTP can also be induced using other LTP inducing protocols such as stimulus-depolarization pairing and spike-EPSP pairing. In whole-cell patch-clamp recording experiments, LTP induced by different pairing protocols is typically NMDA receptor-dependent. Activation of L-VGCCs is not required.

NMDA receptors comprise a variety of subtypes that are composed of different combinations of subunits, typically two GluN1 (NR1) subunits and two GluN2 (NR2) subunits, of which there are four possible subtypes, GluN2A-D (NR2A-D).

In the ACC, it has been shown that LTP, as detected by whole-cell patch-clamp recording, is sensitive to both GluN2A-preferring and GluN2B- preferring antagonists, indicating that tri-heteromers of the NMDA receptor may also be the dominant form of the receptor that contributes to LTP at ACC.

The activation of NMDA receptors leads to an increase in Ca^{2+} levels in dendritic spines, owing to Ca^{2+} entry through NMDA receptors and a consequent Ca^{2+}-stimulated release of Ca^{2+} from intracellular stores [1]. The postsynaptic Ca^{2+} signal is an essential component for the induction of LTP. Electroporation studies indicate that activation of CaM-dependent signaling pathways by Ca^{2+} binding is essential for ACC LTP. The expression of ACC LTP requires AMPA receptor GluA1 subunit. Pharmacological experiments show that allocation of a peptide that mimics the PDZ domain at the C-terminal tail of the GluA1 subunit blocked the early expression of LTP. By contrast, peptides that interfere with interactions with the C-terminal tail of GluA2 (GluR2) or GluA3 (GluR3) do not have any effect. Experiments using genetic knockout mice further support this conclusion. ACC LTP is normal in mice lacking GluA2, whereas it is absent in mice lacking GluA1. Furthermore, a CP-AMPAR antagonist applied at 5 min after the induction of ACC LTP significantly reduced the potentiation. Recent evidence suggests that CP-AMPARs are specifically associated with the PKA-dependent form of LTP and PKA phosphorylation site at serine 845 plays critical roles in ACC LTP [15]. Finally, administration of an inhibitor of PKMζ, zeta inhibitory peptide (ZIP), abolished LTP in the ACC [8] (Fig. 13.1).

Although there are currently less studies of LTP in the IC, similar synaptic mechanisms are likely to be involved [11, 30].

13.3.1 Presynaptic LTP (Pre-LTP)

Recent studies show that another form of ACC LTP (called pre-LTP), an NMDA receptor-independent form of LTP, can also be induced by using paired-pulse low-frequency stimulation. The activation of kainate receptors is critical for the induction. In mice lacking the GluK2 subunit, this pre-LTP is blocked. Furthermore, pre-LTP is also blocked by a potent GluK1-selective kainate receptor antagonist, UBP31060. The activation of the cAMP signaling pathway contributes to the induction of pre-LTP in the ACC neurons. Activation of calcium-stimulated adenylyl cyclase subtype 1 (AC1), but not AC8, is selectively required for ACC pre-LTP [6, 7] and IC [22]. Kainate receptor-dependent pre-LTP in the ACC may also involve FMRP signaling [6, 7].

For the expression of LTP, kainate receptor-dependent LTP is expressed by an increase in the probability of release, P(r), as assessed by changes in paired-pulse facilitation. LTP involves the modulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels leading to a persistent depolarization of presynaptic terminals, which could account for the increase in P(r). There are four HCN channel subunits (HCN1–4), all of which are expressed in both ACC and thalamus.

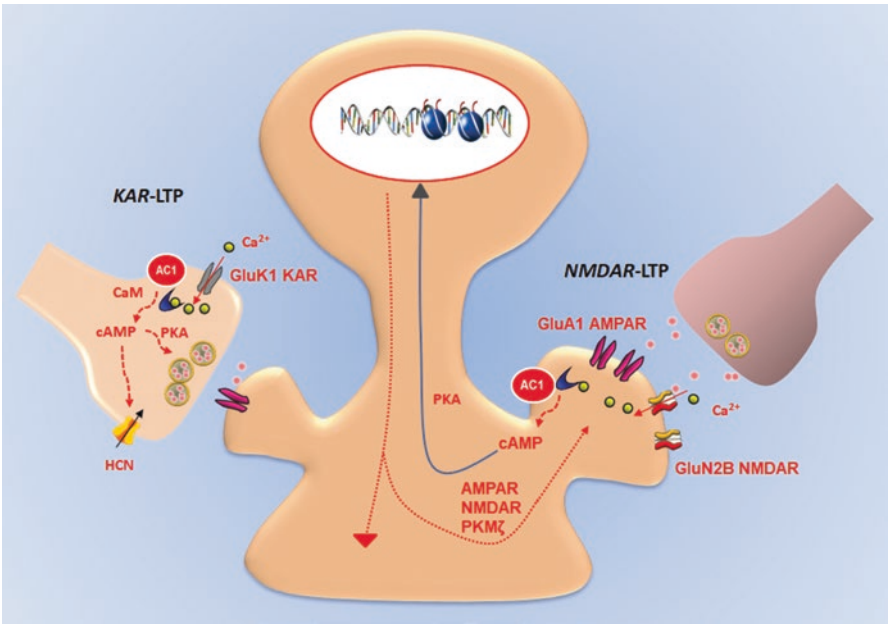

Fig. 13.1 Signaling pathways for the induction and expression of two major forms of LTP in the ACC. For NMDA receptor-dependent LTP, neural activity triggered the release of excitatory neurotransmitter glutamate (Glu: filled circles) in the ACC synapses. Activation of glutamate NMDA receptors leads to an increase in postsynaptic Ca^{2+} in dendritic spines. Both NMDA NR2B and NR2A subunits are important for NMDA receptor functions. Ca^{2+} serves as an important intracellular signal for triggering a series of biochemical events that contribute to the expression of LTP. Ca^{2+} binds to CaM and leads to activation of calcium-stimulated AC1 as well as Ca^{2+}-/CaM-dependent protein kinases (PKC, CaMKII, and CaMKIV). Through various protein-kinase-related intracellular signaling pathways, the trafficking of postsynaptic AMPA receptor as well as other synaptic modifications contributes to enhanced synaptic responses. Activation of CaMKIV, a kinase predominantly expressed in the nuclei, will trigger CREB signaling pathways. In addition, activation of AC1 leads to activation of PKA and subsequently CREB as well. For NMDA receptor-independent LTP, neural activity triggered the release of Glu and subsequent activation of presynaptic kainate receptor. Activation of kainate receptor causes the influx of Ca^{2+} to presynaptic terminal and activation of AC1 as well. Ca^{2+} binds to CaM and leads to activation of calcium-stimulated AC1. Activation of PKA may contribute to the enhancement of glutamate release

13.4 AC1 Acts as a Key Molecule for Triggering LTP

Cyclic adenosine monophosphate (cAMP) is a key intracellular second messenger. The cAMP signaling pathway contributes to learning and memory, chronic pain, emotional fear, and drug abuse. The AC is the enzyme that catalyzes ATP to cAMP. There are two major families of ACs: nine membrane-bound (AC1-9) and one soluble form of AC (sAC). Subtypes of ACs show unique organ and cellular distributions, and the mechanisms leading to the activation of them are different for

subtypes of ACs. These observations support possible distinct physiological functions of each AC isoform in biological systems. Among more than ten subunits, AC1 and AC8 are two of the AC subtypes that respond positively to calcium-calmodulin (CaM). AC1 is more sensitive to a calcium increase than AC8 (more than eight times), indicating that AC1 could play more important role in the production of cAMP. Anatomic studies found that AC1 is highly expressed in cingulate neurons and located in most of the layers of the ACC [20]. Genetic studies using mice lacking AC1 show that gene deletion of AC1 selectively impair ACC LTP, while basal excitatory glutamate transmission in the ACC is not affected. LTP induced by TBS or pairing stimulation is abolished in cingulate pyramidal cells of AC1 knockout mice [9]. By using chemical and biochemical screening, selective chemical inhibitors of AC1 have been identified [17]. In consistent with results from genetic knockout mice, pharmacological inhibition of AC1 in ACC neurons abolishes LTP induced by pairing training [3, 17].

Our recent data also found that AC1 is essential for the induction of late-phase LTP (L-LTP) in the ACC synapses [3]. While in wild-type mice, TBS induced L-LTP that lasted for at least 3–6 h. TBS failed to induce any significant potentiation in ACC slices of AC1 KO mice. Since AC1 is a neuronal selective form of ACs, it is likely that AC1 activity may also contribute to LTP in other pain-related cortical areas such as the prefrontal cortex (PFC), insular cortex, and somatosensory cortex. It has been reported that AC1 activity is required for injury-activated immediate early gene activity in these cortical areas. LTP has been reported in the PFC, somatosensory cortex and IC. Recent studies have reported that AC1 contributes to LTP in the IC [30].

13.5 Requirement of AC1 for Behavioral Sensitization and Spinal Enhancement

Behavioral studies using AC1 knockout (KO) mice have demonstrated that AC1 contributes to chronic pain [20]. Behavioral responses to peripheral injection of two inflammatory stimuli, formalin and complete Freund's adjuvant, were reduced or abolished in AC1 KO mice. However, wild-type and AC1 KO mice were indistinguishable in behavioral tests of acute pain. Using activity-dependent immediate early genes as markers, AC1 is also found to contribute to inflammation-induced activation of CREB. In addition to sensory inputs from the skin, behavioral nociceptive responses in animal models of muscle pain or chronic muscle inflammatory pain were significantly reduced in AC1 KO mice.

The possible roles of AC1 in chronic pain-related plasticity are also reported at areas outside of the cortex. In the spinal cord, AC1 activity is found to be critical for 5-HT-induced spinal facilitation and synaptic plasticity. Application of a low dose of serotonin (5-HT) alone or a co-application of forskolin with 5-HT produced long-lasting facilitation of excitatory synaptic transmission between primary afferent

fibers and dorsal horn neurons. This enhancement requires the recruitment or trafficking of functional postsynaptic AMPA receptors. AC1 is required for 5-HT-induced enhancement using AC1 KO mice. In addition, AC1 also contributes to the activation of the extracellular signal-regulated kinase (Erk) in spinal cord dorsal horn neurons, suggesting that AC1 may link upstream signals to long-term gene regulation and protein synthesis that are linked to synaptic plasticity. Finally, AC1 activity is also found to be required for spinal LTP induced by pairing protocol in spinal dorsal horn neurons [27].

13.6 Discovery of AC1 Inhibitor NB001

Considering the important roles of AC1 in injury-related synaptic plasticity as well as behavioral responses in animal models of chronic pain, it is critical to develop a selective inhibitor for AC1. Using chemical design and screening experiments, a selective inhibitor for AC1, NB001, has been identified. NB001 shows its selective inhibition for AC1 in both human embryonic kidney (HEK) 293 cells, in which AC1 was stably expressed, and in adult mouse neurons. In addition, NB001 produces a dose-dependent inhibition of cAMP production in adult mouse ACC slices, suggesting that NB001 is effective in whole animals. By contrast, NB001 did not significantly affect other isoforms of ACs such as AC5-8 activity at effective inhibiting doses for AC1.

Results from electrophysiological experiments using AC1 KO mice or NB001 are quite similar. NB001 inhibited sensory-related LTP in ACC and spinal cord dorsal horn. Postsynaptic application of NB001 completely blocked the induction of LTP in ACC pyramidal neurons of adult mice. Furthermore, NB001 also prevented the induction of LTP in spinal cord dorsal horn neurons. These findings strongly indicate that AC1 may contribute to chronic pain by playing important roles in injury-related LTP in ACC and spinal cord. Finally, LTP in central synapses contains at least two different major forms: early-phase LTP (E-LTP) and L-LTP. Recent studies using a multiple channel recording system found that NB001 produced powerful inhibition of L-LTP that lasted at least 3 h after the induction.

13.7 NB001 Is Analgesic in Different Animal Models of Chronic Pain

Behavioral studies using AC1 KO mice demonstrate that behavioral allodynia in animal models of neuropathic pain and inflammatory pain was significantly reduced [20, 27]. To confirm the requirement of AC1 activity in these behavioral responses, the effects of NB001 [17, 27] on behavioral allodynia in animal models of neuropathic pain induced by nerve ligation have been examined. Administration of

NB001 (1–5 mg/kg, i.p.) produced significant analgesic effects in different animal models of chronic pain. By contrast, application of NB001 at different dosages did not cause any abnormal behaviors in animals. Animals treated with NB001 showed calm and normal motor functions. Furthermore, emotional responses were also normal after NB001 treatment.

In addition, intraperitoneal injection of NB001 also reduced spontaneous pain in an animal model of IBS as well as cancer pain [23, 4]. These findings suggest that NB001 may serve as a novel analgesic to treat bone cancer pain and visceral pain.

13.8 Conclusion and Future Directions

In summary, integrative experimental approaches have demonstrated that cortical synapses that receive sensory painful information are highly plastic. Major forms of synaptic plasticity discovered in central synapses, including LTP and LTD, are also found in pain-related cortical areas such as ACC and IC. Investigation of cortical LTP mechanisms has provided molecular insights for the induction and expression of chronic pain in the central synapses. Among several key signaling molecules, AC1 has been found to be a key signaling protein for triggering chronic pain-related central plasticity. Inhibiting AC1 activity by using a selective inhibitor NB001 produces analgesic effects in different animal models of chronic pain. In addition to LTP, long-term depression (LTD) has been also reported in the ACC and IC [10, 19]. Peripheral injury causes loss of LTD, offering a new mechanism for cortical excitation. The use of tree shrew, a species with more advanced cortical structures, will facilitate translational research of cortical plasticity (see [12]).

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Chapter 14
Pain-Associated Neural Plasticity in the Parabrachial to Central Amygdala Circuit

Pain Changes the Brain, and the Brain Changes the Pain

Fusao Kato, Yae K. Sugimura, and Yukari Takahashi

Abstract In addition to the canonical spino-thalamo-cortical pathway, lines of recently accumulated anatomical and physiological evidence suggest that projections originating in nociception-specific neurons in lamina I of the dorsal horn or the spinal nucleus of the trigeminal nerve to the lateral parabrachial nucleus (LPB) and then to the central amygdala (CeA) play essential roles in the nociception-emotion link and its tightening in chronic pain. With recent advances in the artificial manipulation of central neuronal activity, such as those with optogenetics, it is now possible to address many unanswered questions regarding the molecular and cellular mechanisms underlying the plastic changes in this pathway and their role in the pain chronification process.

Keywords Central sensitization · Nociception-emotion association · Optogenetics

14.1 Introduction

Pain is "an unpleasant sensory and emotional experience," and chronic pain is "the pain that persists or recurs for longer than 3 months." This long period required for chronic pain to be established might correspond to the time required for the time-dependent plastic changes and their consolidation in the central pain network underlying this "sensory and emotional experience." From the biological point of view, not simply the period itself but rather the central neuroplasticity sequentially taking place during this period should be the primary determinant of the process underlying the chronic pain establishment. Functional brain imaging in human patients and

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electrophysiological recordings in animal models of pain demonstrated that the semi-acute to chronic pain state is closely associated with plastic changes in the "functional modules" of the central pain network, such as pain-associated subcircuits in the anterior cingulate cortex [1], secondary sensory cortex [2], thalamus [3], accumbens nucleus [4], insular cortex [5], medial prefrontal cortex [6, 7], and the central amygdala (CeA) (reviewed in [8, 9]).

Of these structures, the plastic changes in the connection from the parabrachial nucleus to the central amygdala are of particular interest because of the following reasons:

1. The lateral parabrachial nucleus (LPB) is the major target of the nociception-specific neurons expressing Tac1 in the spinal cord lamina I [10] and neurons in the caudal part of the trigeminal spinal nucleus [11]. Therefore, LPB neurons are the first brain neurons that are activated by nociceptive inputs in the brain.
2. This connection relays various types of "primordial sensory information" in addition to nociception, such as visceral sense, body temperature, and appetite [12–14]. Because all these signals transmit information on more or less life-threatening events, it is reasonable that nociception also takes this route to enter the brain thus serving as one of the "alarm signals" [12–14].
3. Unlike the spino-thalamo-cortical pathway that originates in the deeper spinal cord layers, the somatotopy is limited with this connection. This makes it likely that this pathway does not play essential roles in the "sensory" aspect of pain.
4. The CeA, one of the major targets of the projections from the LPB, plays essential roles in emotional memory and responses and also a site for innate and learned fear/threat against aversive events, making it likely that this is the pathway playing a central role in the "emotional" aspect of pain.
5. The synaptic transmission between the LPB and the CeA neurons shows robust synaptic potentiation in various pain models in rodents, suggesting that this pathway is vital to "nociplastic" aspects of the chronic pain.
6. The CeA is one of the "kernel" upstream structures that controls the descending pain regulatory system, making it a "hub" for nociception and pain regulation. Table 14.1 summarizes the reported findings that support the notion that the LPB and CeA are the principal players in the emotional aspect of pain in rodents (Fig. 14.1).

Although the functional significance of this spino-(trigemino-)parabrachio-amygdaloid pathway remains elusive in primates [15], a series of recent clinical studies that followed up yearly changes in brain activity and morphology in chronic back pain patients concluded that an augmented blood oxygenation level-dependent (BOLD) signal during subjective spontaneous pain in the amygdala and medial prefrontal cortex, as measured with functional MRI, provides the cerebral signature for persistent chronic back pain [16].

Table 14.1 Experimental evidence supporting that the spino-parabrachio-amygdaloid pathway underlies the emotional aspect of pain

Findings
Pharmacological inhibition of the LPB during the acquisition attenuates tone cued threat learning [17]
Optogenetic stimulation of the fiber terminals in the CeA of mice expressing channelrhodospin2 (ChR2) in the LPB neurons can induce associative learning in the absence of electrical shock [17]
Large majority (>95%) of the dorsal horn lamina I projection neurons project to the LPB as one of their brain targets [10]
Lumber dorsal horn neurons projecting to the LPB generates action potentials in response to noxious stimulation [18]
Lumber dorsal horn neurons projecting to the LPB show aberrant spontaneous firing in rats with spinal nerve injury [18]
LPB neurons show strong cFos expression after repeated noxious stimulation in the neck and tail and hind limb inflammation [19]
Neurons in the bilateral LPB and right CeA show strong cFos expression 3 h after orofacial inflammation with upper lip injection of formalin [20]
The neurons in the LPB have axonal projections terminating in the capsular and lateral part of the CeA [21]
The axons arising from the LPB forms highly reliable monosynaptic excitatory synapses with the CeA neurons (especially those in the capsular part) [22]
Seventy-eight percent of the neurons (unit recording) in the capsular part of CeA are excited by nociceptive inputs to the body in anesthetized rats [23, 24]
Selective expression of phosphorylated ERK in the right CeA 3 h after hind limb injection of formalin [25]
Increase in newly born neurons in the CeA in the spinal nerve ligated rats and enhanced depressive behaviors [26]
The excitatory synapses between fibers of putatively LPB origin and CeA neurons (especially the capsular part) undergo robust synaptic potentiation in the arthritis, visceral, neuropathic, inflammatory, and muscle pain models [8, 9, 27–31]
Activation of CeA has pro-nociceptive (or antinociceptive, depending on the target neurons) consequences [25, 32–34]

14.2 Synaptic Potentiation in the LPB-CeA (LPB-CeC) Synapse in Pain Models

Since the first pioneering report of synaptic potentiation from Neugebauer's group in an arthritis model of rats [27], robust and large-magnitude synaptic potentiation has been described in various pain models, indicating that this LPB-CeA synaptic potentiation is a commonly observed characteristic in various pain models regardless of their type and etiology. The most important target of LPB fibers is the capsular CeA (CeC, also denoted as CeLC in the earlier literatures), where 78% of the neurons get excited by acute nociceptive simulation. Table 14.2 summarizes the type of pain model, the time at which the potentiation was observed and the underlying mechanisms.

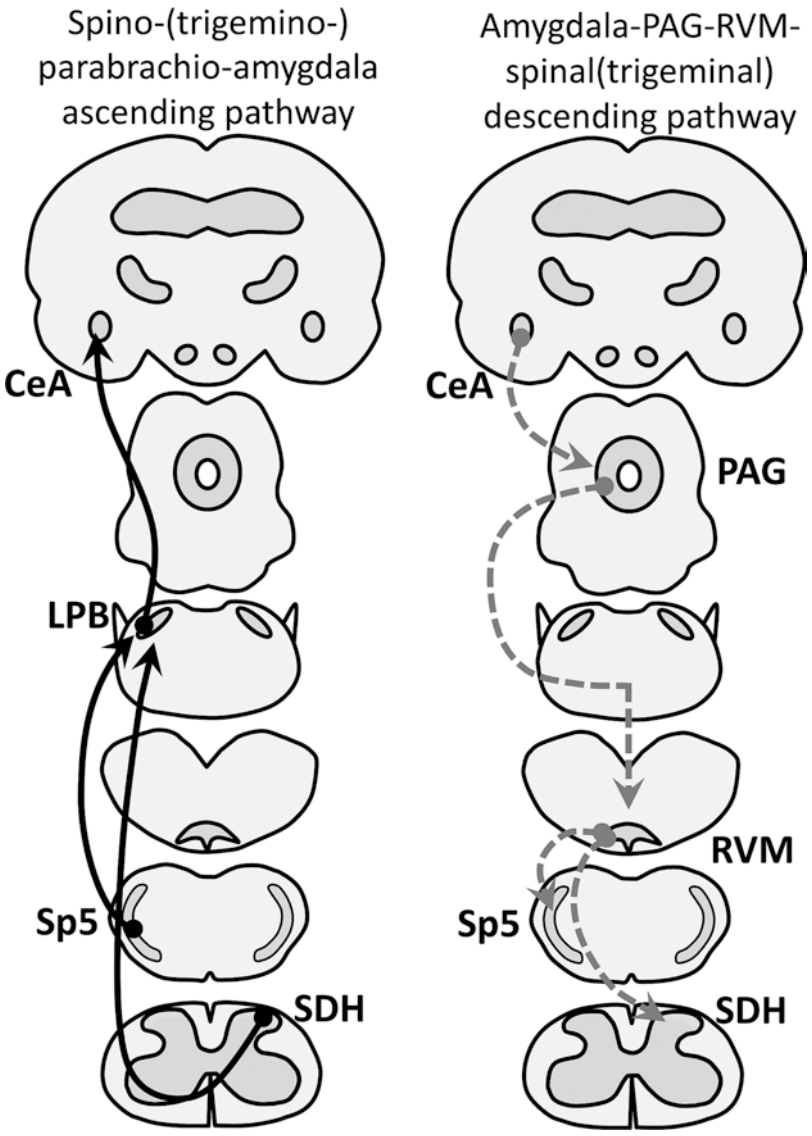

Fig. 14.1 Ascending and descending pain pathways with the CeA as the "hub" of both downstream and upstream flow of the pain-associated information

As shown in Table 14.2, these approaches have clarified how nociceptive inputs of LPB origin affect CeC neuron excitation and advanced our knowledge on how subacute to chronic pain modulates the nociception-emotion link at the first entry point.

Table 14.2 Examples of the LPB-CeC synaptic potentiation in rodents

Pain models	Time after model preparation	Input specificity and synaptic and molecular mechanisms
Kaolin + carrageenam knee injection-induced arthritis [8, 27, 35–37]	6–8 h	↑release; group I and II mGluRs; protein kinase A; corticotropin-releasing hormone; calcitonin gene-related peptide (CGRP)
Zymosan-induced colitis [38]	>6 h	↑LPB-CeC but not basolateral amygdala (BLA)-CeA; ↑postsynaptic excitability; PKA
L5 spinal nerve ligation	6–8 h [39]	↑LPB-CeC (bilateral)
	1 week [29, 30]	↑LPB-CeC (unilateral); ↑BLA-CeC (bilateral); no changes in paired-pulse ratio (PPR). NMDA component is not potentiated; absent in C fiber denervated rats
Streptozotocin-induced diabetics [40]	2 week	↑LPB-CeC (bilateral)
Repeated intramuscular injection of acid [41]	2 h after the second injection	pERK↑
Intraplantar formalin injection [22, 31, 42]	6 h	↑LPB-CeC (right side only); ↓PPR, ↑asynchronous EPSC amplitude; absent in CGRP deficient mice

These studies in Table 14.2 are mostly based on results using patch-clamp recording of membrane currents of CeC neurons and electrical stimulation of the peri-CeA region where the fibers of the LPB origin run in coronal brain sections (except for reference [22]). However, to understand how chronic pain affects the "emotional experience," it is necessary to analyze the output from the CeA network. This, however, has been very challenging with conventional electrical stimulation because of the following three limitations. First, it has risks of stimulating fibers other than those arising monosynaptically from the LPB. Second, because the CeA is mostly composed of GABAergic inhibitory neurons, such peri-CeA stimulation has risks of directly stimulating local GABAergic neurons, and therefore the recordings of excitatory LPB-CeC transmission should be done in the presence of GABA receptor blockers to silence secondary components. This prevents analyzing the secondary signaling after LPB inputs within the CeA local network. Third, because of the rostrocaudal nature of the projections from the CeC or from the lateral CeA (CeL) to the medial CeA (CeM) [43], it is necessary to use horizontal, not coronal, slices to ensure the information from the input point (CeC) to the output point (CeM). However, because of the ventro-dorsal entry of the LPB fibers to the CeC [21], it is impossible to selectively and effectively stimulate them in horizontally sectioned slices.

14.3 Optogenetic Activation of the LPB-CeC Pathway

Use of optogenetics has provided ways to remove these limitations and enabled analysis of the influence of the LPB inputs on the output from the CeA. The first application of optogenetics to the LPB-CeC projection was made by our group using synapsin promoter for channelrhodopsin2 (ChR2) expression in mice [17] and 4 months later using transgenic mice expressing Cre recombinase in neurons with CGRP [13]. These studies consistently demonstrated that activation of the LPB-CeA pathway alone is sufficient to acquire Pavlovian associative learning, thus providing evidence that this pathway indeed carries aversive (likely to be painful) information to the amygdala.

Using coronal and horizontal slice preparations of rats, Sugimura et al. have provided the following novel findings [22]: (1) monosynaptic direct excitatory synaptic transmission from LPB neurons to CeC neurons. Surprisingly, in the CeC, and partly in the CeL and CeM, almost all neurons show robust postsynaptic responses to light stimulation of the LPB fibers. In contrast to the large variation in latency between LPB stimulation and CeC unit firing reported in anesthetized rats in vivo (Fig. 14.2d in Neugebauer and Li [23]), the latency of this monosynaptic connection shown with light stimulation is very stable. (2) The amplitude of this monosynaptic component was larger in the rats that received intraplantar formalin injection 24 h before the slice preparation, which was however observed only in a subset of CeC neurons. The late-firing neurons with A-current-dependent slow depolarization showed this potentiation, suggesting that the pain-associated plasticity is a feature of a subgroup of neurons receiving monosynaptic LPB inputs (Fig. 14.2). (3) Potent and robust feedforward inhibition with a few milliseconds delay and very stable latency was elicited by light stimulation, suggestive of di-synaptic GABAergic neuron involvement (Fig. 14.2). This feedforward inhibition was large in amplitude (i.e., large conductance) with slow desensitization, suggesting that this component is influential in determining the CeC neuron excitation in response to repeated activation of LPB neurons. (4) In contrast to the CeC neurons, the neurons in the CeM, as recorded in horizontal slices, showed asynchronously occurring bombardments of large-amplitude inhibitory inputs in response to single inputs of LPB origin (Fig. 14.2). The network and synaptic organization revealed by analyzing the responses to the optogenetic activation of LPB fibers is depicted in Fig. 14.2.

14.4 Conclusion

Being one of the pivot points of ascending and descending pain-associated information, and also as the well-known site for affective, hormonal, autonomic integration, understanding of the role of the neurons in the LPB and CeA and their synaptic

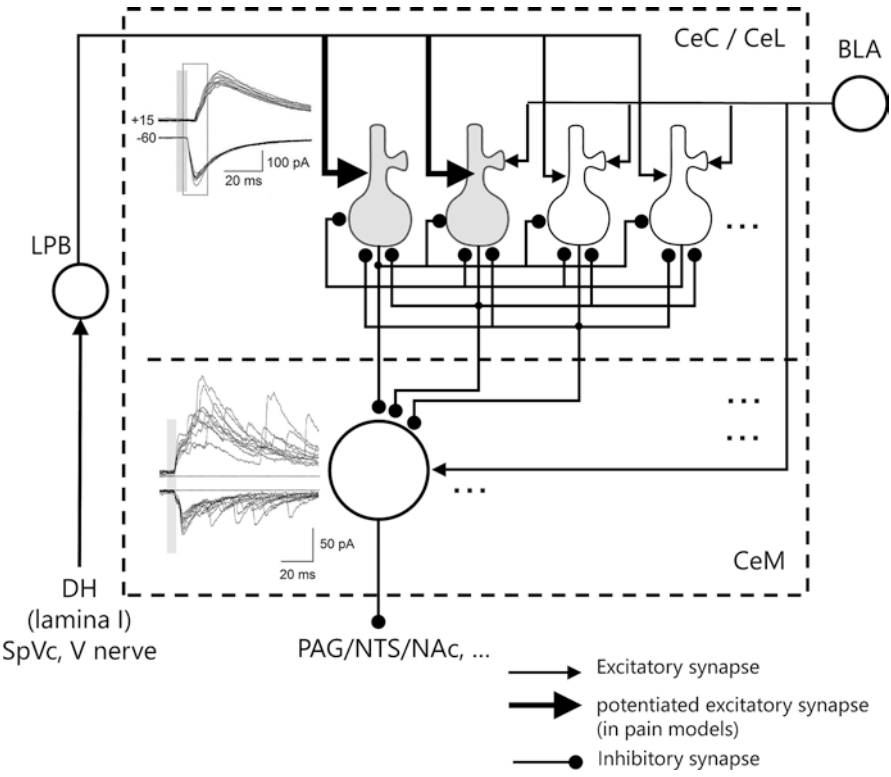

Fig. 14.2 Network and synaptic organization of the CeA. The waveform insets are taken from Ref. [22]

changes in chronic pain models would be the key to understanding the molecular and cellular mechanisms of the chronification of pain as an "emotional experience." Application of recently developed techniques that allows selective manipulation of neurons and pathways would provide us with a clearer view of the network organization and alteration of these circuits in pain-associated neuroplasticity.

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Chapter 15
Electrophysiological Signature of Pain

Zi-Fang Zhao and You Wan

Abstract Pain is a complex neural function involving cognition, sensory, emotion, and memory. Imaging studies have shown that multiple brain regions are actively engaged in the processing of pain. However, roles of each brain regions and their contribution to pain are still largely unknown. Recent studies with electrophysiology especially high-density electroencephalogram (EEG) or multichannel recordings techniques have provided more insights into the dynamics of pain signature. The accumulations of the evidence could facilitate our understanding of pain and provide potential methods for objective pain evaluation and treatment of chronic pain.

Keywords Pain · Neural oscillation · Electroencephalogram

15.1 Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or describe regarding such damage, according to the International for the Study of Pain (IASP) [1]. Pain is a protective function that drives us from potential or existing damage. When pain becomes chronic or

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unnecessary, it could cause an unpleasant feeling to the patient. However, pain is very subjective; it may vary with the different mental processes. Attention [2], emotion, and cognition [3] are related to pain modulation. Functional imaging studies have revealed the existence of a "pain matrix" consisting of multiple functionally coupled brain regions during pain [4]. Primary somatosensory cortex (S1) [5], secondary somatosensory cortex (S2) [6, 7], anterior cingulate cortex (ACC) [8], frontal cortex [9], periaqueductal gray (PAG) [10], and insula [11] are reported to be involved in pain, indicating pain's multimodality. Till now, pain-specific cell or neural oscillation has not yet been found in any of the pain-related regions, suggesting that pain system may not have a center. The interplay between different pain-related regions may be the underlying mechanism of pain.

To study how different brain regions are bonded together during pain, extracellular electrophysiology and electroencephalogram (EEG) provide the best temporal resolution and high spatial selectivity. Field potentials generated by the summation activities of local neurons could reveal the current state of the recorded area [12], and synchrony between field potentials generated by two different area could indicate the functional connectivity between them [13, 14]. By studying the field potentials change during pain, we could learn the dynamics of the pain matrix piece by piece, and it will finally lead to better pain diagnosis and intervention methods.

Here we review the recent electrophysiology evidence of pain. In the first part, we will review the recent advances of research in acute pain. We then summarize the recent findings in chronic pain. In the last part, we will go through the EEG study of a popular drug-free treatment of chronic pain: electrical acupuncture.

15.1.1 Electrophysiology Signature of Acute Pain

Neural oscillations and laser-evoked potentials (LEPs) are the most common focus of the study of electrophysiology of pain. Increased gamma oscillations and suppressed beta oscillations are repeatedly found in parallel with pain [15, 16]. Gamma oscillation is a fast rhythmic activity which is organized in 30–80 Hz epochs and has been considered as a landmark of local information processing [17]. The fast interplay between principal neurons and interneurons generated this high-frequency event. It has been reported that gamma-band activation participates in pain perception. The power of gamma oscillations increases after brief nociceptive laser stimulations in healthy human beings [15, 18] and in normal rats [19], indicating that gamma oscillations are related to experimental acute pain lasting for milliseconds to seconds. Such gamma activity is focused on the somatosensory cortex and positively correlated with the perceived pain intensity measured by the subjective rating of pain sensation on human beings [15] and with convert pain processing in patients with chronic disorders of consciousness [20].

In another study, gamma oscillation recorded by EEG has also been found to have a strong coupling with theta wave shortly after the laser stimulation [19]. The amplitude of gamma oscillations was coupled with the phase of theta oscillations

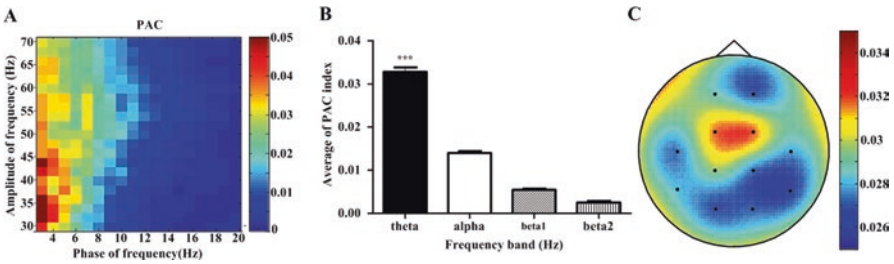

Fig. 15.1 Amplitude of gamma oscillation is modulated by phase of theta oscillation after laser stimulation. (**a**) Averaged phase – amplitude coupling (PAC) between low frequency (3–20 Hz) and high frequency (30–70 Hz) among all channels in rats. (**b**) Phase – amplitude coupling between gamma oscillation (40–70 Hz) and theta (3–7 Hz), alpha (8–12 Hz), beta1 (12–16 Hz), beta2 (16–20 Hz). Phase – amplitude coupling between theta and gamma oscillation was significantly stronger than coupling between other oscillations and gamma oscillation. ***$P < 0.001$. (**c**) Topography of phase – amplitude coupling between theta and gamma oscillations

after perceived nociceptive stimulation (Fig. 15.1). This study provided the evidence that the electrophysiology signature of pain also lies in the interplay of different frequency bands and different brain regions. It also indicates the electrophysiology signature of pain may lie in a high-dimensional feature space.

Although gamma oscillation and theta-gamma coupling have shown a strong correlation to pain, they still cannot be considered solely as a biomarker for pain since it is not an exclusive process. Li et al. have proposed a machine learning-based routine to analyze the laser-induced oscillation of pain [21]. In this study, a generalized linear model was used to identify the contribution of each neural oscillations in four pain-related regions from 336 LFP features. The generalized linear model reached an 86% accuracy in classifying the LFP data to non-painful and painful stimulation trials (Fig. 15.2). An important conclusion of this work is that any single LFP feature is not good enough for pain prediction, and the integrated feature can give a more accurate and robust prediction.

15.1.2 Enhanced Gamma Oscillation in Somatosensory Cortex During Chronic Inflammatory Pain

Gamma oscillation was also shown to participate in the abnormal working memory of chronic pain [22]. However, whether gamma oscillations play a role in the pain, perception in chronic pain is unknown and of great interest. Unlike acute and tonic pain, chronic pain is a pathological, ongoing, and long-lasting condition [1], featured by its persistence and maintenance of pain. Chronic pain is a complex disease with the enhanced and prolonged processing of pain-related sensory, emotion, and cognitive components [23]. Considering gamma activity participates in the sensory, emotional, and cognitive components of pain processing [15, 24, 25], gamma activity

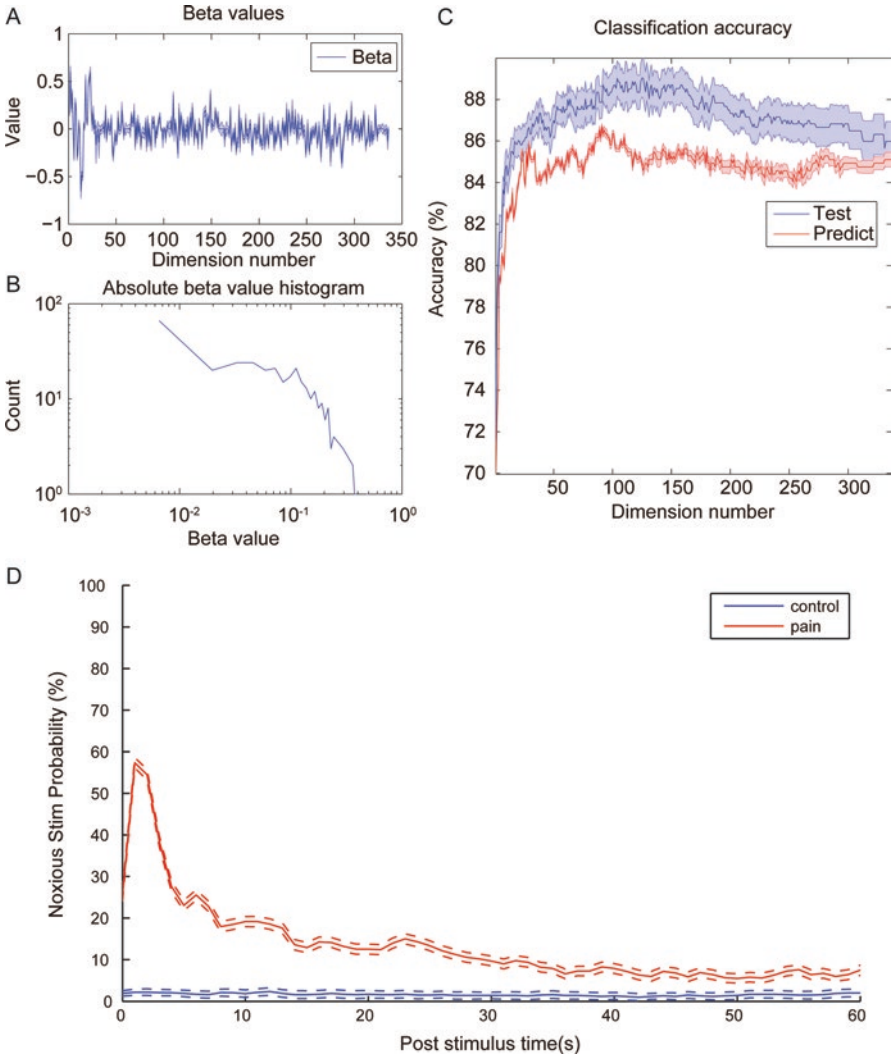

Fig. 15.2 Coefficient values and prediction rate of GLMs. (**a**) β-values of the initial GLM classifier which trained with the whole dataset (blue). Classification accuracy of GLMs trained with the corresponding LFP feature in the test set (red). (**b**) Histogram of absolute β-values. Most features contribute little to the data classification. Only a few features contribute most to the classification. Distribution of coefficient values has a nearly lognormal distribution. (**c**) Prediction accuracy of the GLM models trained with different numbers of most contributing features. Training set (blue) consists of 2962 laser trails from 12 rats, and prediction set (red) consists 296 trails from a separate animal. Accuracy was calculated based on a tenfold cross-validation. Classification accuracy for the test set increases fast with input numbers of features at the beginning and slowly reaches a top accuracy with dimension numbers around 120. Then accuracy starts to drop slowly all the way to around 86%. Shaded area indicates standard error margin. (**d**) Averaged noxious laser stimulation probability vs poststimulus time. Rates were calculated from the most accurate GLM in the previous step. The blue line and the red line represent control stimulation and noxious stimulation, respectively. Dash lines indicate the standard error margin. Significant increase of pain prediction probability appears in a time window of 1–2 s after noxious stimulation, indicating a robust feature of laser-induced pain. Control group kept a low value for the entire period and does not show any increase after the stimulation onset

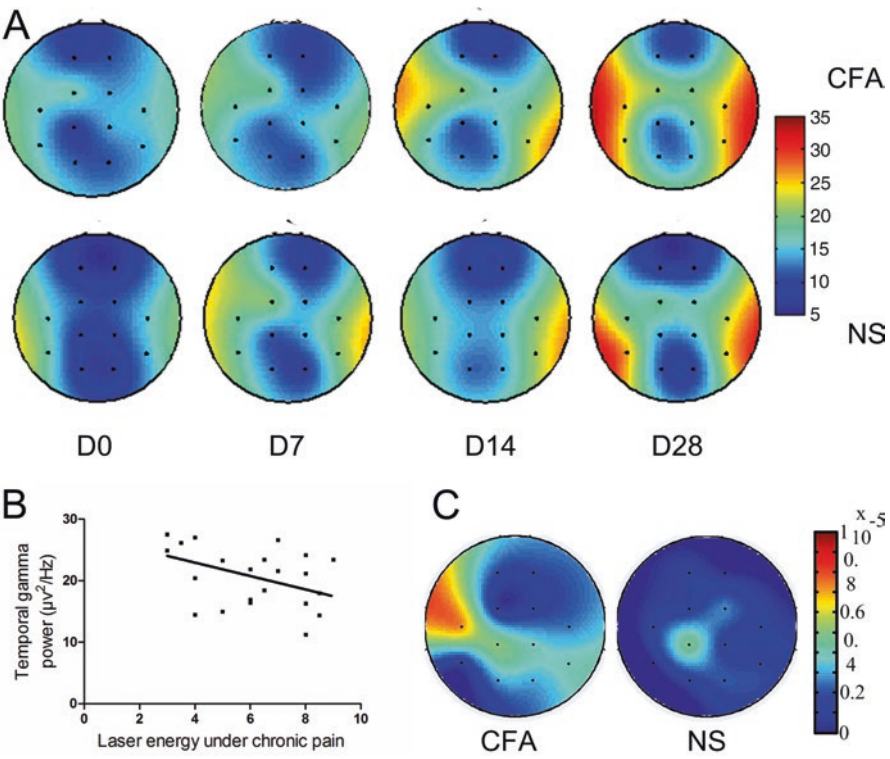

Fig. 15.3 Enhanced gamma power in chronic pain. (**a**) Power spectrum during the development of chronic pain. (**b**) Grand average power (mean ± SEM) of each frequency band in CFA group and in NS control group at different days after CFA injection. Gamma-band power was significantly higher at day 28 in the CFA chronic pain group compared with that in NS control group (*$P < 0.05$). ***C.*** *Gamma* power over channels 3 and 10 was increased in the CFA chronic pain group (upper) compared with that in the NS group (below)

may be also involved in chronic pain. Wang et al. have demonstrated an enhanced spontaneous gamma activity in chronic pain [26]. In their research, they found that ECoG gamma activity increased under chronic inflammatory pain condition in a CFA monoarthritis model of rats. This CFA model mimics the whole processes from the acute stage to the chronic stage of inflammatory pain [27]. Results showed that gamma power gradually reached a significantly high level at the chronic pain states (Fig. 15.3). Similar to the acute pain situation, theta-gamma modulation was also shown a significant increase during inflammatory pain. These findings suggested that the enhanced gamma oscillatory activity was related to long-lasting pain condition.

15.1.3 Extracting Pain Signatures from High Dimensional Data

An accumulating amount of evidence has been pointing to the complexity and high dimensionality of the pain network. However, our tools are getting better. With the advance of the recording devices, more sites can be recorded by electrodes at the same time [28, 29]. It indeed creates a better chance of finding the underlying evidence. However, high-dimensional dataset brings difficulty to data analysis. Field potentials contain complex pattern both in temporal domain and frequency domain. In the high-dimensional dataset, different recording channel also carries spatial information of the location of electrode tips. The difficulty of finding the underlying pattern increases sharply with the dimension numbers; this is referred to as the "curse of dimensionality." The proper mathematical tool must be used to deal with this problem.

When dealing with the electrophysiology data, a very important step is feature extraction. Filtering was usually first applied to extract oscillatory events in different frequency bands. Coherence was commonly used to check the relation between two signals. However, synchronization between two distant brain regions is hard to be detected by linear synchronization algorithms like correlation and coherence. Synchronization likelihood (SL) is a nonlinear synchronization-detecting algorithm and widely used in studies that involve neural signals from two distant brain areas [13]. One drawback of that nonlinear algorithm is the heavy computational burden. Zhao et al. has proposed a graphics processing unit (GPU)-accelerated implement of SL algorithm with optional two-dimensional time-shifting [30]. Results showed that this method could reveal detailed information from original data with synchronization values of two temporal axes: delay time and onset time, thus be used to reconstruct a more detailed temporal structure of neural network consists of distant brain regions (Fig. 15.4).

After the construction of feature space, machine-learning methods like Bayesian inference and generalized linear model can be applied to the data to find the contribution of each feature.

15.1.4 Effect of Transcutaneous Acupoint Electrical Stimulation (TAES) on Pain and Sedation

Electroacupuncture has been considered as a potential treatment for chronic pain [31]. It has been shown that transcutaneous acupoint electroacupuncture (TAES) produced antinociception through the release of endogenous opioid peptides to activate opioid receptors during acute nociception. Electroacupuncture (EA) produced tolerance after its prolonged application. It is reported that 100 Hz EA could reduce mechanical hyperalgesia incomplete Freund's adjuvant (CFA)-induced inflammatory nociception rats [32, 33].

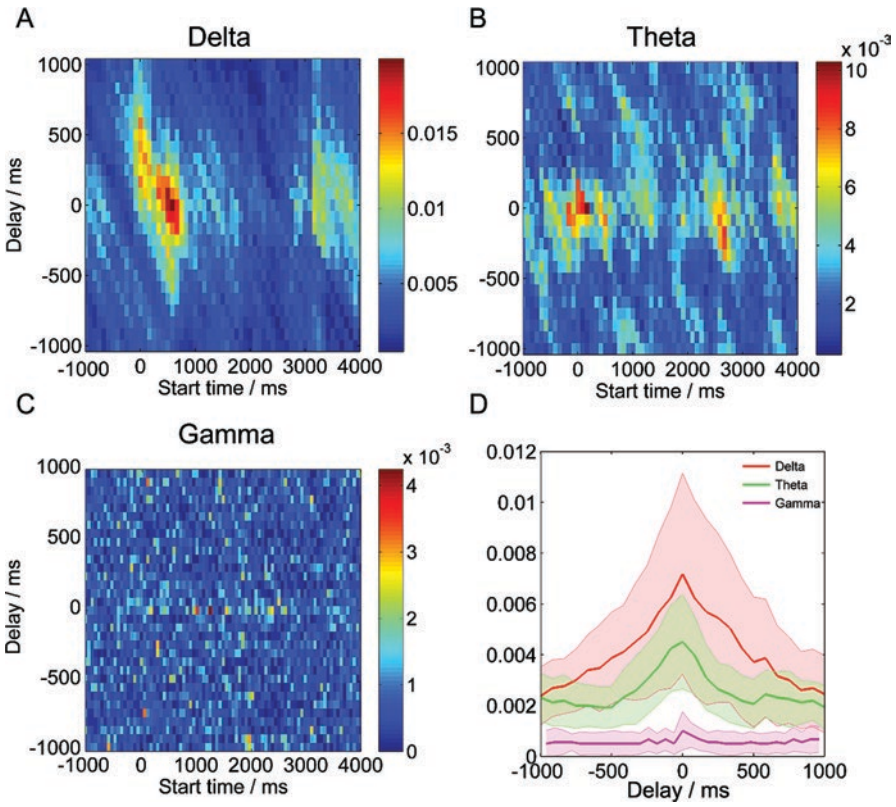

Fig. 15.4 Synchronization likelihood (SL) matrices of S1 and the ACC in several frequency bands and their delay distribution values. (**a**–**c**) SL matrices demonstrating the temporal distribution of notable synchronization events cumulated from all channel pairs between S1 and the ACC in the delta (**a**), theta (**b**), and gamma bands (**c**). (**d**) SL value distribution along the delay axis from these three SL matrices (red, delta; green, theta; purple, gamma). At each step of delay, the SL values of all start times with the same delay were calculated for the median and standard deviation. Each solid line represents the median along the delay axis of each frequency band, and the shadowed area of the same color represents the standard deviation along the delay axis of that band

Wang et al. investigated the changes in spontaneous brain oscillations in the incisional pain and the EA modulation on EEG oscillations [34]. In the incisional pain model, they found that the spontaneous EEG power of delta-frequency oscillation decreased, while the power in the theta, the alpha, and the beta bands increased in rats after plantar incision. Interestingly, no significant change in gamma power was observed. Application of EA decreased the power at high-frequency bands, especially at the beta band, and reversed the enhancement of the cross-frequency coupling strength between the beta and low-frequency bands (Fig. 15.5).

It has been reported that the combined use of TAES and general anesthesia is valid in enhancing the anesthetic effects [35, 36], but its mechanisms are still not clear. Liu et al. investigated the effect of TAES on general anesthesia with an EEG

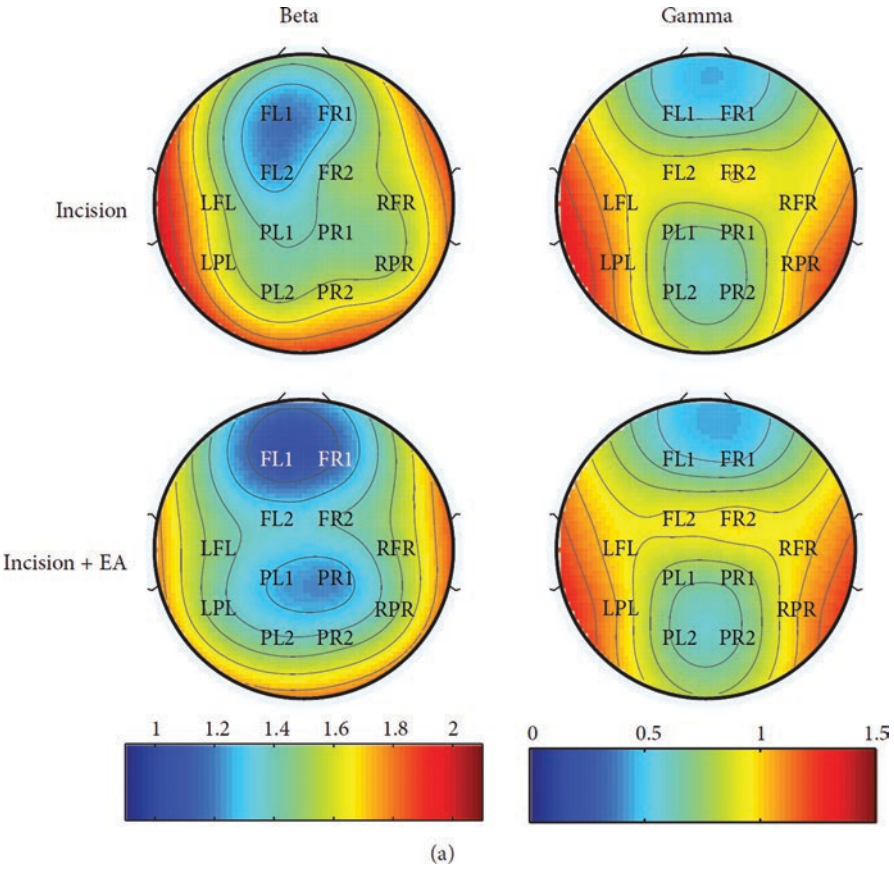

Fig. 15.5 Locations of electrodes with significant EEG power change after EA treatment in incisional pain model of rats. (**a**) Topographic mapping of EEG power at beta band (left) and at gamma band (right) after EA application compared with that before EA application. Averaged EEG power density values were color-coded and plotted at the corresponding position on the planar projection of the epidural surface and interpolated between electrodes (dots). (**b**) Electrodes with statistically significant difference ($P < 0.05$) of power change at the beta band and the gamma band

oscillation analysis on surgery patients anesthetized with propofol [37]. The results showed that after TAES application, the EEG power increased at alpha and beta bands in light sedation of propofol but reduced at delta and beta bands in deep sedation. Also, the EEG oscillation analysis showed an enhancement of synchronization at low frequencies and a decline of synchronization at high frequencies between different EEG channels in either light or deep propofol sedation.

15.2 Conclusions

Recent evidence has shown that neural oscillations carry rich information about the activities of underlying circuits. Electrophysiological studies show that pain generates a rich spatial-temporal pattern of neural oscillations, especially in gamma band and beta band. However, although most of the experiments show a strong statistical result, no single pattern of the oscillation could tell the subject in pain or not. Combing the feature collected from important nodes of the pain network, a machine learning-based classifier could perform a much better guess. With the advance of the recording technologies, we could build a much richer feature space to train the classifiers for a better result. These fundamental researches could path the way to valuable clinical practices like objective pain-scoring and novel pain-controlling methods.

Conflict of Interests The authors declare no conflict of interests.

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Chapter 16
Neuroimaging Studies of Primary Dysmenorrhea

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Abstract Primary dysmenorrhea (PDM), cyclic menstrual pain in the absence of pelvic anomalies, is one of the most common gynecological disorders in reproductive females. Classified as chronic pelvic pain syndrome, PDM encompasses recurrent spontaneous painful ("on") and pain-free ("off") states and is thus a good clinical model to study state- and trait-related changes of pain in the brain. In this chapter, we summarize state-of-the-art neuroimaging studies of primary dysmenorrhea from phenotype and endophenotype to genotype facets. Structural and functional brain alterations associated with primary dysmenorrhea are discussed.

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16.1 Prevalence, Clinical Diagnosis, and Psychological Features of Primary Dysmenorrhea

16.1.1 Importance of Primary Dysmenorrhea (PDM)

Primary dysmenorrhea (PDM) is menstrual pain in the absence of organic pelvic diseases [89]. It is the most prevalent gynecological problem affecting 90% of adolescent girls and more than 50% of menstruating women worldwide, with 10–20% of them experienced severe pain [10]. PDM often results in school or work absenteeism and casts a substantial economic burden on the society [33].

PDM is clinically diagnosed based on medical history and physical examination to exclude pelvic pathology (menstrual pain with pelvic pathology is classified as secondary dysmenorrhea) [60, 100]. It typically begins at or shortly (6–24 months) after menarche. The pain usually starts 1 or, at the earliest, 2 days before the start of the menstrual period and stops 1 or, at the latest, 2 days after menstrual bleeding starts, resulting in menstrual pain that typically lasts for 8–72 h [10, 34, 89]. The current most widely accepted pathogenesis of PDM is the overproduction of uterine prostaglandins, which causes myometrial hypercontractility and uterine vasoconstriction, leads to reduced uterine blood flow, and, ultimately, results in dysmenorrheic pain [34, 60].

PDM is formally coded and classified by International Association for Study of Pain (IASP) and World Health Organization (WHO):

1. 765.X8—under XXIII-15: Primary dysmenorrhea, Group XXIII: Chronic pelvic pain syndromes—"Gynecological System: Internal Pelvic Pain Syndromes" and Section F: "Visceral and Other Syndromes of the Trunk Apart from Spinal and Radicular Pain" in current IASP classification system of chronic pain by IASP
2. N94.4—under N94: "Pain and other conditions associated with female genital organs and menstrual cycle"—N80-N98: "Noninflammatory disorders of female genital tract"—XIV: "Diseases of the genitourinary system" in the current 10th version of International Statistical Classification of Diseases and Related Problems (ICD-10) by WHO
3. MG30.00: Chronic primary visceral pain and GA34.3: Dysmenorrhea under "Female pelvic pain associated with genital organs or menstrual cycle" in the current ICD-11 by WHO as a new diagnostic entity which is formerly neglected in etiologically defined categories [126]

PDM has been considered a genuine model of chronic pain for studying chronic recurrent pain because of its natural "on" (painful) and "off" (pain-free) states. PDM patients (PDMs) suffer from cyclic menstrual pain lasting for days during

each menstrual cycle for years [100]. Notably, dysmenorrhea often co-occurs with chronic pain conditions later in life, including irritable bowel syndrome, painful bladder syndrome, fibromyalgia, chronic headache, chronic low back pain, etc. [10] (further discussed in Sect. 16.3.1).

16.1.2 Quality of Life and Mood in PDM

Dysmenorrhea is associated with decreased self-rated overall health [7] and negative moods [39]. We recently reported that Taiwanese PDMs exhibited significantly lower physical and mental quality of life and higher levels of anxiety, depression, and pain catastrophizing than those of females without PDM [70]. Women with high level of pain catastrophizing may develop negative cognitive appraisal style, which may gradually lead to negative effects such as depression and anxiety and negative pain schema such as pain helplessness [101]. Such alterations sculptured by severe and long-term menstrual pain may ultimately influence women's long-term health through pain chronification as other chronic diseases do.

16.1.3 Pain Sensitivity in PDM

Previous studies reported that Caucasian women with PDM might demonstrate increased pain sensitivity or central sensitization to experimental somatic [3, 5, 48, 51] and visceral stimuli [13] compared with those without PDM. However, no local or generalized hypersensitivity of superficial thermal pain was found in Taiwanese PDMs [70], indicating that different genetic constitutions (caused by ethnicity) may contribute to the diversity of the clinical manifestations of PDM.

16.2 Structural Brain Alterations in PDM

T1-weighted magnetic resonance imaging (MRI) provides a high-resolution contrast of soft tissues, such as gray matter and white matter in the brain. Structural brain alterations are often studied by measuring gray matter volume, white matter volume, cortical thickness, white matter microstructure, and fiber tract organization. Previous studies on chronic pain have reported reduced gray matter volume, reduced cortical thickness, and altered white matter microstructure in pain processing regions such as the cingulate cortex, insula, and thalamus [21, 117, 123]. Comparing with neuroimaging evidence from other chronic pain conditions, there are relatively few studies that demonstrate structural brain alterations in PDM. In this session, we introduce current findings of gray matter alterations [81, 129, 131] and white matter alterations [79, 80] in PDM.

When studying brain alterations, participants with brain anomalies or abnormalities, which might stem from abnormal development, injury, or disease, should be excluded from neuroimaging studies. It is noteworthy that there is a higher prevalence of incidental brain anatomy findings (normal variants) in PDMs, especially cavum septum pellucidum [73]. However, it is unlikely to link these incidental findings to developmental factors since, from organogenesis perspective, uterus and brain stem from different embryological origins [108]. Nevertheless, incidental findings in PDMs should be considered when interpreting future brain findings of PDM.

16.2.1 Gray Matter Alterations in PDM

A widely used technique for gray matter volume investigation is voxel-based morphometry (VBM). VBM is an automatic, unbiased, semiquantitative analysis technique and has become a standard whole-brain exploratory analysis procedure [49]. However, it measures cortical tissues collectively, which did not differentiate among other features, such as cortical thickness, cortical surface area, and cortical folding, for a better discriminative capability of anatomical boundaries [147].

Previous PDM studies using VBM analysis demonstrated that recurrent menstrual pain is associated not only with trait-related [129] but also state-related structural brain alterations [131]. Short-lasting cyclic menstrual pain changes the gray matter volumes in brain regions related to pain information processing and modulation (including somatosensory cortex, insula, and cingulate cortex), as well as in regions that regulate estrogen level (hypothalamus). PDMs showed decreased gray matter volume (hypotrophic changes) in the secondary somatosensory cortex (SII)/posterior insula, mid-anterior insula, and medial prefrontal cortex (mPFC). The SII/posterior insula and mid-anterior insula receive sensory input from visceral organs and play key roles in integrating sensory information with pain affect [31, 36]. The posterior insula responses to viscerosensory afferents and is sensitive to physiological stress [107]. The mPFC exerts massive visceromotor control outputs and is largely involved in subjective affective experience [63]. These decreases of gray matter volume might be the consequence of compensatory inhibitory responses toward excessive cyclic viscerosensory input of menstrual pain [104, 129, 131].

In contrast, PDMs showed increased gray matter volume (hypertrophic changes) in the hypothalamus, hippocampus, periaqueductal gray (PAG), and anterior cingulate cortex (ACC). The hypothalamus is involved in menstrual pain through several potential pathways [129], including the hypothalamic–pituitary–gonadal (HPG) axis, the hypothalamic–pituitary–adrenal (HPA) axis, and the spino-bulbo-spinal loop. First, the hypothalamus responds to elevated estrogen levels through the HPG axis [146]. Estrogen is essential in regulating the synthesis of uterine prostaglandins in both qualitative and quantitative ways [53]. Periodically increased estrogen levels result in elevated levels of prostaglandins, which together contribute to the overproduction of prostaglandins, causing menstrual pain in PDMs [33, 60].

Second, hypothalamus and hippocampus are key regions in the HPA stress regulation system. PDM is thought to be intensified by heightened emotional stress [138] that is generated by abnormal hippocampal feedback to the hypothalamus [61, 90, 133]. Third, dysfunction of the spino-bulbo-spinal loop and its cortical feedback (including the hypothalamus, PAG, amygdala, hippocampus, ACC) may lead to enhanced negative mood and emotions associated with pain perception [122]. In short, gray matter volume increases in PDMs may underpin reactive pain modulation and the regulation of endocrine function.

On the other hand, using cortical thickness measurement, Liu et al. [81] found that PDMs had increased global mean cortical thickness and regional cortical thickness compared to healthy female controls. These cortical regions included the orbitofrontal cortex, superior temporal cortex, precuneus, posterior cingulate cortex, primary and secondary somatosensory cortex, insula, and parahippocampus. Their findings were similar to previous VBM studies reported [129, 131]. All these findings demonstrate that protracted nociceptive input could result in a combination of decreased pain inhibition or increased pain facilitation in PDMs.

16.2.2 White Matter Microstructural Alterations in PDM

Diffusion-weighted MRI has encouraged the analysis for microstructural properties of white matter. Diffusion tensor imaging (DTI) is a promising technique to noninvasively quantify the white matter tract organization and microstructural feature in human brains [8]. Fractional anisotropy, mean diffusivity, radial diffusivity, and axial diffusivity are common DTI measurements [80]. Fractional anisotropy estimates the directional preference of molecular diffusion of water and reflects white matter integrity. Mean diffusivity estimates molecular diffusion rate and may reflect the inflammatory swelling. Radial diffusivity estimates the diffusion rate in the transverse direction and axial diffusivity estimates the diffusion rate along the main tract; they may reflect changes in membrane permeability and myelination [1, 119]. These directional estimates endow us with the possibilities of performing diffusion tractography to trace the pathways of underlying fiber bundles.

Contradictory findings of increased or decreased fractional anisotropy in PDM have been reported, while consistent findings of mean diffusivity and radial diffusivity are reported by Liu et al. [80] and Dun et al. [40]. Liu et al. [79] focused on white matter microarchitecture alteration in the cingulum bundle using tract-based analysis method and found decreased FA in the dorsal-posterior and parahippocampal section. Posterior cingulum cortex has a pivotal role in the default mode network (DMN) [14]. Several studies reported abnormal morphology or function of DMN in PDMs [129, 139, 142], which may suggest maladaptive neuroplasticity of the endogenous pain control systems.

Tract-based spatial statistics (TBSS) [118] is a skeleton projection algorithm that could mainly detect the deficits in white matter tract. Previous studies using TBSS demonstrated white matter microstructural abnormalities in PDMs [40, 80], including

alterations in the corpus callosum, longitudinal fasciculus, corona radiate, internal capsule, fornix, thalamic radiation, and external capsule.

Corpus callosum, which is the brain's core commissural white matter bundle, plays an essential role in the interhemispheric communication [113]. Abnormal interhemispheric transfer in the corpus callosum may result in augmented pain perception. White matter microstructural anomalies in the corpus callosum may disrupt structural connectivity and may affect PDMs in integrating pain modulation and sensory information [80].

Longitudinal fasciculus connects the anterior to posterior cerebral parts, including the frontal, parietal, and occipital cortices [128]. Alterations in the superior longitudinal fasciculus have been reported in pain-related disorders [37, 75] and PDM [80], implicating disrupted functions in the affective and cognitive processing.

Internal capsule locates at the inferior medial part of each hemisphere. It consists of ascending and descending fiber tracts that convey information between cortical and subcortical regions and the spinal column. External capsule contains cortico-cortical association fibers and conveys information pertaining to the emotional component of pain perception [75]. White matter microstructure alterations in the internal and external capsules might implicate abnormal sensation and pain arising from the uterine, which could further cause abnormal hypometabolism in the sensorimotor and emotional regions [130].

Corona radiata is the most prominent projection fibers of the cerebral cortex. It is a white matter sheet containing both ascending and descending axons. Anterior corona radiata passes through the limbic-thalamo-cortical circuitry and is implicated in top-down emotional regulation [109]. Tu et al. [130] suggested that the disinhibition of thalamo-orbitofrontal-prefrontal network in PDM might increase negative emotion and thus might result in pain generation and heightened pain sensitivity.

In summary, both gray and white matter abnormalities in primary dysmenorrhea implicate that PDM manifests characteristics common to chronic pain that may pose impacts on functional neuroplasticity.

16.3 Functional Brain Alterations in PDM

16.3.1 Resting-State fMRI Studies in PDM

Among all the neuroimaging techniques, resting-state functional magnetic resonance imaging (rs-fMRI) is widely used to study brain function in the absence of explicit input or output [12, 44]. The rs-fMRI measures blood oxygen level-dependent (BOLD) signals during rest and noninvasively reveals the manifestation of spontaneous neuronal activity, allowing us to study the intrinsic functional connectivity of the brain [44]. In chronic pain patients, such task-free brain activity reflects a combination of spontaneous thought processes and ongoing neural and

physiological maintenance processes involved in ongoing pain [32, 64]. Variability in brain activity can provide insights into brain health, pain sensitivity, and the capacity for brain plasticity [105].

To further unravel functional brain networks, resting-state functional connectivity (FC) has been developed by measuring the temporal correlation of low-frequency spontaneous fluctuations in the BOLD signals between different brain regions [12]. Resting-state FC may reflect brain state of readiness to engineer an instant mind operation [46], system memory out of intensive short-term training [72], and sustained long-term learning and plasticity [76, 124]. Functional connectivity studies have also revealed abnormal brain networks (brain states) in various chronic pain conditions, shedding light on the pathophysiological mechanisms underlying different facets of pain chronification, as well as the neural bases for neurocognitive conditions (mind states) of chronic pain [6, 27, 58, 65, 85, 94].

Based on the established functions of particular brain regions (regions of interest; ROIs), seed-based functional connectivity is developed to calculate the temporal correlations between neural signals in an ROI (the "seed") and those in all other regions [44]. The descending pain modulation system (DPMS) consists of neural substrates and pathways from the cerebral cortex to the spinal cord and modulates different dimensions of pain, including sensation, cognition, and emotions [17]. Periaqueductal gray (PAG) is one of the most critical neural substrates of the DPMS, functioning as its critical hub [20]. Based on the established engagement of PAG in the overall brain alterations associated with PDM [129], we focused on the PAG and employed a seed-based resting-state FC approach through fMRI to address the functional dynamics of the PAG-seeded FCs in the DPMS.

The neural networks between the PAG and pain-related brain regions have been revealed in studies of brain anatomy [20], resting-state fMRI [62], and diffusion tensor imaging [77]. PAG connects directly and indirectly with rostral ventromedial medulla (RVM) to act on pain facilitation (ON cells) and pain inhibition (OFF cells) in pain downstream transmission [95]. Homeostatic regulation shifts dynamically between pain facilitation and inhibition, resulting in either augmented or diminished central sensitization of pain [57] and leads to inappropriate inhibition or facilitation of ascending pain signals [35]. This feature is shared among many chronic pain disorders [11, 15, 114, 120]. On the other hand, DPMS might be pre-injured and confer a vulnerability toward chronic pain [35].

We recently reported that PDMs demonstrated maladaptive functional hypo-connectivity (hypo-FC) between PAG and many critical regions of the DPMS but adaptive/reactive functional hyper-connectivity (hyper-FC) between PAG and the sensorimotor cortex [139]. Furthermore, the PAG-FCs demonstrated predictive values for the overall quality of life in PDMs. The higher the correlation strength of the PAG-sensorimotor FC, PAG-ventrolateral prefrontal cortex FC, and PAG-posterior parietal cortex (PPC) FC, the lower the physical well-being. Maladaptive FCs in the DPMS, including FCs of the PAG-mPFC and PAG-supplementary motor area (PAG-SMA), found in young PDMs may underpin the central susceptibility to the development of chronic pain disorders later in life [139]. PAG-mPFC hypo-FC is

common in functional pain disorders, while PAG-SMA FC alteration is shared by chronic pelvic pain syndromes [67].

Maladaptive PAG-seeded functional connectivity in the DPMS altered by long-term menstrual pain may be the cause that dysmenorrhea often co-occurs with many functional disorders and chronic pain conditions later in life. These co-occurrences include painful bladder syndrome [26], irritable bowel syndrome [2], fibromyalgia [48], temporomandibular joint disease, chronic fatigue syndrome, chronic headache, low back pain, and many others [10]. Notably, all these functional pain disorders have pronounced female predominance [92], and their highest prevalence rates usually occur after the age of 30 [84]. In contrast, the prevalence of PDM peaks much younger [121]; newly diagnosed PDM usually occurs before the age of 30 in Taiwanese females [96]. The high comorbidity of PDM with many chronic pain conditions later in life suggests that possible maladaptive functionality of DPMS may occur in young PDMs, predisposing them vulnerable to functional pain disorders. This point is of particular importance because pain and stress early in life can predict a reduced quality of life and severe or chronic pain later in life [10]; furthermore, early life injury (i.e., PDM) may create an imbalance in the DPMS [35].

Our findings of alterations in the PAG-seeded FC tie together previous reports of altered metabolism and gray matter structure in the DPMS and are in line with the results of our structural neuroimaging studies of PDM (see Sect. 16.2). We previously reported *trait*-related decrease in regional gray matter volume in the DMN (including mPFC and precuneus) and PPC [129] and *state*-related increase in regional gray matter volume in the primary somatosensory cortex in PDM [131]. In seed-based FC, we observed *trait*-related hypo-FCs of PAG-DMN and PAG-PPC and *state*-related hyper-FC of PAG-sensorimotor FC in PDM. Therefore, structural changes may be coupled with alterations in the resting network organization in corresponding brain regions.

16.3.2 Resting-State MEG Studies in PDM

Magnetoencephalography (MEG) is a noninvasive neurophysiological technique that uses highly sensitive sensor arrays to directly capture tiny changes in the magnetic fields produced by small changes in the brain's electrical activity [54, 84]. MEG possesses superior temporal resolution and good spatial resolution [54], which complements the coarse temporal resolution of fMRI and coarse spatial resolution of electroencephalography (EEG) [4]. Thus, MEG is an excellent tool to study the regularity and irregularity of brain signals, such as brain rhythms and brain dynamics, respectively. In addition, various source estimation methods can be applied to investigate neural sources in the brain, such as dipole fitting, minimum current estimation, and beamformer techniques. Evaluating different facets of central changes in PDMs using various signal analysis techniques helps us to advance our understanding of chronic pain and further provides clinically useful information and improves diagnostic utility of chronic pain.

16.3.2.1 Resting-State Brain Rhythms Alterations in PDM

Neural synchronization is temporally precise interactions between neural assemblies, telling us how regular neuronal populations synchronize rhythmically at distinct frequency bands each with specific functions [87]. To further understand pain-related rhythms in PDM, neural oscillations indexes such as phase synchronization [42, 111] and cross-frequency coupling [19, 59] are considered.

The phase of neural oscillations at a given frequency band reflects a network's excitability due to cyclic fluctuations and is affected by the precise discharge time of neurons [42], offering insights to link putative neural mechanisms to sensory perception [16, 132]. Phase synchronization is considered to promote neural communication and neural plasticity [42].

The analysis of brain rhythms and oscillations has been applied to localize pain-related activity in narrow or wide frequency bands during resting state [25, 66, 71, 93]. Spontaneous low (delta, theta, and alpha) and high (beta and gamma) frequency oscillations have been associated with the sensory, affective, and evaluative representations of pain processing [97–99]. Recently, we used a beamformer method [23] to localize theta activity during painful and pain-free states in PDMs [71]. We observed elevated theta activity in regions related to sensory and emotional processing, which might be related to thalamocortical dysrhythmia in chronic pain [110, 112, 136]. Our findings suggested the role of theta oscillation in the encoding of complicated processes related to perception and context of menstrual pain experience [71].

On the other hand, cross-frequency coupling is calculated as the statistical relationship between oscillatory activities across two different frequency bands. Phase-amplitude coupling (PAC), in particular, may be the most frequently reported cross-frequency coupling in pain studies. PAC reflects the interaction between phase and amplitude, where the envelope of the faster oscillations is modulated by the phase of the slower oscillations [19, 22, 78].

Although currently there are no studies of cross-frequency coupling in PDM, Liu et al. [78] demonstrated that during painful laser stimulations, increased gamma local field potentials in the right amygdala and hippocampal significantly coupled with the phases of theta and alpha oscillations. Hippocampus and amygdala, two significant elements in the limbic structure, are fundamental in the generation of emotion and emotional memory [69, 106]. Thus, gamma low-frequency coupling is speculated to be a basic mechanism of integrating the sensory and affective dimensions of pain [78].

Overall, analyzing brain rhythms is a straightforward and objective tool to study the mechanisms involved in menstrual pain and the encoding of menstrual pain experience. Further studies utilizing quantitative analysis of oscillations and synchrony in PDM are invited.

16.3.2.2 Resting-State Brain Dynamics Alterations in PDM

The brain is a complex system encompassing both regular and irregular neural activities. The temporal irregularity and unpredictability of neural signals at multiple temporal scales can be regarded as neural complexity, which may reflect the adaptability/flexibility of the nervous system and the information processing between neurons [86, 125, 137]. Measuring nonlinear temporal variability of brain signals using entropy measures would provide a useful metric of brain dynamics [47], which would fundamentally complement the information revealed by spectral-based analysis.

Multiscale entropy (MSE) [28, 29] measures the irregularity of time-varying signals by calculating sample entropy [103] over multiple time scales. Loss of complexity is often reported in neuropsychiatric diseased and aged groups; increased complexity has been seen in healthy and recovery conditions [52, 74, 143]. Recently, MSE analysis has also been applied to pain [82, 116, 134] and PDM studies [66, 83].

We recently developed a model to predict subjective spontaneous pain level by decoding resting-state MEG signals of PDMs during painful state (menstruation) [66]. We found that brain complexities in the precuneus and posterior cingulate cortex, which are key regions of the DMN, were the most selected features in predicting subjective spontaneous pain level. In addition, we compared the brain complexity during pain-free state in PDMs and healthy female controls using resting-state MEG signals to investigate trait-related changes in neural adaptability after long-term menstrual pain [83]. PDMs demonstrated loss of complexity in pain-related sensory, affective, and evaluative regions. General loss of brain complexity in pain-related regions revealed by nonlinear dynamical analysis might suggest a loss of neural adaptability and efficiency [47] in response to chronic recurrent pain and might correspond to low-frequency alterations in chronic pain [83].

To sum up, changes in resting-state MEG activity in PDMs [66, 71, 83] may reveal abnormalities of the underlying neurophysiological mechanisms involved in the sensory, cognition, and emotion components of long-term menstrual pain experience. Brain rhythms alterations in PDMs might have resulted from alterations in regional gray matter volume or cortical thickness [81, 129, 131], white matter structural connectivity [79, 80], and functional connectivity [139, 142] within the pain modulatory system. Accordingly, these findings support the idea that there are alterations in multiple interactively connected networks that receive inputs from various parallel nociceptive pathways, including the sensorimotor network, default mode network, salience network, and limbic regions.

16.4 Interactions of Genotype Polymorphisms and Long-Term Menstrual Pain

The perception of pain and the clinical response to analgesics vary among people; genetic factors, at least partially, may explain the variability in the experience of pain [18]. Therefore, genetic testing in pain may enhance the selection, dosing, and

evaluation of medical treatment [127]. Recent evidence suggests a role of single-nucleotide polymorphism (SNP), a substitution at a specific position in the genome, in the serotoninergic, dopaminergic and catecholaminergic systems [18]. Persistent alteration in histone methylation at the promoter region of brain-derived neurotrophic factor (BDNF) has been linked to depression and could also be relevant in chronic pain [30]. In the same vein, studies have revealed a wide variety of risk alleles for chronic pain, including mu-opioid receptor (OPRM1), catechol-O-methyltransferase (COMT), serotonin receptor 2A (5HTR2A), and solute carrier family 6, member 4 (SLC6A4; serotonin transporter) [91].

Imaging genetics, using neuroimaging as endophenotypic assays to evaluate genetic variations [55], is a new strategy in brain science developed during the past decade. Genetic impact on the brain endophenotype (intermediate phenotype) can be more significant than its impact on behavioral phenotype [102]. Thus, brain imaging bridges the mechanistic gap between genotype and phenotype [35]. Furthermore, only a proportion of patients with acute pain go on to develop chronic pain; both genotype (innate mechanisms) and disease at critical developmental periods (acquired mechanisms) are crucial risk factors [35]. In PDM, maladaptive PAG-seeded FC was found in the DPMS that may eventually contribute to the comorbidity of various chronic pain disorders later in life; we also reported that such maladaptive neuromodulation of DPMS might have genetic attributions [140, 141].

16.4.1 The BDNF *Val66Met Polymorphisms Influence the Functional Connectivity Dynamics of the DPMS*

BDNF modulates the formation, maturation, and plasticity of neuronal synapses [50], including those at the spinal and supraspinal levels of pain circuitry [88]. Therefore, BDNF is crucial in central sensitization and chronic pain [68]. The *BDNF* Val66Met (rs6265) is a SNP which leads to valine (Val)-to-methionine (Met) substitution at codon 66. This functional polymorphism has been shown to reduce activity-dependent BDNF secretion [24, 41] and influences cortical pain processing [38, 135].

The *BDNF* Val66Met polymorphism is associated with diverse functional expressions of the DPMS in healthy subjects. Individuals with different *BDNF* Val66Met genotypes demonstrate different functional dynamics of the DPMS upon long-term recurrent menstrual pain [140]. The Val/Val PDMs mainly engage the ascending pain-sensory system (adaptive neuroplasticity), while the Met/Met PDMs exhibit significant PAG-limbic structure FCs, indicating a predisposition of pain chronicity (maladaptive neuroplasticity) [56].

Our reasoning is corroborated by the anatomical and physiological evidence in the PAG. BDNF-containing projecting neurons, especially those in the ventrolateral PAG, project to RVM and release BDNF that might participate in the descending pain modulation [145]. Analgesic effect has been observed by infusing BDNF into rats' PAG-dorsal raphe [45]. In addition, healthy subjects with Met alleles showed a less progressive reduction of ERP responses to experimental pain [38], whereas low

back pain patients with Met alleles showed heightened sensitivity to experimental pain [135]. This effect may occur through neural plasticity affected by neurotrophin polymorphism or through direct neurotransmitter-like effect of BDNF [38]. Moreover, we had previously described the possible genetic association of *BDNF* Val66Met polymorphism with the susceptibility to PDM [70].

16.4.2 The OPRM1 *A118G Polymorphisms Are Associated with the DPMS*

On the other hand, OPRM1 remarkably mediates the analgesic effects of opioids in the central nervous system [43]. The *OPRM1* A118G (rs1799971) is a SNP which leads to an adenine (A)-to-guanine (G) substitution at codon118 in the human *OPRM1* gene. It is associated with reduced *OPRM1* gene expression [148], heightened pain sensitivity [144], and increased analgesics consumption [115].

The *OPRM1* A118G polymorphism influences the functional connectivity dynamics in the DPMS during menstrual pain (but not during pain-free state) in PDMs, suggesting that menstrual pain experience might be an epigenetic factor that interacts with genetic polymorphism [141]. The AA homozygous PDMs exhibited an active cortical modulation, while G-carriers PDMs showed decreased functional connectivity and dysregulation in the DPMS.

In summary, genetic polymorphisms modify the functional dynamics of DPMS and may underlie altered pain processing or predict differential efficacy of analgesics. Indeed, PAG, the critical hub in the DPMS, is enriched with opioidergic neurons [43], and its ventrolateral subregion is vital for opioid-mediated analgesia [9]. The interactions between genetic polymorphism and DPMS are plausible neurological mechanisms underlying intersubject variations in pain experience and may eventually predispose PDMs to the vulnerability of chronic pain conditions.

16.5 Conclusions

In this chapter, we reviewed neuroimaging studies in females with primary dysmenorrhea. Structural brain alterations in PDM include alterations in gray matter volume, cortical thickness, and white matter microstructure. Functional brain alterations in PDM include alterations in resting-state functional connectivity, resting-state brain rhythms, and resting-state brain dynamics. Genotype polymorphisms currently reported in PDM include the *BDNF* Val66Met polymorphism and the *OPRM1* A118G polymorphism. These alterations highlight the state- and trait-related assaults of long-term menstrual pain experience on our most complex organ, the brain. We suggest the early diagnosis of PDM and appropriate treatment or pain medication to prevent pain chronification that predisposes PDMs to the development of chronic pain disorders later in life.

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Chapter 17
Brain Reward Circuit and Pain

Moe Watanabe and Minoru Narita

Abstract Pain plays an important role in alerting the body to potential tissue injury and drives behavior that protects the body from further harm. In contrast, chronic pain does not serve this function and instead only provides a persistent sensation of pain and a negative experience. The mesolimbic dopaminergic system has been recognized to play a central role in motivated behaviors, including various types of reward and pleasure. Many dopaminergic neurons may release multiple neurotransmitters, and the physiological role of the co-release of these transmitters has been revealed incrementally. However, it was not yet clear whether the mesolimbic dopaminergic system and small molecules released in the nucleus accumbens (N.Acc.), the input region of mesolimbic dopaminergic neurons, are involved in pain modulation. Recently, we revealed that the mesolimbic dopaminergic system and small molecules released in the N.Acc. could contribute to pain modulation. In this review, we provide an overview of the relationship between pain and the brain reward circuit using a combination of optogenetics, electrophysiology, and in vivo microdialysis/mass spectrometry integrated system.

Keywords Dopamine · Ventral tegmental area · Nucleus accumbens · Morphine

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17.1 Introduction

The mesolimbic dopaminergic system is an important pathway that mediates "reward" and modulates positive or negative emotion [12]. The brain reward circuit regulates motivation and incentive and thus controls which actions an individual will take to obtain a reward. Circuits that respond to pain partly overlap those associated with pleasure, including the mesolimbic dopaminergic system [22]. These observations suggest that stimuli that promote positive emotions may be able to reduce pain and that pain may reduce reward-associated behaviors (Fig. 17.1). Indeed, it has been shown that pleasant music, food, or fragrances alleviate pain [37, 39], while pain may reduce the motivation to seek natural rewards [5]. Although it seems that there are complicated mechanisms behind the balance between pain and pleasure, not enough is known about the involvement of specific pathways or molecules. This review examines the functional role of the brain reward network in pain modulation.

17.2 Intractable Pain: Neuropathic Pain and Cancer-Associated Pain

Pain leads to a diminished quality of life (QOL) for patients by producing a negative emotional state [28], which has been recognized as an important factor in evaluating the effectiveness of medical treatment [6]. However, it can be difficult to manage pain, including neuropathic and cancer-associated pain. Neuropathic pain is initiated or caused by a primary lesion or pathological changes in the peripheral or

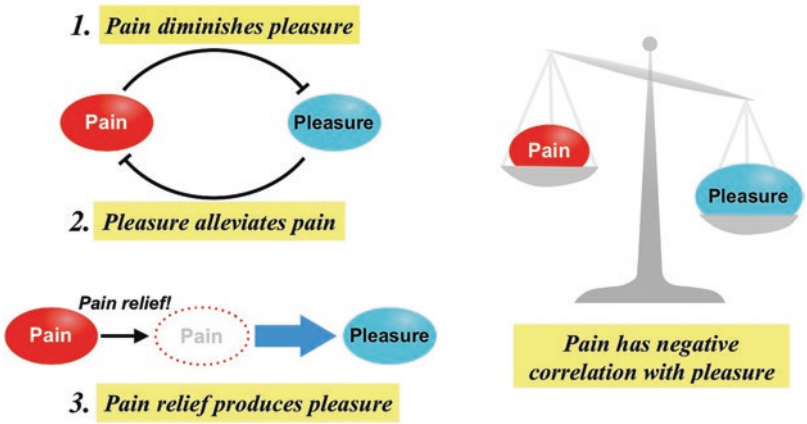

Fig. 17.1 Physiological and functional balances between pain and pleasure. These schematics illustrate the negative correlation between pain and pleasure as follows. *1*. Pain diminishes pleasure. *2*. Pleasure alleviates pain. *3*. Pain relief produces pleasure

central nervous system and induces hypersensitivity to both noxious (hyperalgesia) and non-noxious stimuli (allodynia). Patients with neuropathic pain suffer from a multitude of symptoms including difficulty sleeping, and sleep deprivation leads to depression and anxiety, which in turn make the pain worse. Since general analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids are often unable to control neuropathic pain, more effective measures for the management of neuropathic pain are required.

On the other hand, bone cancer pain, one of the most common types of pain in patients with advanced cancer, has been suggested to result from the contributions of structural, inflammatory, and neuropathic components. This pain is often found with the metastasis of tumor cells beyond the primary tumor. The metastasis of various types of cancer cells to skeletal bones can cause spontaneous pain and hyperalgesia. Tumor growth in bone directly injures the nerve fibers that innervate the bone, which induces pain [26].

17.3 Mesolimbic Dopaminergic System and Its Dysfunction Under Chronic Pain

The mesolimbic dopaminergic system originates in the A10 region of the ventral tegmental area (VTA) and mainly projects to the nucleus accumbens (N.Acc.). These neurons play key roles in positive and negative reinforcement, approach behavior, decision-making, working memory, stimulus salience, incentive salience, and the expression of positive emotions such as pleasure and happiness [1, 7, 10, 11, 20, 40, 41]. According to the electrophysiology experiments, mesolimbic dopaminergic neurons display characteristic firing patterns, tonic and phasic firing, each of which plays a specific role in regulating various types of behavior, such as learning, motivation, and decision-making. Tonic firing consists of a slow and regular firing pattern of around 5–6 Hz. In addition, this tonic dopamine release modulates the intensity of the response to phasic dopamine release by its effect on the background level of extracellular dopamine. On the other hand, phasic firing consists of a rapid series of action potentials with a short inter-spike interval (burst-firing pattern of over 30 Hz) [16]. In particular, phasic firing is observed following unpredicted or better-than-expected rewards [3, 30]. However, by contrast, some dopamine neurons in the VTA were phasically excited and other dopamine neurons were suppressed by acute noxious stimuli [14]. It was supported by the cellular heterogeneity of dopamine neurons in the VTA [31], suggesting the dopamine neuronal firing is regulated by the various types of cells in the VTA. A growing body of evidence suggests that a hypodopaminergic state is observed under chronic pain [23, 35, 38], which is consistent with increased and decreased pain in patients with Parkinson's disease and schizophrenia, respectively [8, 25, 43, 44]. Additionally, pain often accompanies other psychiatric diseases including major depressive disorder, which is characterized by anhedonia [13, 17].

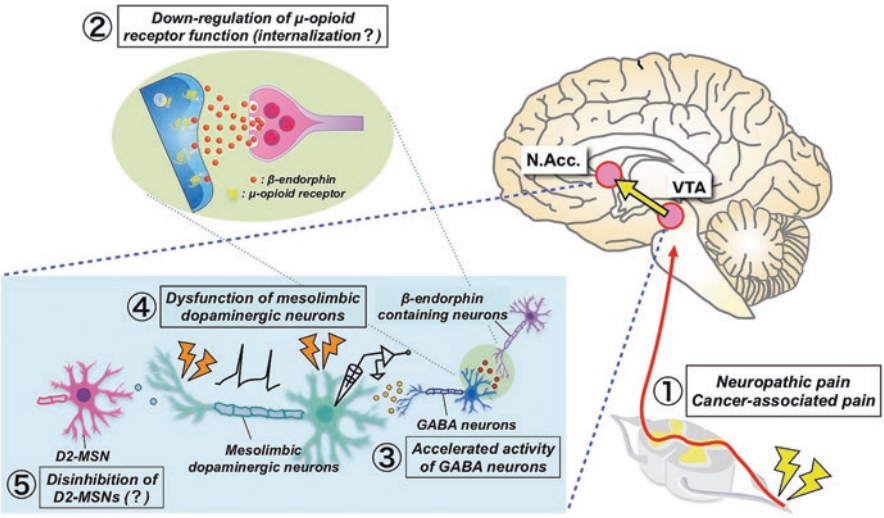

Fig. 17.2 Dysfunction of mesolimbic dopaminergic neurons caused by chronic pain. These schematics illustrate the possible mechanism of the suppression of the phasic activity of mesolimbic dopaminergic neurons under the chronic pain state

Decreased tonic dopamine levels in the N.Acc. cause a loss of inhibition of dopamine D_2 receptor signaling, which may promote hypersensitivity to pain [9, 38] as well as increased impulsivity [36]. Furthermore, the sustained release of β-endorphin in the VTA after sciatic nerve injury may lead to downregulation of VTA μ-opioid receptor function, resulting in the increased activity of GABAergic μ-opioid receptor (MOR)-expressing neurons and subsequently the inhibition of mesolimbic dopamine neurons [34] (Fig. 17.2). Indeed, we used patch-clamp electrophysiology combined with injection of retrograde fluorescent tracer to label the VTA neurons terminating in the N.Acc. A retrograde red fluorescent tracer, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI) was injected into the N.Acc. on the side contralateral to the sciatic nerve ligation (SNL) or intrafemoral bone marrow implantation of osteosarcoma cells (bone cancer). We performed patch-clamp electrophysiology to DiI-labeled VTA to N.Acc. projecting neurons in the VTA and characterize the impact of the intrinsic neuronal excitability of DiI-labeled neurons from SNL or the bone. DiI-labeled neurons in the VTA were confirmed as dopaminergic neurons by co-labeling for the marker of dopaminergic neurons, tyrosine hydroxylase (TH). As a result, in response to current injection, the intrinsic neuronal excitability of DiI-labeled dopaminergic neurons was significantly lower in the SNL and bone cancer groups than in each control groups at all levels of current tested without significant differences in other electrophysiological properties [45] (Fig. 17.2). In line with our results, other groups also showed that the firing of VTA neurons was significantly decreased, and oppositely, the firing of dopamine D_2 receptor-positive medium spiny neurons (D2-MSNs) in the N.Acc. was increased under chronic pain following spared nerve injury (SNI) [38] (Fig. 17.2).

These results were consistent with observations of decreased activity of VTA dopamine neurons and decreased activation of the N.Acc. in response to a noxious stimulus in patients with chronic pain [2, 27].

17.4 Activation of the Mesolimbic Dopaminergic System Modulates Chronic Pain

While pain and opioids can activate the mesolimbic dopaminergic system [14, 48], sustained pain or prolonged periods of drug consumption have the opposite effect [47]. Opioids remain one of the most effective pain-relieving medicines. These drugs produce reinforced behaviors and elicit a positive mood and euphoria through, in part, context-dependent phasic dopamine signals from the VTA to the N.Acc. [42] as well as the prefrontal and anterior cingulate cortices [21]. On the other hand, pain and pain relief are salient stimuli that promote motivation for protective or escape behaviors to achieve safety [15]. The relief of pain activates the VTA dopaminergic pathway and thus is rewarding [33]. In our recent study, we performed the selective activation of mesolimbic dopaminergic neurons using optogenetic methods to investigate a causal link between activity of VTA-N.Acc. dopaminergic neurons and neuropathic pain following SNL. Optogenetics is a smart technique to specifically express light-sensitive opsins in the targeted cells combined with light exposure with spatiotemporal precision, which can directly elicit electrical current across cellular membranes in response to light and control the activity of targeted cells. First, we generated transgenic mice expressing a light-activated nonselective cation channel, channelrhodopsin-2 (ChR2), in VTA- N.Acc. dopaminergic neurons using in vivo Cre-loxP approach. To specifically express the ChR2 in dopaminergic neurons, adeno-associated virus vector (AAV) carrying the ChR2 gene with the flip-excision (FLEX) switch system was injected into the VTA of TH-cre mice. We next demonstrated the functional activity of ChR2 in mesolimbic dopaminergic neurons by in vivo microdialysis study and confirmed that the levels of dialysate dopamine in the N.Acc. were significantly increased by optical stimulation of the N.Acc. Under these conditions, specific optical activation of mesolimbic dopaminergic neurons reverses evoked allodynia in mice with neuropathic pain. Moreover, activation of mesolimbic dopaminergic neurons also alleviates cancer-associated pain [45], suggesting that activation of mesolimbic dopaminergic neurons may be an effective strategy for treating at least two types of intractable pain. Interestingly, in each control groups, the activation of VTA dopaminergic neurons does not influence the threshold of physiological thermal pain. These findings are consistent with observations in human subjects that increased levels of dopamine pharmacologically do not modulate the responses to noxious stimuli in healthy volunteers [4]. Moreover, it has been reported that the cell-type-specific activation of D2-MSNs in the N.Acc. worsens the allodynia following SNI, and suppression of D2-MSNs alleviates the allodynia following SNI [38]. Thus, these VTA dopaminergic cells

and MSNs in the N.Acc. may enable selective pharmacological modulation of pathological pain, without interfering with physiological pain or abnormal consequences of reward including addiction.

17.5 Identification of Small Molecules That Modulate the Sensation of Pain

Neurotransmitters are endogenous small molecules that act as chemical messengers in synaptic transmission. They are released from presynaptic nerve terminals and used by neurons to communicate with each other and with target cells by binding to specific receptors. Several criteria are generally used to identify neurotransmitters: (i) they must be synthesized or stored in vesicles in presynaptic nerve terminals of releasing cells, (ii) they must be released from presynaptic neurons in response to membrane depolarization, (iii) they must produce specific responses at postsynaptic cells by binding to specific receptors, and (iv) there must be some mechanism for their deactivation, e.g., reuptake into presynaptic neurons or glia or metabolization by enzymes [17, 23]. Neurotransmitters can be classified as classical neurotransmitters or neuropeptides. Classical neurotransmitters include choline (acetylcholine), monoamines (dopamine, noradrenaline, serotonin, etc.), amino acids (gamma-aminobutyric acid (GABA), glutamate, etc.), and purines (adenosine, adenosine triphosphate, etc.). Neuropeptides include opioid peptides (pro-enkephalin, pro-opiomelanocortin, pro-dynorphin, etc.), hypothalamic-releasing factors (corticotrophin-releasing hormone, growth hormone-releasing hormone, somatostatin, etc.), pituitary hormones (adrenocorticotropic hormone, α-melanocyte-stimulating hormone, prolactin, etc.), neurohypophyseal peptides (oxytocin, vasopressin), neuronal and endocrine peptides (calcitonin gene-related peptide, vasoactive intestinal peptide, etc.), circulating peptides (angiotensin, bradykinin), gastrointestinal and brain peptides (cholecystokinin, neurotensin, substance P, etc.), gastrointestinal and pancreatic peptides (glucagon, etc.), and so on. Many neurons can release multiple neurotransmitters. Recently, the physiological role of the co-release of multiple transmitters has been revealed incrementally. For example, the co-release of dopamine and glutamate in dopaminergic neurons is required for some dopamine-dependent behaviors [28]. On the other hand, various small molecules, including several amino acid derivatives and peptides, play roles in neural communication as not only classical neurotransmitters but also neuromodulators [18], and their synaptic concentrations alter the excitability of specific neural circuits. Since chronic pain alters neuronal excitability in the N.Acc. [2], extracellular small molecules in the brain, including small peptides, as well as classical neurotransmitters, may play important roles in modulating the sensation of pain. However, molecules with the potential to modulate pain had not been identified. We thus applied a mass spectrometry (MS)-based metabolomics technique coupled with microdialysis to analyze extracellular metabolites released from cells in the brain and identified

small molecules that are endogenously released in the N.Acc. to modulate pain and analgesia [45]. This combined microdialysis/MS system could reveal the changes in the extracellular metabolome during the application of pain stimuli or the administration of analgesics. This approach allowed us to identify N-acetylaspartylglutamate as an endogenously released neurotransmitter in the N.Acc. and to explore its relevance to the modulation of nociception [45].

17.6 Conclusion

Although the relationship between pain and reward circuits has been contemplated for many years, it was only directly proved fairly recently [31, 32, 44]. In addition, studies have revealed that these circuits show cellular heterogeneity [30] and the role of a co-transmitter in reward- or pain-associated behavior [28]. However, further research is required to clarify the complex mechanisms that underlie modulation of the brain reward system by pain stimuli.

Since cancer patients now live longer due to advances in cancer treatment, they must now face the challenge of severe and chronic tumor-induced pain. Bone cancer pain is one of the most difficult of all chronic pains to fully control, as the efficacy of analgesics that are commonly used to treat bone cancer pain is limited by significant adverse side effects. Taken together, we conclude that targeting of the central reward network may make it possible to modulate distinct aspects of neuropathic and cancer pain at the peripheral-brain interface.

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Chapter 18
Involvement of P_2X_7 Receptors and BDNF in the Pathogenesis of Central Poststroke Pain

Yung-Hui Kuan, Hsi-Chien Shih, and Bai-Chuang Shyu

Abstract Central pain is commonly found in patients with neurological complications that are associated with central nervous system insult, such as stroke. It can result directly from central nervous system injury. Impairments in sensory discrimination can make it challenging to differentiate central neuropathic pain from other types of pain or spasticity. Central neuropathic pain may also begin months to years after the injury, further obscuring the recognition of its association with past neurologic injury. This chapter focuses on the involvement of P_2X_7 receptor and brain-derived neurotrophic factor (BDNF) in central poststroke pain (CPSP). An experimental animal model is introduced that assesses the pathogenesis of central neuropathic pain, and pharmacological approaches and neuromodulatory treatments of this difficult-to-treat pain syndrome are discussed.

Keywords P_2X_7 receptor · Brain-derived neurotropic factor · Central poststroke pain · Cytokines · Thalamic hemorrhage · Medial thalamus · Anterior cingulate cortex

18.1 Central Neuropathic Pain

The International Association for the Study of Pain defines neuropathic pain as pain that is caused by a lesion or disease of the somatosensory nervous system [1]. Neuropathic pain resulted from origins that affect the central nerves system (CNS) termed central neuropathic pain, which can result from any type of injury to the CNS. Central neuropathic pain is most commonly a sequela of stroke, multiple sclerosis, or SCI [2]. Central neuropathic pain is often found months or years after the original insult to CNS, which is very challenging to treat and may not respond

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to pharmacological agents that are routinely used for peripheral neuropathic pain. Thus, understanding the pathogenesis of different central neuropathic pain is thus a vital task for medical researchers, physicians, and specialists.

18.2 Central Poststroke Pain

Stroke is one of the top five causes of death in the United States [2, 3] and responsible for 1 in 20 deaths [2, 4]. Stroke is also one of the leading causes of disability in the United States [2, 5]. Central poststroke pain (CPSP) is a neuropathic pain syndrome that is associated with somatosensory abnormalities following stroke and the most common form of central neuropathic pain [6, 7]. Because of the difficulty managing the condition and potential under diagnosis, studies that address detailed mechanisms of CPSP are not well established, thus resulting in limited treatment options [8, 9].

Articles have reported that the lateral and medial pain pathways have been shown to rule the homeostasis of pain processing in coding the strength of CPSP [10–13]. Reports have shown that stroke patients with dysfunction in the lateral thalamus exhibited a disruption of inhibition of signaling to the medial thalamus (MT), resulting in mechanical allodynia and thermal hyperalgesia [14, 15], which can be viewed as a disinhibition disorder.

The descending pain modulation system, including the dorsolateral prefrontal cortex, rostral anterior cingulate cortex (ACC), amygdala, hippocampus, periaqueductal gray (PAG), and rostral ventromedial medulla, comprises a network that regulates nociceptive processing [16]. Human functional magnetic resonance imaging (fMRI) studies have provided important evidence that the spinothalamic tract (STT) and MT-ACC pathway might be involved in CPSP [17–20]. A recent human fMRI study dissociated differences in thalamic subregions in CPSP reporting that the VPL but not VMpo plays a crucial role in CPSP [27]. Another fMRI study indicated that the contralateral somatosensory cortex and bilateral mid-/posterior insula, anterior insula, and posterior cingulate were activated during exposure to acute pain stimulation [20], concluding that the STT and MT-ACC pathway may be critically involved in CPSP symptoms.

18.3 Animal Model of CPSP

To assist the understanding of CPSP, development and characterization of animal models that mimic CPSP is the first hurdle to discovering the underlying mechanisms of this disease and possible therapeutics. Following in this section, we introduce the development of an animal model of CPSP. We will discuss our observations with studies that utilized this model of a lateral thalamic hemorrhage in rats to generate CPSP, which we believe is useful for studying the neuropathology and

physiology of CPSP and developing potential therapies. We will also discuss our findings of studies that utilized the same animal model of CPSP focusing on the involvement of P_2X_7 receptors and brain-derived neurotrophic factor (BDNF) in poststroke inflammation and the pathophysiology of CPSP.

18.4 Preparation of Animal Model of CPSP with Targeting ATP and BDNF Receptors

Male Sprague Dawley rats (250–300 mg) from laboratory animal suppliers were utilized. All surgical procedures were performed according to previously described methods [21] with slight modifications. Experimental rats were maintained under anesthesia with 1% isoflurane during surgery. Body temperature was maintained at 36.5–37.5 °C with a homeothermic blanket system, and the animals were injected with type IV collagenase (0.125 U/0.5 μl saline) in the region of right ventral posterior medial nucleus (VPM)/ventral posterior lateral nucleus (VPL) of the thalamus (coordinates: 3.0–3.5 mm posterior, 3.0–3.4 mm lateral to bregma, 5700–6000 μm depth). Sham control animals were injected with 0.5 μl sterile saline only. An intravenous catheter was implanted in the femoral vein, tunneled subcutaneously, and fixed to the back for chronic perfusion of the P_2X_7 receptor antagonist Brilliant Blue G (BBG; 50 mg/kg), began 6 h after hemorrhagic lesion induction, once daily for the following 3 days and a total of four times. To investigate effects of BDNF in vivo, the rats received an acute microinjection of artificial cerebrospinal fluid (1 μl/min), denatured TrkB-Fc (dTrkB-Fc, 1 μg/μl/min), and TrkB-Fc (1 μg/μl/min), with a 30 gauge stainless-steel cannula in the MT (2.5 mm posterior, 1.5 mm lateral to bregma, 4.2 mm depth) during electrophysiological recording under isoflurane anesthesia. For the group of rats that received chronic microinjections under awake conditions, a 27 gauge stainless-steel cannula was implanted in the VB (2.5 mm posterior, 2.5 mm lateral to bregma, 5.2 mm depth) and conglutinated with dental resin under the same anesthetic conditions. On day 36, dTrkB-Fc, TrkB-Fc (1 μg/μl/day), or Tat cyclotraxin-B (CTX-B; 0.5 μl solution, 10 μg/μl/day; Tocris, Bristol, United Kingdom) was applied at a rate of 0.3 μl/min per injection using a 30 gauge stainless-steel cannula in the VB once daily followed by a 5-day rest period (days 36–40).

18.5 Behavioral Tests that Are Typically Performed to Evaluate CPSP Conditions

18.5.1 von Frey Test

The animals were placed on an elevated mesh platform for 30 min before testing, and filaments were gradually applied with ascending, graded force to determine the minimal force that elicited a limb withdrawal response. The threshold was defined

as the average of three minimal forces that were measured in consecutive trials, each separated by 5 min.

18.5.2 Plantar Test

The plantar test was performed by placing the rats in a transparent Plexiglas box for 30 min before testing. Radiant heat (IITC 390G Plantar Test, IITC Life Science, Woodland Hills, CA, USA) was delivered through the glass floor. Before each test, the heat-generating apparatus was calibrated to the same power level. The hindpaw was directly stimulated by the infrared light source to assess withdrawal responses. The paw-withdrawal latency in response to thermal stimulation was measured. Each rat was tested in three trials with the right and left hindpaws, respectively. The inter-trial interval was 5 min.

18.6 Electrophysiological Recordings to Observe Neuronal Activity

Electrophysiological recordings were performed 5 weeks after CPSP induction. The rats were maintained under anesthesia with 1.5% isoflurane. Multichannel probes (NeuroNexus) were used to record extracellular field potentials in the right ACC (2.5 mm anterior, 1 mm lateral to bregma) and right MT (2.2–3.5 mm posterior, 0.5–1.0 mm lateral to bregma). An Ag–AgCl reference electrode was placed in the nasal cavity. The left sciatic nerve was isolated and implanted with a cuffed electrode to deliver constant-current pulses (Model 2100, A-M Systems) for sciatic nerve stimulation (SNS). The minimal effective pulse was measured as the 1 × threshold stimulation for each experimental animal. Neuronal activity in the MT was recorded using 2-, 5-, 10-, and 20-fold increases in the threshold current. The sampling rate of the recorded analog signals was 6 kHz for the field potential data and 24 kHz for the unit data. All of the electrophysiological data were processed using a multichannel data acquisition system (Tucker-Davis Technologies).

18.7 Immunostaining and Cell Count Analysis to Evaluate Changes in Tissue Composition

After electrophysiological recording, the animals were sacrificed and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline, and the brains were removed and postfixed overnight at 4 °C. The brains were incubated with a

30% sucrose solution prior to cryosectioning (30–40 μm sections). The ACC, primary somatosensory cortex (S1), MT, and VPL/VPM cryosections were divided into three sets. One set of sections was stained with cresyl violet, and the other two sets were stained with the following primary antibodies: rabbit anti-P2X7 (1:50, ATTO-550, Alomone Labs) and mouse anti-CD11b (1:100, Serotec), followed by secondary Alexa Fluor-488 goat anti-mouse IgG (H + L) antibody (1:400, Life Technologies) and DAPI staining (1 μg/ml, Life Technologies).

After immunohistochemistry, four sections with visible lesions from the center were chosen for image analysis. Stacks of images at 2 μm increments in depth were collected using a confocal microscope (LSM780, Zeiss) with Zen software (Zeiss) and either a 20× air objective (NA 0.7) for automatic full-section scans or 40× oil objective (NA 1.3–1.4) for small-field single-cell distinguishable images. The continual disruption of tissue organization and/or the loss of staining were identified as the lesion area. The edges of the lesion were marked in individual sections, and 200 μm distances from the edges of the lesion were chosen as the distant field, the area of which (μm^2) was the region of interest (ROI), measured using ImageJ software with calibrated parameters from the image acquisition. Signals >15 μm accompanied by a DAPI signal were counted as positive signals. The cell counts were manually performed within the distant field using ImageJ 1.47 software (National Institutes of Health).

18.8 Gene Transcript Analysis: Reverse-Transcription Polymerase Chain Reaction for the Determination of Selected Cytokines and Neurotropic Factor

RNA samples from sham control and CPSP rat brain tissues from peri-lesion sites were collected and processed with designed probes that flanked rat tumor necrosis factor α (TNF-α), IL-6, IL-1β, and BDNF for reverse transcription polymerase chain reaction (RT-PCR). Crude extracts of total RNA were obtained from each experimental animal using Trizol reagent (Invitrogen). Reverse transcription was performed with 0.5 μg total RNA using designed probes and Superscript III (Invitrogen) in a 20 μl reaction mixture. Quantitative PCR amplification was performed for all of the samples in a reaction volume of 50 μl that contained 1 × standard PCR buffer and 1 U Platinum *Taq* DNA polymerase (Invitrogen). The quantitative PCR product samples were analyzed using agarose gel electrophoresis. Each experiment is consisted of TNF-α, IL-6, IL-1β, and BDNF targets and was performed in triplicate. The internal sham controls consisted of GAPDH, and negative sham controls consisted of the omission of the reverse transcriptase reaction or no cDNA template.

18.9 Digital Data Processing and Statistical Analysis

All of the electrophysiological data were transformed and processed using MATLAB (MathWorks). Prominent evoked oscillations based on the method for the current source density (CSD) analysis of evenly spaced multichannel extracellular field potentials were used to display the evoked ACC response [22]. Unit activity that was recorded from the MT was digitally filtered to obtain high-frequency spike activity in response to SNS. All of the statistical data were analyzed using unpaired Student's *t*-tests, one-way analysis of variance (ANOVA), and two-way ANOVA using SPSS software. Values of $p < 0.05$ were considered statistically significant.

18.10 CPSP Features and Molecules that may Effectively Modulate CPSP Conditions

18.10.1 CPSP Rats Exhibit Allodynia and Hyperalgesia, Which are Eliminated by P_2X_7 Receptor Antagonism and BDNF Receptor Blockade

Rats with thalamic lesions developed significant hyperalgesia to mechanical and thermal pain stimulation beginning 1 week after surgical CPSP induction, which persisted for at least 5–6 weeks compared with the control group (Fig. 18.1).

18.10.2 CPSP Rats Exhibit Elevated CD11b, P_2X_7 Receptor, Selected Cytokine, and BDNF Levels in Peri-lesion Sites

Increases in the immunoreactivity of the reactive microglia marker CD11b and P_2X_7 receptors were found largely in peri-lesion sites in CPSP rats, whereas their immunoreactivity remained at basal levels in the contralateral site (data not shown) and sham control rat brains. Significant increases in the counts of cells that were immunoreactive to both CD11b and P_2X_7 receptors were observed in selected regions of interest (ROI; Fig. 18.2).

Fig. 18.1 (continued) time points with the CPSP groups (showing contralateral hindpaw data only: $n = 25$; $^{**}p < 0.01$, $^{*}p < 0.05$, two-way ANOVA followed by Tukey post hoc test). (**e**) Changes in mechanical (left) and thermal (right) thresholds after microinjections of dTrkB-Fc, TrkB-Fc, and CTX-B in CPSP animals. $^{\#}p < 0.05$, control-TrkB-Fc group compared with CPSP-TrkB-Fc group or CPSP-CTX-B group (mixed two-way ANOVA followed by post hoc test): $^{\#}p < 0.05$, control-TrkB-Fc group compared with CPSP-dTrkB-Fc group (mixed two-way ANOVA followed by post hoc test). (Figure adapted or modified from (**a**–**c**) Kuan et al. [38] (**d**) Kuan et al. [39, 40], and (**e**) Shih et al. [41])

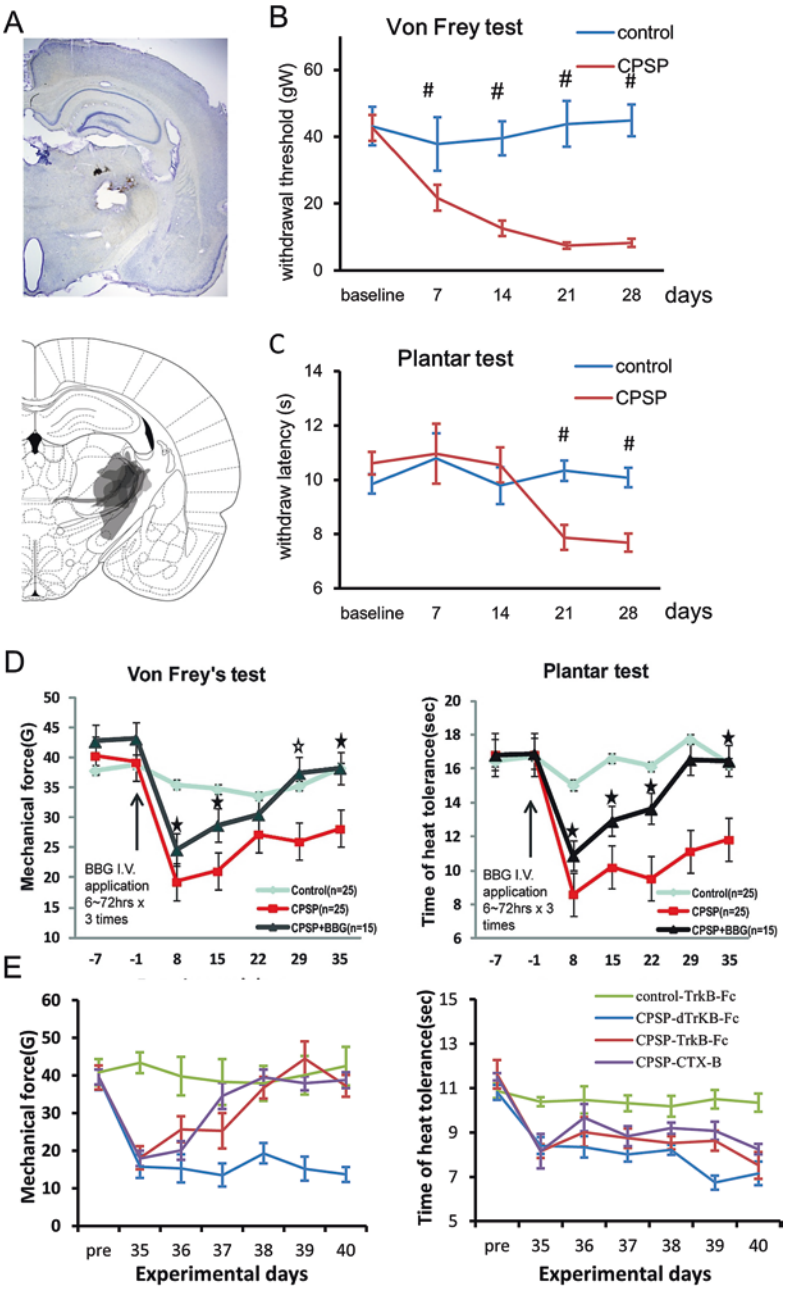

Fig. 18.1 CPSP rats exhibit allodynia and hyperalgesia, which were eliminated by P_2X_7 receptor antagonism and BDNF receptor blockade. (**a**) One month post-thalamic hemorrhage induction, a lesion scar or hollow space remained. One week after the lesion, the mechanical von Frey test (**b**) and thermal plantar test (**c**) of allodynia were performed once a week for 5 weeks. (**d**) Effects of BBG treatment on von Frey test (left) and plantar test (right) in both hindpaws compared with corresponding

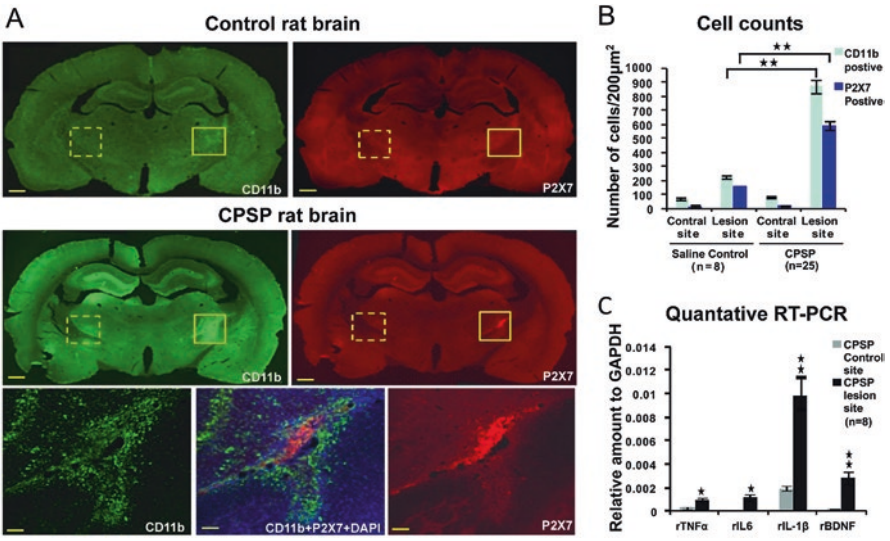

Fig. 18.2 CPSP rats exhibit elevated CD11b, P_2X_7 receptor, selected cytokines, and BDNF levels in peri-lesion sites. (**a**) Immunofluorescent analysis of CPSP rat brains. (*Upper panel*) The control rat brain had only slight elevations of the reactive microglia marker CD11b (left, *green*) and normal basal levels of P_2X_7 receptors (right, *red*). (*Middle panel*) CPSP rat brain with significantly elevated levels of the reactive microglia marker (*green*) and P_2X_7 receptor (*red*) along the lesion site around the right VPM/VPL of the thalamus. (*Lower panel*) Overlapped enhancement of CD11b and P_2X_7 receptors around the lesion site under higher magnification. Scale bar = 500 μm for upper and middle panels, 50 μm for lower panel. Solid-line square, peri-lesion site on the right. Dashed-line square, control site on the left. (**b**) Quantification of CD11b and P_2X_7-positive cells. The ROI of each brain slice was selected as the squares on the right and left sides that are shown in Fig. 18.2a. $^{**}p < 0.01$, compared with corresponding parameter in control group. (**c**) Quantitative RT-PCR results of selected cytokines (TNF-α, IL-6, IL-1β) and BDNF. The levels of these cytokines were elevated in CPSP rat brains compared with control brains ($F_{3,16} = 28.324$, $^{*}p < 0.05$, $^{**}p < 0.01$, two-way ANOVA followed by Tukey post hoc test). (Figure adapted or modified from Kuan et al. [39, 40])

18.10.3 CPSP Rats Exhibit Enhancement of the Noxious Response in the MT-ACC Pathway, Which was Suppressed by P_2X_7 Receptor Antagonism and BDNF Receptor Blockade

Thalamocingulate circuitry in the CNS is known as a medial pain processing pathway. Changes in nociceptive sensitivity in the forebrain under conditions of neurogenic pain likely result from aberrant neuronal activity along this pathway, reflected by thalamocortical dysrhythmia [23–26]. To test the hypothesis that persistent pain in CPSP rats is accompanied by changes in nociceptive sensitivity, multichannel electrodes were used to record evoked neuronal activity in the ACC and MT in response to SNS. Our study found that CPSP rats exhibited enhancement of the noxious response in the MT-ACC pathway, which was suppressed by a P_2X_7 receptor antagonist and BDNF receptor blocker (Fig. 18.3).

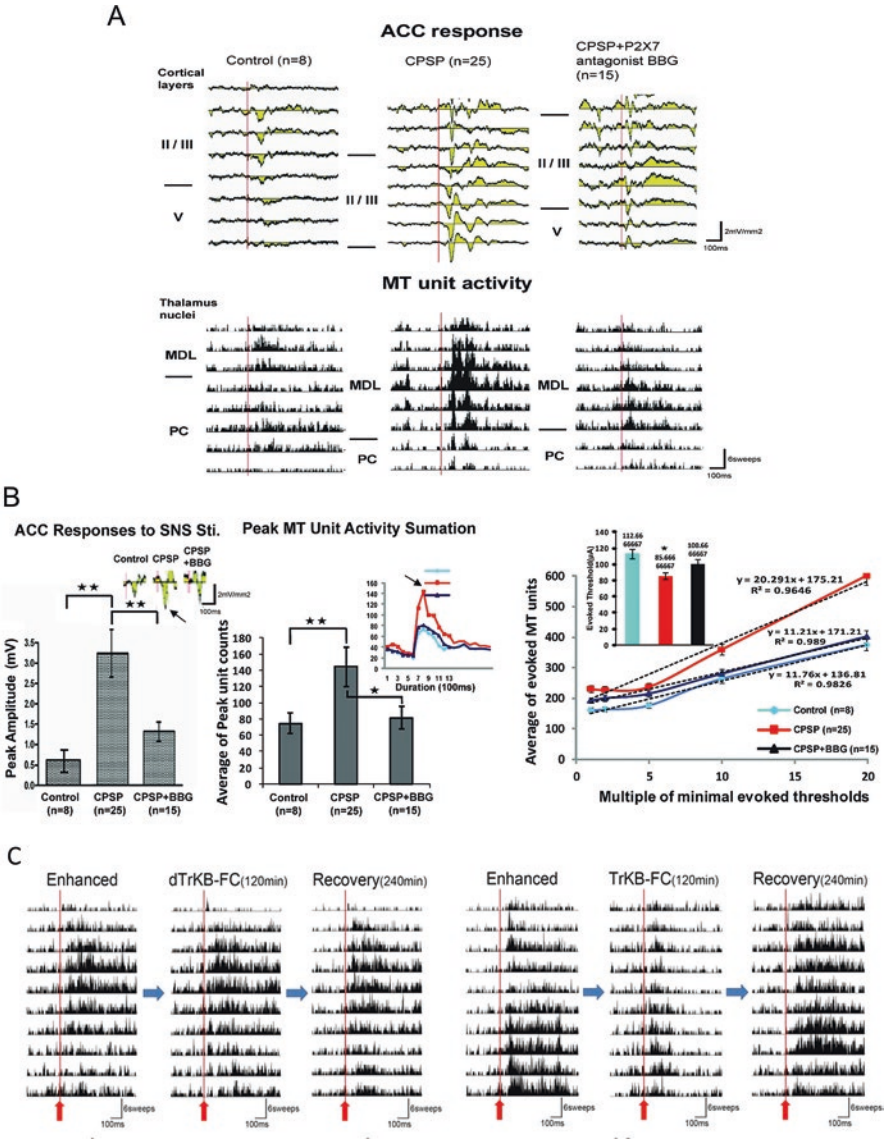

Fig. 18.3 CPSP rats exhibit enhancement of the noxious response in the MT-ACC pathway, which was suppressed by P_2X_7 receptor antagonism and BDNF receptor blockade. (**a**) Representative traces of evoked ACC CSD and MT unit activity following SNS. CPSP rats exhibited a stronger response to SNS. BBG-treated CPSP rats had a lower CSD and lower MT unit activity compared with the previously tested CPSP rats. (**b**) Quantification of ACC and MT neuronal activity in control rats and CPSP rats with and without BBG treatment. BBG-treated CPSP rats exhibited a significantly lower CSD peak amplitude (inset, arrow) of the ACC sink current and integrated MT unit activity (inset, arrow) compared with CPSP rats. One-way ANOVA followed by Tukey *post hoc* test: $*p < 0.05$, $**p < 0.01$, $F_{2,38} = 38.221$ (left panel), $F_{2,38} = 29.245$ (right panel). (**c**) Influence of BDNF and BDNF scavenger TrkB-Fc injection in the MT. Noxious responses were recorded in CPSP animals. The wind-up noxious response was not influenced by dTrkB-Fc. One to 2 h after the TrkB-Fc injection, the enhanced noxious response in the MT decreased and recovered after 180–240 min. (Figure adapted or modified from (**a**, **b**) Kuan et al. [39, 40] and (**c**) Shih et al. [41])

18.10.4 CPSP Rats Exhibit Enhancement of Spontaneous MT Activity, Which was Suppressed by BDNF Receptor Blockade

In our studies, we also observed significant changes in spontaneous electroencephalographic activity in CPSP rats, which strongly indicated the manifestation of persistent pain-related behavior. BDNF levels were not reduced by treatment with the P_2X_7 receptor antagonist BBG. This indicates the involvement of a separate BDNF-mediated mechanism in CPSP. To examine the influence of BDNF after CPSP, the BDNF scavenger TrkB-Fc was infused into the MT. Denatured TrkB-Fc (dTrkB-Fc) was infused in the control group. The evoked response in the MT was not significantly altered by dTrkB-Fc in control rats. TrkB-Fc infusion for 60–120 min attenuated the MT evoked response, which then recovered after 240 min. Both the fast component and late component significantly decreased after TrkB-Fc infusion (Fig. 18.4).

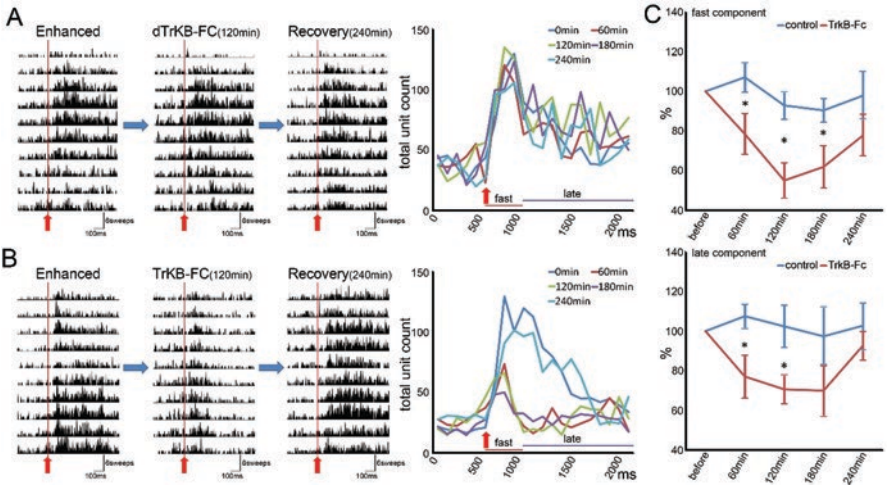

Fig. 18.4 CPSP rats exhibit enhancement of spontaneous medial thalamus activity, which was suppressed BDNF receptor blockade. (**a**) The enhanced MT nociceptive response was not influenced by the injection of denatured TrkB-Fc at any time period of the 240-min recording. Summed numbers of MT multiunit response before and after the injection at different time periods were not significantly different. The stimulation time was at 600 ms (red arrow). The overall recording period was 2100 ms. The summed time bin was 100 ms. (**b**) Sixty minutes (at 120 min) after the TrkB-Fc injection, the enhanced MT nociceptive response on CPSP decreased, and the enhanced nociceptive response recovered after 240 min. (**c**) Effect of denatured TrkB-Fc or TrkB-Fc treatment on the fast component and late component of evoked MT nociceptive responses. Four hours after the injection, the fast component (between 90.4 ± 5.8% and 107.1 ± 7.1%) and late component (between 97.4 ± 10.4% and 107.6 ± 5.8%) were not influenced by denatured TrkB-Fc. The fast component (5.0 ± 8.6% at 120 min, 61.9 ± 10.4% at 180 min) and late component (between 70.7 ± 7.0% at 120 min) significantly decreased after the TrkB-Fc injection. Fast component: $F_{1,10} = 6.69$, $^*p < 0.05$ at 60, 120, and 180 min (mixed two-way ANOVA followed by post hoc test). Late component: $F_{1,10} = 4.62$, $^*p < 0.05$ at 60 and 120 min (mixed two-way ANOVA followed by post hoc test). (Figure adapted or modified from Shih et al. [41])

18.10.5 P2X7 Antagonism and BDNF Receptor Blockade Prevented or Reversed the High Coherence Coefficients of MT-ACC Spontaneous Local Field Potentials in CPSP Rats

Persistent pain states under conditions of neurogenic pain may result from "thalamocortical dysrhythmia" [26]. Evaluating the effects of BBG and TrkB-Fc treatments on alterations of spontaneous cortical EEG oscillations helps to study such phenomena. Local field potentials in the ACC were evaluated in control rats, CPSP rats, and CPSP rats that were either treated with the P_2X_7 receptor antagonist BBG or received an intra-MT injection of the BDNF receptor blocker TrkB-Fc (Fig. 18.5).

18.10.6 Involvement of P_2X_7 and BDNF in Different Series of Sequential Signaling Effects on Microglia and Neuronal Activity in CPSP Rats

Combined our studies and other reports of the post-tissue damage signaling response, we postulated the net involvement of P_2X_7 receptors and BDNF, a schematic diagram of cellular mechanisms that are hypothesized to underlie the pathophysiology of CPSP is shown in Fig. 18.6.

18.11 Conclusion

This chapter described a particularly common type of central neuropathic pain, CPSP, and discussed the effects of treatment with a P_2X_7 receptor antagonist and BDNF receptor blocker on nociceptive behaviors and aberrant neuronal activity in the thalamocingulate pathway. Notably, the early targeting of P_2X_7 receptors could act as an immunosuppressant that inhibited inflammatory damage to brain tissue and prevented the occurrence of CPSP as the application of P_2X_7 receptors antagonist reduced the CPSP elevation of inflammatory cytokines. The alterations of thalamic neuronal excitability in CPSP could be rescued by treatment with a BDNF receptor blocker. Both P_2X_7 receptor antagonism and BDNF receptor blockade altered abnormal spontaneous EEG activity and coherence, which are strongly indicative of thalamocortical dysthymia.

A rat model of CPSP has been successfully applied in several studies and shown to be useful for elucidating the underlying causes of CPSP that are associated with lateral thalamic lesions. CPSP rats exhibited persistent sensitized behaviors with lower thresholds and greater neuronal responses to noxious stimulation and alterations of spontaneous EEG patterns within various frequency bands. This model consists of brain tissue damage, and its wound progression causes innate inflammatory

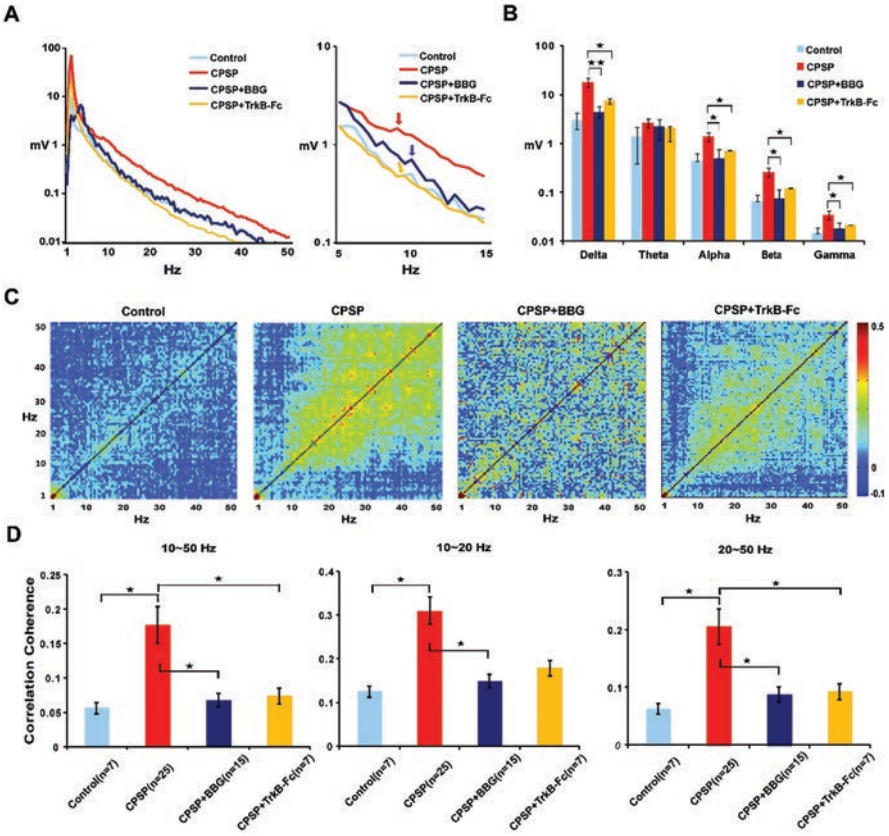

Fig. 18.5 P_2X_7 receptor antagonism and BDNF receptor blockade prevented or reversed high coherence coefficients of MT-ACC spontaneous local field potentials in CPSP rats. (**a**) Averaged power density of local field potentials (LFPs) shown in left panel in control group (light blue line, $n = 7$), CPSP group (red line, $n = 25$), CPSP+BBG group (dark blue line, $n = 15$), and CPSP+TrkB-Fc group (yellow line, $n = 7$). Averaged LFP spectrum peaks in CPSP+BBG group and CPSP+TrkB-Fc group revealed a shift that was similar to the frequency spectrum in the control group. (**b**) The enhanced spontaneous LFP oscillation in the CPSP+BBG group and CPSP+TrkB-Fc group was shifted similarly to the control group in the alpha, beta, and gamma bands ($F_{3,27} = 11.16$, 9.20, 7.56, 8.29, 9.24 for delta, theta, alpha, beta, and gamma bands, respectively, $^*p < 0.05$, $^{**}p < 0.01$, one-way ANOVA followed by Tukey post hoc test). (**c**) Color maps of averaged correlation coherence for spontaneous LFP oscillation between 10 and 50 Hz differed in the CPSP group. BBG and TrkB-Fc treatments shifted the LPF correlation coherence similarly to control animals (control, $n = 7$; CPSP, $n = 25$; CPSP+BBG, $n = 15$; CPSP+TrkB-Fc, $n = 7$). (**d**) Comparisons of averaged LFP correlation coherence values between 10 and 50 Hz, 10 and 20 Hz, and 20 and 50 Hz. (*Left panel*) Mean value of correlation coefficient between 10 and 50 Hz in the control group (0.056 ± 0.015, $n = 7$), CPSP group (0.177 ± 0.021, $n = 25$), CPSP+BBG group (0.067 ± 0.019, $n = 15$), and CPSP+TrkB-Fc group (0.074 ± 0.020, $n = 7$). (*Middle panel*) Mean value of correlation coefficient between 10 and 20 Hz in the control group ($r = 0.125 \pm 0.024$, $n = 7$), CPSP group ($r = 0.31 \pm 0.046$, $n = 25$), CPSP+BBG group ($r = 0.149 \pm 0.036$, $n = 15$), and CPSP+TrkB-Fc group ($r = 0.135 \pm 0.032$, $n = 7$). (*Right panel*) Mean value of correlation coefficient between 20 and 50 Hz in the control group ($r = 0.073 \pm 0.015$, $n = 7$), CPSP group ($r = 0.206 \pm 0.03$, $n = 25$), CPSP+BBG group ($r = 0.088 \pm 0.023$, $n = 15$), and CPSP+TrkB-Fc group ($r = 0.093 \pm 0.023$, $n = 7$). Different band ranges were analyzed separately by one-way ANOVA ($^*p < 0.05$). (Figure adapted or modified from Kuan et al. [39, 40] and Shih et al. [41])

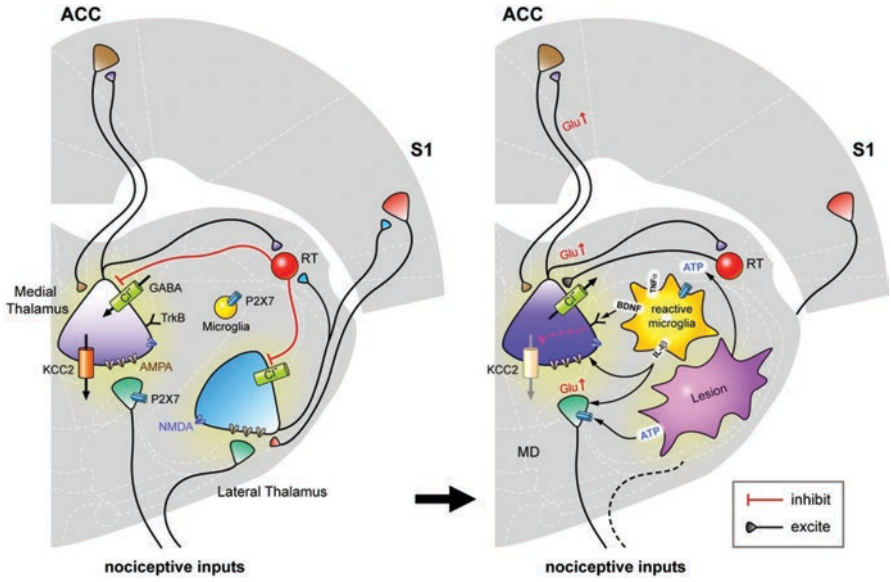

Fig. 18.6 Schematic illustration of P_2X_7 receptor and BDNF involvement in different series of sequential signaling effects on microglia and neuronal activity in CPSP. Combining our data, we postulate that, in a normal physiological state, P_2X_7 receptors are found on glutamatergic nerve terminals and microglial cells in the central nervous system. Glutamate that is released by neuron bursting is partially removed by transporters on adjacent astrocyte processes and also excites AMPA receptors on these processes, which then release ATP. The remaining ATP acts on presynaptic P_2X_7 receptors to facilitate glutamate release in the absence of neuropathic/neuroinflammatory insult. The positive feedback of terminal glutamate release that triggers astrocyte ATP release and leads to further glutamate release through the activation of P_2X_7 receptors is then sufficient to allow normal action potentials to elicit postsynaptic action potentials. Basal BDNF levels do not alter ionic flow through KCC2 channels and GABA receptors via TrkB receptor modulation. In a pathological state, such as CPSP, these traumatized cells at the lateral thalamic lesion site release a high amount of intracellular ATP, and a higher amount of BDNF and IL-1β is secreted by reactive microglia into the surrounding tissues, including synaptic clefts. Subsequently, ATP and IL-1β enhance glutamate release, resulting in a higher frequency of neuron bursting along the thalamocingulate pathway. Elevated BDNF levels result in overbinding to TrkB receptors, thus inducing the downstream inhibition of KCC2 and inversion of the flow of chloride ions through the GABA receptor, which then contributes to the disinhibition of lateral thalamus inhibitory inputs to the medial thalamus. This effect also results in a higher frequency of neuron bursting along the thalamocingulate pathway. After the onset of CPSP, P_2X_7 receptor antagonism can block presynaptic P_2X_7 receptor activation, and BDNF blockade may reduce the disinhibition, resulting in the efficient suppression of the hyperexcitability of MT and ACC neurons in response to nociceptive stimulation. The early targeting of P_2X_7 receptors upon the occurrence of stroke may block the overexpression of activated microglia and prevent the greater release of IL-1β, thus resulting in the prevention of CPSP. (This diagram adapted or modified from Kuan et al. [39, 40] and Shih et al. [41])

responses that may reflect the initiation of persistent pain. Electrophysiological assessment along the thalamocingulate nociceptive pathway provides solid neurological evidence that MT neuron hyperexcitability and greater cingulate responses may underlie the hyperalgesia and reduction of exploratory movements that are observed in nociceptive pain-related behavior. Abnormal thalamic bursting activity was observed in patients who suffered from central pain, and an imbalance of the lateral and medial thalamic interaction was proposed [27, 28]. The present chapter reports profound changes in the threshold and sensitivity of thalamic and cortical responses and spontaneous cortical EEG, strongly supporting the hypothesis that the deafferentation that results from lateral thalamic lesions may alter medial thalamic neuronal excitability [28, 29]. The alterations of spontaneous EEG patterns in conditions of CPSP suggest that both cingulate cortical and thalamic neurons in the medial pain pathway may contribute to persistent pain-related behavior, manifested as alterations of locomotor activity and neurogenic central pain that results from thalamocortical dysrhythmia, as proposed by Llinás [23].

P_2X_7 receptor expression was elevated in CPSP brains at peri-lesion sites and associated with CNS immunoresponsive cells (i.e., reactive microglia). The adenosine triphosphate (ATP)-induced activation of P_2X_7 receptors led to the rapid maturation and release of IL-1β from proinflammatory microglia suggesting that activation of this ATP receptor after hemorrhage plays an important role in the progression of CPSP [30]. Combining that the P_2X_7 receptor activation have been reported to facilitate the release of glutamate by mobilizing Ca^{2+} in the terminals [31–33] and the progression of this persistent pain could be regulated by inhibition of P_2X_7 receptors, we thus postulate, such phenomena may result from the prevention of ATP activation and thus lead to a reduction of glutamatergic facilitation, hence preventing the further release of proinflammatory cytokines, particularly IL-1β. The BBG treatment approach shortly after stroke induction may prevent excessive inflammation and the resulting hyper-release of cytokines from sites of trauma. Thus, the early targeting of P_2X_7 receptors after neuronal injury may have neuronal protection effects [34]. These results support our hypothesis and lead us to conclude that the CPSP condition is linked to the activation of P_2X_7 receptors through the release of abundant intracellular ATP to the extracellular space following thalamic cell damage. The increase in intracellular ATP levels then facilitates nociceptive input signals along the thalamocingulate pathway, thus chronically enhancing innate inflammation that is caused by IL-1β secretion.

In addition to the involvement of P_2X_7 receptors in the increase in nociceptive responses in the thalamocingulate pathway, our studies also found that BDNF mediates thalamic hypernociceptive responses in CPSP. The BDNF mRNA in the MT increased after CPSP, and the MT nociceptive responses were inhibited by an acute injection of BDNF receptor blocker into the MT.

The shift of low-frequency bands and increase in the EEG correlation coefficient in neurological patients with chronic pain was reported to be caused by thalamocortical dysrhythmia oscillation [35, 36]. The abnormal cortical oscillation pattern was thought to be attributable to over-enhancement of the GABA system in the thalamus [26, 37]. Consistent with this possibility, we found that the role of the GABA system

is altered in CPSP (data not shown). Characteristics of EEG oscillation patterns and coherence coefficients in the CPSP group were highly similar to CPSP patients [23, 26, 29]. The results of TrkB-Fc treatment confirmed that an alteration of the GABAergic system could be one of the main reasons for thalamocortical dysrhythmia. The high correlation coefficient between 10 and 50 Hz in CPSP animals was completely abolished by BBG treatment indicating that P_2X_7 receptor antagonism may alter glutamatergic transmission. This brought hints of that the glutamatergic system may also play an important role in thalamocortical dysrhythmia oscillation after CPSP. The abolishment of correlation coefficient between 20 and 50 Hz was differed between the effect of $P2X_7$ receptor or BDNF, such differed effects of BBG and TrkB-Fc treatments on CPSP thalamocortical dysrhythmia indicate that the mechanisms of P_2X_7 receptors and BDNF are different in CPSP.

The animal model of CPSP that was introduced in this chapter has strong preclinical value. It opens insights into drugs that target P_2X_7 receptors and BDNF receptors that may be applied clinically as either early treatments at the initial onset of stroke or delayed treatments until the generation of CPSP in the subacute to chronic phase. For stroke patients who already suffer from CPSP, our results also indicate that targeting P_2X_7 receptors and BDNF TrkB receptors may have antinociceptive effects by suppressing or blocking neuronal hyperexcitability and reversing abnormal oscillations. The cell count results demonstrated that the early treatment of stroke patients with a P_2X_7 receptor antagonist can prevent the activation of microglial P_2X_7 receptors in peri-lesion tissue, thus reducing the release of regional inflammatory cytokines and associated neuronal damage. Our preliminary tests have shown that both site-directed and systemic infusions of the BNDF receptor blocker BBG did not have apparent toxicity in experimental animals, suggesting its potential as a clinical therapeutic candidate in acute stroke or other brain injury.

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Chapter 19
Melatonin: A New-Generation Therapy for Reducing Chronic Pain and Improving Sleep Disorder-Related Pain

Tavleen Kaur and Bai-Chuang Shyu

Abstract Melatonin is an endogenous neurohormone that is produced in most living organisms, including unicellular and multicellular organisms, plants, vertebrates, and nonvertebrate animals. In diurnal animals, endogenous melatonin functions as a neurohormone and contributes to circadian rhythms. In nocturnal animals, endogenous melatonin no longer functions as a contributor to circadian rhythms. Circadian rhythms control the timing, quantity, and quality of hormones and neurotransmitters that the body produces and eventually secretes. An imbalance of these events creates disturbances in circadian rhythm. During disturbances of circadian rhythm, the body produces hormones, chemicals, and neurotransmitters in aberrant amounts or at the wrong time of day. The human circadian system is synchronized with physiological functions and metabolism. Many studies have reported that exogenous melatonin has analgesic and neuroprotective effects in chronic pain. Considering that chronotherapy may be beneficial for the treatment of chronic pain, the present review describes the properties, possible mechanisms, and function of melatonin in chronic pain.

Keywords Melatonin · Circadian rhythms · Neuroprotection · Chronotherapy · Chronic pain · Central poststroke pain

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19.1 Introduction

Melatonin, also called *N*-acetyl-5-methoxytryptamine, is a neurohormone. The biosynthesis of melatonin in the pineal gland is regulated by the suprachiasmatic nucleus (SCN) and is synchronized to the light/dark cycle of the environment. Melatonin is known to contribute to the biological clock [34]. Circadian rhythms control the timing, quantity, and quality of hormones and neurotransmitters that the body produces and eventually secretes. When out of balance, the quantity, quality, and timing of hormone and neurotransmitter secretion are disrupted, thus causing disturbances of circadian rhythm. During such disturbances of circadian rhythm, the body produces hormones, chemicals, and neurotransmitters in aberrant amounts or at the wrong time of day [76]. Chronotherapy may be beneficial for the treatment of diseases and related pain states.

Melatonin acts through melatonin 1 (MT_1) and MT_2 receptors in mammals and has been shown to be involved in nonreceptor-mediated functions [133]. Melatonin participates in circadian rhythms and other physiological functions, including mood states [15]. Under normal physiological conditions, melatonin has been shown to play a pivotal role in pain regulation. Both pain perception and melatonin secretion have a circadian component [29].

The administration of melatonin as an analgesic and anxiolytic agent has been proven effective for the treatment of fibromyalgia, migraine, and irritable bowel syndrome [130]. Melatonin also exerts neuroprotective effects in various animal models of brain injury (e.g., spinal cord injury and traumatic brain injury) [91]. Selective MT_2 receptor partial agonists have been reported to have analgesic properties by modulating brainstem descending antinociceptive pathways [72]. Clinically, melatonin is known to exert antioxidative, anxiolytic, analgesic, antihypertensive, anti-inflammatory, and oncostatic effects [130]. Accumulating evidence from prospective studies suggests an association between night work and the risk of breast cancer, most likely because of the suppression of melatonin [109]. Different animal models of pain have resulted in conflicting results of melatonin treatment, such as a lower pain threshold (i.e., paw-lick latency) but greater tolerance (i.e., escape latency) in the hot plate test in mice [100]. Most animal experiments have shown that melatonin influences pain pathways or the levels of signaling chemicals that regulate pain, but such conclusions are still debatable.

19.2 Properties of Melatonin

19.2.1 Melatonin in Plants and Animals

Melatonin is found in almost every plant and organism, but not all endogenous melatonin is related to chronobiological function. Several species exhibit diurnal peaks or a lack of rhythmicity, such as various bacteria, fungi, and rodents (e.g., rats

and mice). Although precise determinations of the functions of melatonin in many of these organisms are lacking, and the roles of melatonin are still unknown, investigations in some species have allowed more detailed conclusions to be drawn. Nonvertebrate melatonin is not necessarily circadian and thus does not always peak at night, although nocturnal maxima are frequently found [50].

Diversity in melatonin signaling or perhaps the absence of melatonin signaling (e.g., during the daytime) may be a reason for the existence of diurnally peaking melatonin rhythms or arhythmicity. Many studies have suggested that the major biosynthetic pathway is identical between nonvertebrates and vertebrates. Circadian rhythms with nocturnal maxima have been reported for nonvertebrates, especially in dinoflagellates [48, 103], several unicellular green algae [49], *Neurospora* [11], and several species of insects, including *Drosophila* [18]. In plants, the involvement of melatonin in photoperiodism is conceivable but requires further investigation. Food can be a source of melatonin, with possible implications for ecophysiology, human nutrition, and pharmacology [50]. In aerobic nonvertebrates, melatonin might have antioxidant properties, although evidence of its contribution at physiological levels is lacking. Animal experiments suggest that endogenous melatonin has an analgesic effect, but human data have been controversial. The majority of human studies suggest that exogenous melatonin eases pain, improves sleep, and improves symptoms of depression [137].

19.2.2 Melatonin Receptor Distribution

The presence and distribution of melatonin receptors that are involved in nociceptive transmission have been identified in different parts of the nervous system. Autoradiographic studies have localized these receptors to the hypothalamus, thalamus, anterior pituitary, dorsal horn of the spinal cord, spinal trigeminal tract, and trigeminal nucleus [117, 126, 127]. Receptor-binding sites for melatonin are located throughout the spinal cord in dorsal horn regions, with a high density in the superficial lamina. Both MT_1 and MT_2 receptor mRNA and protein are found in dorsal horn regions, indicating their localization in second-order neurons [94]. These melatonin receptors have been found in lamina I–V and X of both the ventral and dorsal horns of the lumbar and thoracic segments of the spinal cord, which are the main regions that are involved in pain transmission [94, 136]. Spinal melatonin enhanced the antinociceptive effect of morphine, suggesting that melatonin and MT_1 and MT_2 receptors that are in sensory regions of the spinal cord play a role in modulating pain transmission [136]. Behavioral electrophysiological studies have shown that although melatonin's actions are complex in nature, their effects on spinal nociception are predominantly inhibitory [65, 92].

19.2.3 Pharmacology of Melatonin

Melatonin undergoes extensive first-pass metabolism with varying bioavailability. Melatonin is a highly lipophilic substance with a consequently high volume of distribution, and 70% of plasma melatonin is albumin-bound. Ninety-nine percent is metabolized in the liver to 6-hydroxymelatonin by the cytochrome enzyme CYP1A2, conjugated to form sulfates and glucuronides. The remaining 1% is unchanged and excreted in urine. The elimination half-life in humans is approximately 45 min [77].

19.2.4 Toxicity and Adverse Effects of Melatonin

Melatonin is relatively nontoxic. Animal studies have shown that the maximum dose that can be given in vivo without any adverse effects or death is 200 mg/kg in pregnant rats throughout the entire pregnancy and 800 mg/kg in mice. Melatonin has been administered in many clinical studies in both adults and newborns without serious adverse effects. Doses of 1000 mg daily for 1 month have been given, and the only reported adverse effect was drowsiness. The most often reported side effects are headache, dizziness, nausea, and drowsiness. Interestingly, a previous study reported that abnormally high melatonin levels might cause a disease called growing pain [78]. A meta-analysis concluded that melatonin is safe for short-term use [17]. This meta-analysis found that the most common side effects of melatonin in this setting were headache, dizziness, nausea, and drowsiness. Adverse effects of melatonin premedication were evaluated in detail in a systematic review [134]. Adverse events were evaluated in terms of psychomotor impairment, sedation, disorientation, and amnesia. Even large doses of melatonin in healthy volunteers do not impair memory or visual sensitivity [68]. However, melatonin lacks amnestic effects. Memory recall both immediately after surgery and 24 h postoperatively remained unaffected in patients who were treated with melatonin. Melatonin caused more sedation than placebo, but melatonin-induced sedation was significantly less than midazolam [97]. These authors concluded that compared with midazolam, melatonin has similar anxiolytic efficacy but less psychomotor impairment and fewer side effects [77].

19.3 Melatonin in Neuropathic Pain

Pain that is caused by lesions or disease of the somatosensory nervous system is called neuropathic pain. Neuropathic pain can develop after nerve injury when deleterious changes in injured neurons occur. There are two kinds of neuropathic pain: peripheral and central [64]. Long-term neuropathic pain can cause central pain

sensitization, resulting in exaggerated pain in response to mildly painful stimuli (i.e., hyperalgesia) or pain in response to innocuous stimuli (i.e., allodynia) [63, 64]. Neuropathic pain refers to pain that is caused by nerve damage. Painkillers, such as morphine, nonsteroidal anti-inflammatory drugs (NSAIDs), and paracetamol, that are effective for the relief of nociceptive and inflammatory pain are ineffective for the relief of neuropathic pain. This is because the underlying mechanisms that cause neuropathic pain following nerve injury are different from those that induce nociceptive and acute inflammatory pain. Current neuropathic pain treatment includes antidepressants or anticonvulsants that boost signaling in the brain to inhibit pain signaling at the level of the spinal cord. The detailed mechanisms by which these drugs alleviate neuropathic pain are diverse, but the net effect is to dampen pain signals. Furthermore, these drugs have substantial side effects [47]. The absence of effective treatment is at the root of the high prevalence of chronic pain and neuropathic pain. Therefore, alternative treatments for neuropathic pain that are more effective and have fewer side effects are needed.

Melatonin has been shown to reduce neuropathic pain in animal models. In a mouse model of neuropathic pain, in which mice underwent tight ligation of the sciatic nerve, melatonin reduced paw-withdrawal latencies, which is a measure of thermal hyperanalgesia, but had no significant effect on withdrawal thresholds, a measure of mechanical allodynia [122]. Melatonin also exerted antiallodynic effects in rats that were subjected to L5–L6 spinal nerve ligation [8]. This antiallodynic effect was prevented by MT_2 receptor antagonists (luzindole and 4P-PDOT) and a nonselective opioid receptor antagonist (naltrexone). These results support the notion that the antinociceptive effects of melatonin are associated with MT_2 and opioid receptor activation. A recent study found that melatonin reversed formalin-induced flinching behavior and tactile allodynia in diabetic rats. The selective MT_2 receptor antagonist K-185 dose-dependently blocked this effect [10]. Intrathecal (3–100 μg) and oral (37.5–300 mg/kg) melatonin administration decreased tactile allodynia that was induced by spinal nerve ligation in rats, whereas intrathecal/oral administration of MT_2 and opioid receptor antagonists dose-dependently blocked the antiallodynic effect of melatonin [8]. Ulugol et al. [122] reported that intraperitoneal (30, 60, and 120 mg/kg) and intracerebroventricular (0.001, 0.01, and 0.1 nmol) melatonin exerted an antihyperalgesic effect but not an antiallodynic effect in mice through the L-arginine-nitric oxide (NO) pathway and opioid system. Hyperalgesia and allodynia are mediated by different nerve fibers, and small-diameter unmyelinated high-threshold C fibers were more sensitive to melatonin.

Interestingly, Zurowski et al. [138] reported an opposite result, in which the intraperitoneal administration of exogenous melatonin (100 mg/kg) abolished mechanical allodynia but not thermal hyperalgesia in a chronic constriction injury model in rats. This effect was significantly blunted by naloxone and the MT_1/MT_2 receptor antagonist luzindole. These authors concluded that the influence of melatonin on the process of thermal hyperalgesia involves an anti-inflammatory effect rather than opioid system activation [25]. It has been reported that melatonin dose-dependently improved behavioral hypersensitivity in rats by attenuating phosphorylated p38 mitogen-activated protein kinase (MAPK) levels and the release of

proinflammatory cytokines. Pinealectomy, which causes a reduction of endogenous melatonin levels, exacerbated these effects. Although most studies have reported analgesic effects of melatonin, there is controversy with regard to its use because of the many mechanistic pathways through which melatonin exerts its effects [70].

19.4 Melatonin and Inflammation

The most common cause of pain that is attributable to noxious stimuli is inflammation, and the treatment of inflammation is quite challenging. Frequently used morphine-based drugs, NSAIDs, and acetaminophen, while efficient for the treatment of pain, often have undesirable side effects [13, 37], including addiction and dependence, constipation, confusion, sleep disturbances, ulcers, gastro-inflammation, and respiratory depression [54, 96]. Chronic pain is often unresponsive to conventional medications and can cause anxiety and fear, which further aggravate pain [128]. Therefore, a better understanding of normal physiological defense mechanisms against pain is needed, and alternative therapies for inflammatory pain need to be developed that have fewer side effects [130].

Melatonin has been suggested to play an important role in the regulation of pain under normal physiological conditions. Both pain perception and melatonin secretion are circadian in nature [102]. Melatonin has also been shown to influence pain perception [115]. Melatonin's effect on pain has been demonstrated in various animal models of inflammatory pain [104]. A previous study used the formalin test to assess nociceptive behavior, such as paw licking and flinching, compared with nonspecific behaviors, such as self-grooming and locomotor activity [10]. A subcutaneous injection of 5% formalin produced a licking response of the injected hindpaw in a biphasic pattern, involving rapid and brief withdrawal of the affected paw. The two phases of the licking/flinching response are phase 1 (0–9 min) and phase 2 (10–60 min). Melatonin decreased licking and flinching responses in both phases [51, 104]. In another study that injected formalin, intrathecal melatonin administration reduced the flinching response during both phases, thereby attenuating both the facilitated state and acute pain that were evoked by formalin [111]. Similarly, in carrageenan-induced inflammation in rats, melatonin (0.5 and 1.0 mg/kg, i.p.) increased nociceptive thresholds [35]. Melatonin reduced inflammatory pain, likely by blocking the production of NO through inducible NO synthase (iNOS) and NO-cyclic guanosine monophosphate (cGMP) signaling pathways [35, 51]. However, higher nociceptive responses were found in mice during the dark period compared with the light period, and the melatonin receptor antagonist luzindole and functional pinealectomy reversed these changes [100].

It was found that an MT_2 receptor antagonist and selective δ-opioid receptor antagonist completely or partially reduced the antinociceptive effect of melatonin [10]. This suggests that MT_2 and δ-opioid receptors play important roles in these effects. In contrast, Ray et al. [104] found that the melatonin antagonist prazosin attenuated the licking response at a low dose (0.5 mg/kg) but increased the licking

response at a high dose (1 mg/kg) [104]. In a study, the MT_1 receptor antagonist luzindole did not inhibit but rather enhanced the antinociceptive effect of melatonin and supported the role of MT_2 receptors rather than MT_1 receptors in pain modulation [104]. These contradictory findings suggest that melatonin at low (i.e., physiological) doses mainly acts on membrane receptors, whereas higher (i.e., pharmacological) doses act on nuclear receptors or other receptors, thus leading to different results [70]. Further studies are needed to elucidate the mechanism of action of melatonin in inflammatory pain.

19.5 Melatonin in Fibromyalgia

Fibromyalgia syndrome (FMS) is a disorder that is characterized by chronic muscular pain, fatigue, and tenderness at particular sites of the body, called tender points. It is more frequent in women than in men. Fibromyalgia syndrome is currently an incurable disease and considered a chronic illness [131].

A previous study evaluated the effects of different doses of melatonin, alone or combined with fluoxetine, for the management of FMS. The following groups were formed: 20 mg/kg fluoxetine, 5 mg melatonin, 20 mg fluoxetine + 3 mg melatonin, and 20 mg fluoxetine + 5 mg melatonin. The treatments were given for 8 weeks. Melatonin (3 or 5 mg/day) combined with 20 mg/day fluoxetine significantly reduced fibromyalgia scores compared with pretreatment scores. The authors concluded that melatonin administration, alone or combined with fluoxetine, was effective in the treatment of FMS [52]. In another study, melatonin treatment alone or combined with amitriptyline was more effective than amitriptyline alone in modifying the endogenous pain-modulating system [52].

According to previous clinical studies, the most prevalent complaints in patients with FMS are sleep disturbances, fatigue, and chronic pain, and these symptoms might be a consequence of the disruption of melatonin secretion [30]. The administration of 6 mg/day melatonin in patients with FMS normalized sleep/wake cycles, normalized diurnal activity, decreased pain, decreased fatigue, and improved behavioral symptoms of depression. Furthermore, FMS patients have been reported to have deficiencies of serotonin, melatonin, cortisol, and cytokines, which are fully regulated by circadian rhythms. The mechanism of action that underlies this finding is unknown, but it may involve impairments in melatonin synthesis that are caused by low tryptophan/serotonin availability and possibly serotonin antibodies. This could explain the lack of restorative sleep and could be a mechanism of dysfunctional pain modulation [129]. A previous study evaluated circadian rhythms in 10 women with fibromyalgia and 12 healthy control women. Both groups had similar circadian rhythms in self-reported alertness. Although pain and stiffness were significantly higher in women with fibromyalgia compared with healthy women, no circadian rhythms in either parameter were detected. The authors suggested that abnormalities of circadian rhythmicity are not a primary cause of fibromyalgia or its symptoms [62, 129].

Patients with FMS had 31% lower melatonin secretion than healthy subjects during hours of darkness. This may contribute to impairments in sleep at night, fatigue during the day, and alterations of pain perception [129]. The analgesic effects of melatonin may be mediated by opioids [117] and γ-aminobutyric acid (GABA)ergic systems [89]. However, dissociating the influence of each neurobiological system in human experimental and clinical studies is not possible [67]. Indeed, only the net effect can be assessed. Previous studies have also suggested that additional pathways play a role in the analgesic actions of melatonin, such as nuclear signaling pathways, receptor-independent radical-scavenging systems, and inhibition of the release of proinflammatory cytokines at peripheral sites. Melatonin has multiple actions, including modulating the sleep/wake cycle and hypnotic-like effects [123]. Melatonin improves sleep and rest and decreases anxiety that is caused by sleeplessness. Additionally, melatonin synchronizes neurotransmitter circadian rhythms, including GABA, benzodiazepine, dopamine, and glutamate [4, 60].

One beneficial effect of melatonin in FMS may involve the normalization of neurotransmitter rhythms and the sleep/wake cycle [52]. Melatonin also has antistress properties and influences the hypothalamic-pituitary-adrenal axis, which may account for some of its effects in FMS. Data on the anti-inflammatory role of melatonin have been reported [81], as well as its inhibition of macrophage/monocyte activation, including reductions of both iNOS and proinflammatory cytokines. Altogether, these effects of melatonin may be beneficial for the treatment of FMS.

The effects of melatonin that have been observed in FMS patients, together with its minimal side effects and the fact that it decreases the toxicity and increases the efficacy of many drugs [105], suggest that melatonin treatment alone or combined with other therapies may have significant value for the pharmacological management of FMS.

19.6 Melatonin in Alzheimer's Disease-Related Pain

Melatonin was reported to be an efficient antioxidant and cognitive enhancer in Alzheimer's disease (AD). Alzheimer's disease decreases cognitive function by decreasing synaptic function in the hippocampus and cerebral cortex [93]. It is also characterized by mitochondrial dysfunction at the molecular level [33]. Melatonin has been shown to prevent oxidative stress and restore mitochondrial function in AD [116].

People who suffer from AD also suffer from common causes of acute and chronic pain. Some AD patients also have neuropathic pain, but the main mechanism of such pain in AD patients is still unclear. The efficacy of analgesic drugs for the treatment of dementia-related pain has not been thoroughly investigated.

19.7 Melatonin in Irritable Bowel Syndrome

Individuals with irritable bowel syndrome (IBS) have a relatively poor quality of life. This condition is associated with abdominal pain, flatulence, constipation, diarrhea, and sleep disturbances. Among the putative pathogenesis of IBS, gut dysmotility results in pain and abnormal defecation. The latter is likely caused by an effect of abnormal gut water secretion. The interaction between abnormal gas accumulation, abdominal pain, and bloating remains controversial [23]. Visceral hypersensitivity and its modification along with the central transmission are common characteristics of IBS [23]. Research shows that melatonin is secreted in the gastrointestinal tract, has strong antioxidant and anti-inflammatory properties, and is involved in the regulation of intestinal motility [24]. These mechanisms likely contribute to the possibility that melatonin can relieve symptoms in IBS patients.

A previous double-blind, placebo-controlled study of female subjects administered 3 mg melatonin per day, which was reported to improve bowel symptoms in IBS patients [73]. In another study, melatonin reduced abdominal and rectal pain in IBS patients [113]. Furthermore, melatonin was shown to inhibit smooth muscle motility, possibly by stimulating certain receptors in the gastrointestinal tract, regulating cellular calcium-activated potassium channels, and blocking nicotinic receptors [112, 113]. Although the precise mechanism of action is unknown, melatonin has been shown to provide symptom relief in IBS patients. Furthermore, melatonin was also reported to increase colonic transit time [73]. Although many studies favor the use of melatonin for the treatment of IBS symptoms, the precise types of IBS that were investigated in these previous studies were unclear. Therefore, further studies should be conducted with large sample sizes, and melatonin doses should be optimized based on the severity and type of IBS. Overall, melatonin may relieve IBS symptoms.

19.8 Melatonin in Chronic Migraine

The human biological clock is situated in the SCN in the hypothalamus. The biosynthesis of melatonin in the pineal gland is regulated by the SCN and synchronized to the light/dark cycle [98]. The SCN regulates many physiological signals, including the levels of corticosteroids and melatonin. The homeostatic functions of the hypothalamus influence several physiological functions, such as the regulation of body temperature, blood pressure, blood glucose levels, hunger, and satiety [3]. A positron-emission tomography study reported activation of the hypothalamus during spontaneous migraine attacks [32]. According to this study, hypothalamic dysfunction was involved in migraine attacks, with the involvement of the retino-hypothalamic-pineal axis [6]. Migraine is primarily a headache-related disorder that is characterized by moderate to severe recurrent episodes of headache combined with gastrointestinal, neurological, and autonomic symptoms. Migraines have a tendency

to recur in daily, weekly, monthly, or even seasonal patterns [5]. The periodicity of migraines indicates that the master biological clock influences the susceptibility to migraine, but a pivotal role for this master clock has not been proven [55]. The melatonergic system may play a role in the pathogenesis of migraine.

Tabeeva et al. [121] reported that agomelatine may be effective as a prophylactic treatment for migraine because of its specific mechanism of action and similarity to melatonin. Guglielmo et al. [46] reported two cases of patients with migraine who were successfully treated with agomelatine. One patient presented comorbid depression, whereas the other patient had no comorbidities. A Brazilian study suggested that melatonin may effectively prevent migraines, in which 3 mg melatonin was more effective than 25 mg amitriptyline, placebo, and other analgesics [43]. Melatonin was also more tolerable than amitriptyline. Amitriptyline treatment is associated with adverse events, including daytime sleepiness and weight gain [99]. Some researchers inferred that migraine and sleep are related [59].

Migraine patients also report significantly more subjective sleep problems, including insomnia, tiredness, global sleep problems (as determined by the Pittsburgh Sleep Quality Index), and pain-related sleep difficulties [36]. Sleep has been reported to allay migraine attacks [55]. As early as the nineteenth century, patients with sleep disorders were found to be more prone to morning headaches [71], in addition to patients with both chronic [108] and episodic [6] migraine. Melatonin is effective, relatively inexpensive, safe, and tolerable for patients with sleep insufficiency or patients who are sensitive to other drugs and may be a drug of choice for the treatment of migraine.

19.9 Melatonin in Chemotherapy Pain

Chemotherapy-induced neuropathic pain is a debilitating and common side effect of cancer treatment. Mitochondrial dysfunction that is associated with oxidative stress in peripheral nerves has been implicated in the underlying mechanism [9]. Melatonin is a free-radical scavenger, antioxidant, and immunomodulatory agent. In both in vitro and in vivo studies, melatonin was shown to protect healthy cells from radiation-induced and chemotherapeutic drug-induced toxicity [124]. Furthermore, several clinical studies have reported that melatonin treatment, either alone or combined with traditional therapy, resulted in a favorable toxicity ratio in the treatment of human cancers [69].

Chemotherapy-induced neuropathic pain causes tingling and pain sensation to touch and cold temperatures, often making everyday activities, such as fastening buttons or walking barefoot, painful [95]. The condition can persist beyond the treatment period and after the cancer is cured [118]. Patients who received melatonin during taxane chemotherapy had a lower incidence of neuropathy [9, 22, 90]. In a rat model of paclitaxel-induced painful peripheral neuropathy, pretreatment with oral melatonin (5, 10, and 50 mg/kg), given as a daily bolus dose, limited the devel-

opment of mechanical hypersensitivity and the subsequent development of pain [40]. Importantly, melatonin did not interfere with the anticancer effects of chemotherapy. The researchers found a reduction mitochondrial damage, which may be key to preventing chemotherapy-induced neuropathic pain [40]. In vitro, paclitaxel caused a 50% reduction of mitochondrial membrane potential and metabolic rate, independent of concentration (20–100 μmol/L). Mitochondrial volume was dose-dependently increased by paclitaxel (200% increase at 100 μmol/L). These effects were prevented by co-treatment with 1 μmol/L melatonin. This indicates that melatonin may act within mitochondria and thus reduce mitochondrial damage and neuropathic pain that are caused by paclitaxel. Melatonin has been shown to limit chemotherapy-induced neuropathic pain by protecting nerve cell mitochondria during chemotherapy.

19.10 Melatonin in Preoperative Anxiety and Postoperative Analgesia

The most common issues that are related to surgical procedures are preoperative anxiety and postoperative pain, and these conditions are related to each other. Surgery and anesthetic procedures can disrupt circadian rhythm [41, 56]. Disruptions of circadian rhythm and higher cortisol secretion after surgery may disrupt rapid eye movement (REM) sleep [39]. Although anesthetics and benzodiazepines are given to relieve pain and anxiety, these treatments have several side effects, including psychomotor illness, excessive sedation, disorientation, amnesia, and poor sleep quality [77].

Melatonin has recently received attention with regard to its anxiolytic, hypnotic, and pain-controlling effects. Exogenously administered melatonin has hypnotic properties in humans [80]. Most clinical studies that investigated the preoperative anxiolytic effects of melatonin found a significant reduction of anxiety compared with placebo. Caumo et al. [20] reported that melatonin and clonidine were comparable in terms of anxiolysis and postoperative morphine consumption and more effective than placebo. Ismail and Mowafi [53] found that oral 10 mg melatonin premedication had anxiolytic, analgesic, and intraocular pressure-reducing effects compared with placebo in patients who underwent cataract surgery under topical anesthesia. They found that sublingual premedication with 0.05 mg/kg melatonin resulted in preoperative anxiolysis and sedation without impairing cognitive function or psychomotor skills or affecting the quality of recovery [77]. These authors concluded that melatonin had similar anxiolytic efficacy as midazolam but less psychomotor impairment and fewer side effects. Melatonin has also been used in various surgical procedures, including tourniquet-related pain and cataract surgery to relieve pain and anxiety [114].

Midazolam and clonidine premedication was associated with significantly more postoperative delirium than placebo. Melatonin premedication (5 mg orally at night and 90 min before surgery) was associated with significantly less delirium than placebo [120].

In a double-blind, placebo-controlled study, preoperative treatment with melatonin significantly decreased pain and anxiety during the first 36 h after surgery [21]. In a study of patients who underwent elective hand surgery, premedication with melatonin reduced anxiety and enhanced intraoperative and postoperative analgesia [86]. In a rat model of postoperative pain, intrathecal co-administration of low doses of melatonin and morphine attenuated mechanical and thermal hyperalgesia [136]. In animal studies, adjunct treatment with melatonin potentiated the anesthetic effects of thiopentone, ketamine, and isoflurane [16]. Premedication with 0.2 mg/kg melatonin also reduced the doses of propofol and thiopental [77].

Melatonin levels that are altered both during and after surgery affect the regulation of melatonin release from the pineal gland and disrupt circadian rhythm, consequently decreasing REM sleep and reducing wakefulness during the daytime period after surgery compared with before surgery sleep/wake cycle disturbance [42]. Various drugs that are commonly used for anesthesia are also reported to alter melatonin secretion. Benzodiazepines [82], NSAIDs [88], clonidine [87], corticosteroids [31], and β-blockers [119] decrease plasma levels of melatonin. However, opioids may increase plasma melatonin levels by stimulating serotonin-*N*-acetyltransferase [44].

Even large doses of melatonin do not impair fine motor skills, memory, or visual sensitivity in healthy volunteers [68]. A detailed systematic review by Yousaf et al. [134] supported these findings. Melatonin is a natural hormone that does not influence the duration of REM sleep. Therefore, it is an attractive drug of choice for perioperative procedures because of its anxiolytic, sedative, chronological, anti-inflammatory, and antinociceptive properties. Physiologists and anesthesiologists have used melatonin for anxiolysis, the prevention of delirium, analgesia, the prevention of oxidative stress, and hypnosis.

19.11 Melatonin in Rheumatoid Arthritis

To date, the role of melatonin in rheumatoid arthritis is unclear. One study suggested that rheumatoid arthritis patients had significantly higher melatonin secretion at night compared with healthy individuals. Blood serum melatonin levels were higher during the morning in patients who had a shorter disease history [2, 28]. Inflammatory cytokines, such as interleukin-1 (IL-1) and IL-6, were shown to be secreted into the peripheral blood flow after melatonin stimulation. Melatonin was also detected in synovial liquid in rheumatoid arthritis patients [27]. Melatonin inhibited the activity of matrix metalloproteinase, which is involved in joint destruction in rheumatoid arthritis patients [107]. The role of melatonin in rheumatoid arthritis-related chronic pain needs to be investigated further.

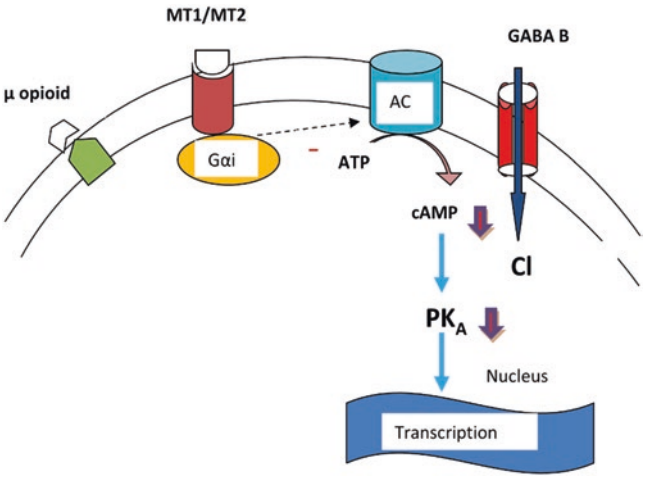

Fig. 19.1 Melatonin exerts antinociceptive effects by acting through G_i-coupled MT_1/MT_2 receptors, GABA receptors, and opioid receptors in the thalamus and somatosensory cortex by inhibiting adenyl cyclase and decreasing cyclic adenosine monophosphate (cAMP)

19.12 Proposed Mechanism of Action of Melatonin

19.12.1 MT_1 and MT_2 Receptors

Melatonin has two high-affinity G-protein-coupled membrane receptors (MT_1 and MT_2) that belong to the ML1 membrane-associated binding site in mammals. Another melatonin membrane-associated melatonin-binding site, ML2, includes relatively low-affinity nuclear receptors (RZR/ROR) and calmodulin (Fig. 19.1). Previous studies found that melatonin receptors are expressed in various areas of the brain, such as the thalamus, hypothalamus, spinal trigeminal tract, and trigeminal nucleus. In rats, MT_1 and MT_2 receptors are expressed in the ventral and dorsal horn of thoracic and lumbar regions of the spinal cord [136]. Pain regulatory mechanisms are also managed by melatonin receptors in lamina I–V and X of the spinal cord [114].

19.12.2 Opioid Pathway

The opioid system may be related to the antinociceptive effect of melatonin. Pinealectomy was reported to eliminate the antinociceptive effect of melatonin, whereas the nonselective opioid receptor antagonist naloxone hampered the day/night cycle of nociception in mice [57]. δ- and μ-opioid receptors are found in the bovine pineal gland [101], suggesting that there might be some correlation between opioid receptors and the pineal axis. However, another study found that melatonin

agonists or antagonists did not bind to opioid receptors but increased the release of β-endorphin through its receptors [110]. Opioid receptor antagonism attenuated the analgesic effect of melatonin and the interaction between melatonin and the endogenous opioid β-endorphin [122, 138]. Therefore, melatonin may also have nonreceptor actions. Intraperitoneal melatonin administration increased pain thresholds and increased endogenous endorphin release in perfusate from the central gray in rats, indicating that melatonin may enhance endorphin release [135]. However, in contrast, it was found that pinealectomized rats showed significantly higher levels of β-endorphin as compared to control animals [12]. The localization of melatonin receptors at the principal site of endorphin synthesis and release in the pituitary gland (in the median eminence and pars tuberalis) strongly suggests that melatonin may have actions on these sites [85]. Although still controversial, a relationship likely exists between melatonin, opioid receptors, and β-endorphins.

19.12.3 Nitric Oxide Pathway

Nitric oxide is synthesized from arginine by a family of NOSs. Nitric oxide is a gas that diffuses freely through membranes of target cells to activate cGMP. Nitric oxide acts as a second messenger that is involved in neurotoxicity and neurotransmission [19]. Melatonin reduced inflammatory pain, possibly by blocking the production of NO by iNOS and NO-cGMP signaling pathways [35, 51]. Previous reports suggested that NO is involved in the pain-modulating effects of melatonin in the formalin test, and the mechanism likely involves the NO-cGMP-protein kinase G-K^+ channel pathway [122]. A previous study reported that melatonin blocked protein nitration by affecting iNOS expression in "paw tissue," thus helping reduce tissue damage and inflammation [38]. Nevertheless, evidence suggests contradictory roles of NO in pain modulation [38, 58]. Callsen-Cencic et al. [19] found that spinal neuronal NOS expression critically depended on the type of afferent fibers that were activated by specific lesions and the intensity and duration of inputs to the spinal cord [19]. The role of NO in the pain-modulating effects of melatonin requires further investigation.

19.12.4 GABA and N-Methyl-D-Aspartate Receptors

The human biological clock is situated in the SCN in the hypothalamus. The biosynthesis of melatonin in the pineal gland is regulated by the SCN and synchronized to the light/dark cycle [98]. Neurons in the SCN express GABA neurotransmitters and receptors [84], and GABA is an essential component of the regulation of SCN function. Melatonin stimulates GABA [106] and may suppress hyperneuronal

excitability by acting through GABA receptors. Therefore, melatonin may be useful for the treatment of chronic pain by mediating the GABAergic pathway and dampening hyperexcitability that is associated with pain.

N-methyl-D-aspartate (NMDA) receptors are critically involved in the pain-regulating effects of melatonin, especially in peripheral and central pain sensitization [26]. Spinal cord synaptic potentiation plays an important role in the development and maintenance of chronic pain, whereas NMDA receptors play an important role in spinal pain transmission [65, 83]. Melatonin dose-dependently inhibited spinal pain transmission [92]. Melatonin attenuated mechanical hyperalgesia and downregulated NMDA receptors in a rat model of temporomandibular joint inflammatory pain [125].

19.12.5 Free-Radical Scavenging and Mitochondrial Dysfunction

Melatonin prevents mitochondrial dysfunction under conditions of oxidative stress. Melatonin has been shown to play a role in mitochondrial homeostasis and exert protective effects in various diseases, such as AD and Parkinson's disease, a common characteristic of which is mitochondrial dysfunction [1]. Such neuroprotection that is afforded by melatonin may help inhibit nociceptive neurons and inflammation that is associated with oxidative stress, thus reducing pain. The protective action of melatonin involves free-radical scavenging and the stimulation of mitochondrial enzymes, such as superoxide dismutase. Melatonin time-dependently enhanced the activity of mitochondrial respiratory complexes I and IV in the brain and liver in rats [79]. Melatonin also has antioxidant properties to reduce oxidative damage by restoring reduced glutathione levels, restoring glutathione peroxidase activity, and scavenging hydroperoxides [66].

19.12.6 Neuroprotective and Antiapoptotic Actions: ERK/MAPK Pathway

The extracellular signal-regulated kinase/MAPK pathway represents a major component of melatonin signaling during oxidative stress. Several studies suggested that melatonin activates the ERK1/2 pathway [132] and promotes cell-survival signaling. Previous studies reported that the involvement of melatonin in microtubule assembly in AD depends on the ERK1/2 MAPK signaling component, possibly as a consequence of the MT_1 receptor-dependent regulation of protein kinase A [14]. Melatonin can prevent mitochondrial damage and the accumulation of reactive

oxygen species, thereby influencing intrinsic pathways that are involved in apoptotic signaling, including Bcl2/Bax, which controls the actions of cardiolipin and cytochrome c. When cells were exposed to melatonin, the ERK1/2 pathway was activated, the oxidation of cardiolipin was inhibited, and cytochrome c release into the cytoplasm was inhibited, thus preventing DNA damage [74].

A previous study exposed U937 leukemia cells to UVB light, which caused mitochondrial damage and reactive oxygen species overproduction. Treatment with pharmacological concentrations of melatonin prevented the damage and apoptotic signaling of mitochondria, and the mechanism depended on ERK1/2 activation [75]. Another study found that chronic exposure to melatonin in vivo stimulated neuroprotection in rats that were subjected to ischemia-reperfusion, accompanied by the coactivation of ERK-MAPK and Jun N-terminal kinase (JNK) [61]. Melatonin stimulated the activation of ERK1/2 and decreased the production of the stress kinases p38 MAPK and JNK [61]. Therefore, by decreasing mitochondrion-derived, reactive oxygen species-dependent Ras activation, melatonin might help activate the transcription factor nuclear factor-κB to protect against neuronal damage and neuroinflammation, thus alleviating pain (Fig. 19.2).

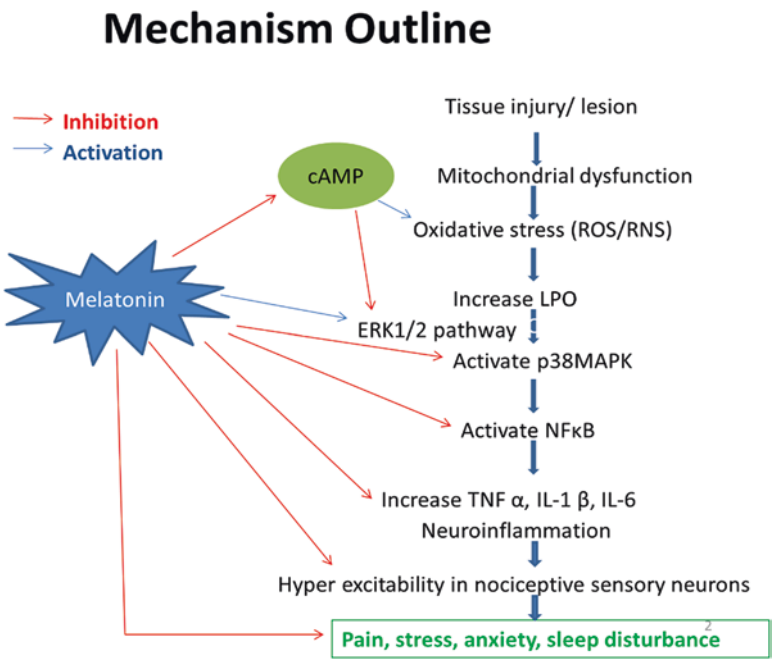

Fig. 19.2 Melatonin activates the ERK1/2 pathway, thereby decreasing mitochondrion-derived, reactive oxygen species (ROS)-dependent Ras activation, preventing neuroinflammation, and alleviating pain

19.13 Conclusion

Melatonin is produced by the pineal gland. It helps regulate circadian rhythm and other physiological functions, such as sleep and mood, in humans, but its function may vary in other organisms. Exogenous melatonin administration has been shown to be an effective treatment for many pain-related conditions in both humans and animal models of pain perception and surgical procedures. Melatonin is most commonly administered orally (e.g., sublingual or intraperitoneal). It appears to act through MT_1 and MT_2 receptors and has free-radical scavenging activity. It also interacts with different pathways, including NMDA, opioid, ERK/MAPK, and NO systems. Melatonin is currently sold as a dietary supplement and not as a drug per se in the United States. Therefore, US Food and Drug Administration (FDA) regulations are not applicable to melatonin. The FDA has given melatonin an orphan drug status for circadian rhythm disturbances. In Europe, 2 mg prolonged-release melatonin is accepted for older individuals ($\geq$ 55 years of age) for the treatment of insomnia [7]. Many other countries, such as Canada, the United Kingdom, and many European countries, have banned the sale of over-the-counter melatonin [45]. Melatonin should be studied further as a circadian regulator and for the treatment of chronic pain.

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Chapter 20
Central Poststroke Pain, Comorbidity, and Associated Symptoms in Animal and Human Models

Bai-Chuang Shyu and Andrew Chih Wei Huang

Abstract The objective of the present review paper was to comprehensively introduce the pain symptom and comorbidities of depression, anxiety, and learning and memory dysfunctions in the central poststroke pain (CPSP) of human and animal models. CPSP is a disease in which the lesion or dysfunction of the spinothalamocortical circuits is due to thalamic stroke hemorrhage. According to previous literature, CPSP patients experience impaired explicit and implicit learning and memory in addition to the pain symptom. Moreover, there are associated depression and anxiety comorbidities for CPSP. However, the data from some clinical studies were not supportive of the notion that CPSP patients also experienced induced comorbid depression and anxiety. On the other hand, the motor function test was likely to be inconsistent in terms of the results of human and animal models. The review paper provides some implications for further development of animal models for examinations of CPSP comorbidities of depression, anxiety, learning and memory dysfunction, and motor functions, aside from the central pain symptom. In human models, some conflicting data related to comorbid depression, anxiety, explicit and implicit learning memory, and motor dysfunctions should be re-elucidated in further studies.

Keywords Central poststroke pain · Comorbidity · Learning and memory · Depression · Anxiety

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20.1 Pain and Comorbid Symptoms for Central Poststroke Pain

Central poststroke pain (CPSP) is defined as the lesion or dysfunctions of any neural substrate of the spinothalamocortical pathway due to various clinical symptoms such as hyperalgesia or allodynia [6]. Patients with thalamic stroke hemorrhage revealed the important diagnostic symptom of somatosensory abnormalities [3]. According to previous findings, the prevalence of CPSP patients is approximately up to 8% for stroke disease [6, 9, 10]. Aside from the major symptom of pain, a lot of comorbidities or associated symptoms occurred in patients with thalamic hemorrhage. For example, some clinical studies have reported that stroke hemorrhage patients also experience movement dysfunctions very often [12]; moreover, stoke hemorrhage has been shown to interfere with both explicit memory and implicit memory [1]. A previous clinical report suggested that stoke hemorrhage appeared as amnesia in learning and memory function [2]. Additionally, thalamic hemorrhage was demonstrated to induce sensorimotor deficits and neuropsychological disturbances [11]. Some studies of CPSP clinical patients have shown that CPSP might be associated with major depression disorder and anxiety symptoms [3, 5, 16]. Therefore, CPSP patients may experience not only hyperalgesia or allodynia in pain perception but also dysfunction in learning and memory, motor deficits, depression, and anxiety comorbidities. Until now, a few researchers have investigated the issue of whether the thalamic lesion-induced central neuropathic pain would be associated with other comorbidities or symptoms in addition to pain in animal models.

Altogether, the purpose of this paper is to comprehensively introduce a variety of comorbidities of CPSP including learning and memory, motor function, depression, and anxiety in human models of clinical trials. Also, we reviewed the previous evidence of animal models of CPSP in which collagenase was used to destroy the ventrobasal complex of the thalamus, as well as evidence regarding whether the lesions of the ventrobasal complex of the thalamus (i.e., CPSP rats) affect comorbid behaviors including learning and memory and motor function.

20.2 CPSP Comorbidity: Learning Memory

20.2.1 Human Models in CPSP Learning and Memory

The multiple systems hypothesis of learning and memory suggests that the types of learning and memory can be divided into explicit memory and implicit memory [17, 20, 21]. Explicit memory is required to process the contents of memory on a conscious level; however, implicit memory does not process information of memory in consciousness [20, 21]. Instead, implicit memory underlies unconscious processing to produce the formation of learning and memory [20, 21]. Little research has been conducted to learn how CPSP patients exhibit performance in implicit and explicit

learning and memory. To examine this issue, a previous study investigated whether lesions in the thalamus with infarction or hemorrhage disrupted implicit learning and memory as well as explicit learning and memory in human models. These authors demonstrated that focal infarction or hemorrhage did not affect subjects' intellectual and executive functions; however, it interfered with psychomotor speed, attention, explicit memory, and implicit visuomotor sequential learning and memory [1]. Another study reported that a patient with stroke hemorrhage in the anterior portion of the thalamus showed amnesia syndrome for the lower scores of the Wechsler memory questionnaire [2]. This study indicated that the explicit memory was deficient following the anterior thalamus stroke hemorrhage. Therefore, the human studies demonstrated that thalamic stoke hemorrhage (i.e., CPSP) interfered with both explicit and implicit learning and memory.

20.2.2 Animal Models in CPSP Learning and Memory

On the other hand, there is similar evidence that in animal models, the thalamic lesions imitate the clinical data of thalamic stroke, indicating that animal models demonstrated that the thalamus is also involved in learning and memory [13, 14, 19]. For example, destroying the ventroposterial nucleus of the thalamus with ibotenic acid was found to reduce heart rate-conditioned learning in rabbits [13]. Using the electrophysiological recording approach, rats were trained to learn tactile discrimination in which a tactile stimulus was contingent with the reward and the primary somatosensory thalamocortical circuits showed hyperactivity for this kind of reward-related conditioned learning [14]. Utilizing microinjection of ibotenic acid to destroy the parvocellular part of the ventral posteromedial nucleus of the thalamus (and amygdala) appeared to interfere with an emetic drug lithium chloride-induced conditioned taste aversion [19]. Previous studies of animal models have suggested that in animal models, thalamus lesions might be associated with learning and memory.

Recently, our study examined whether collagenase-induced lesions in the ventrobasal complex of the thalamus affect the formation of learning and memory during the acquisition or retrieval phase to imitate an animal model of stroke hemorrhage in the clinic [18]. In the present study, rats with lesions of the ventrobasal complex of the thalamus (i.e., the CPSP group) showed lower withdrawal time compared with the sham group, indicating that rats with lesions of the ventrobasal complex experienced hyperalgesia in the right and left paws during the acquisition phase (Table 20.1). Moreover, during the retrieval phase, rats in the CPSP group revealed shorter withdrawal time, indicating that CPSP rats demonstrated hyperalgesia regardless of right and left paws during the retrieval phase (Table 20.2). Furthermore, we found that the CPSP rats with lesions of the ventrobasal complex of the thalamus exhibited morphine-induced conditioned place preference, suggesting that in the CPSP rats, the extinction effect induced by morphine during the acquisition phase decreased (Fig. 20.1). However, with regard to spatial learning, CPSP did not affect

Table 20.1 (**a**) Left paw and (**b**) right paw tests from Experiment 1 during the acquisition phase before the VBC lesion. The data show the mean ± SEM withdrawal time (in seconds) between the sham and CPSP groups for the baseline, week 1–5, and total phases

		Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Total
(A)								
Sham group	Mean	2.53	2.83	2.53	2.66	2.49	2.67	13.17
	SEM	0.11	0.16	0.10	0.10	0.08	0.11	0.30
CPSP group	Mean	2.53	2.40*	2.21*	2.05*	2.50	2.40	11.58*
	SEM	0.06	0.08	0.07	0.06	0.33	0.09	0.41
(B)								
Sham group	Mean	2.47	2.63	2.53	2.39	2.10	2.15	11.79
	SEM	0.09	0.13	0.15	0.11	0.08	0.11	0.36
CPSP group	Mean	2.31	2.15*	1.93*	1.79*	1.69*	1.89*	9.44*
	SEM	0.07	0.05	0.05	0.06	0.04	0.08	0.15

CPSP central poststroke pain
*$p < 0.05$, compared with sham group. "Total" indicates the combined mean withdrawal time for all weeks

Table 20.2 (**a**) Left paw and (**b**) right paw tests from Experiment 2 during the retrieval phase after the VBC lesion. The data are expressed as the mean ± SEM withdrawal time (in seconds) between the sham and CPSP groups for the baseline, week 1–4, and total phases

		Baseline	Week 1	Week 2	Week 3	Week 4	Total
(A)							
Sham group	Mean	1.93	2.29	2.31	2.37	2.01	8.98
	SEM	0.11	0.15	0.07	0.12	0.12	0.30
CPSP group	Mean	2.02	2.25	2.19	1.94*	1.79	8.17*
	SEM	0.12	0.11	0.06	0.11	0.08	0.22
(B)							
Sham group	Mean	1.84	2.20	2.17	2.25	2.22	8.84
	SEM	0.11	0.07	0.07	0.07	0.10	0.17
CPSP group	Mean	1.92	1.76*	1.88*	1.55*	1.56*	6.75*
	SEM	0.10	0.10	0.07	0.10	0.08	0.20

CPSP central poststroke pain
*$p < 0.05$, compared with sham group. "Total" indicates combined mean withdrawal time for all weeks

total distance traveled, time spent, and velocity during the acquisition phase when compared with the control; thus, the CPSP rats, in which the ventrobasal complex of the thalamus was destroyed, did not show a change in performance in spatial learning (Fig. 20.2). Regarding the conditioned place preference effect on the retrieval phase, CPSP rats with lesions of the ventrobasal complex of the thalamus showed morphine-induced conditioned place preference, but the sham rats did not. The results indicated that during the retrieval phase, lesions of the ventrobasal

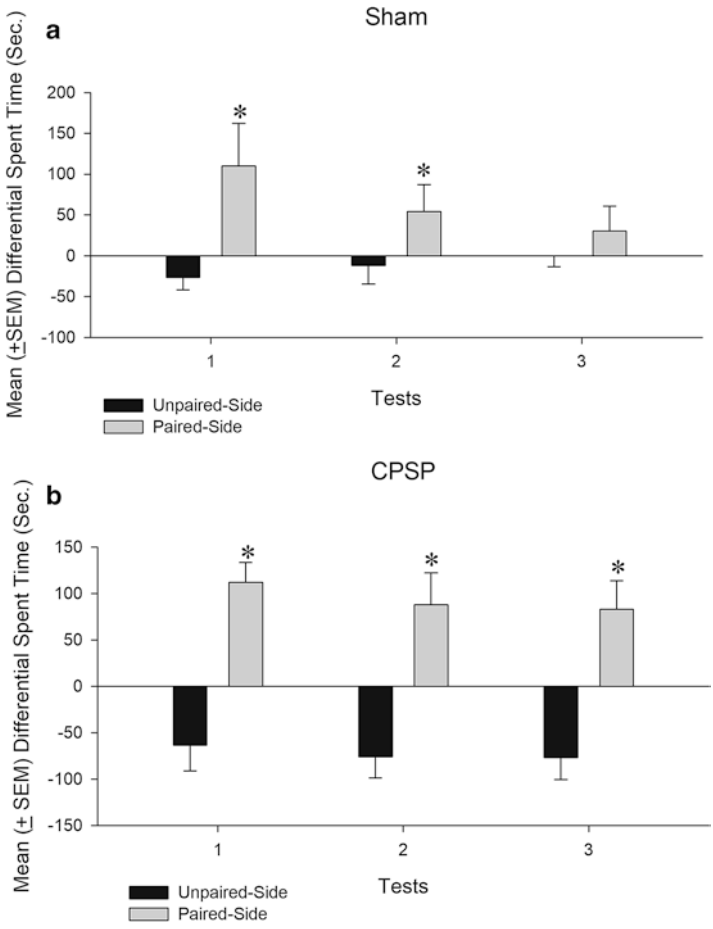

Fig. 20.1 Mean ± SEM differential time spent (in seconds) in the unpaired and paired sides in tests 1–3 in the (**a**) sham group and (**b**) CPSP group during the acquisition phase. CPSP, central post-stroke pain. *$p < 0.05$, compared with unpaired side

complex of the thalamus also remained resistant to the effect of morphine-induced conditioned preference (Fig. 20.3). During the retrieval phase, the performance of spatial learning including total distance traveled, time spent, and velocity was not influenced by the group with lesions of the ventrobasal complex of the thalamus, indicating that CPSP rats can retain normal functions of spatial learning and did not exhibit altered performance of spatial learning (Fig. 20.4). Therefore, rats with collagenase-induced lesions of the ventrobasal complex of the thalamus imitated CPSP symptoms during the acquisition and retrieval phases. The results showed that the right and left paws produced a lower withdrawal response, and in CPSP rats,

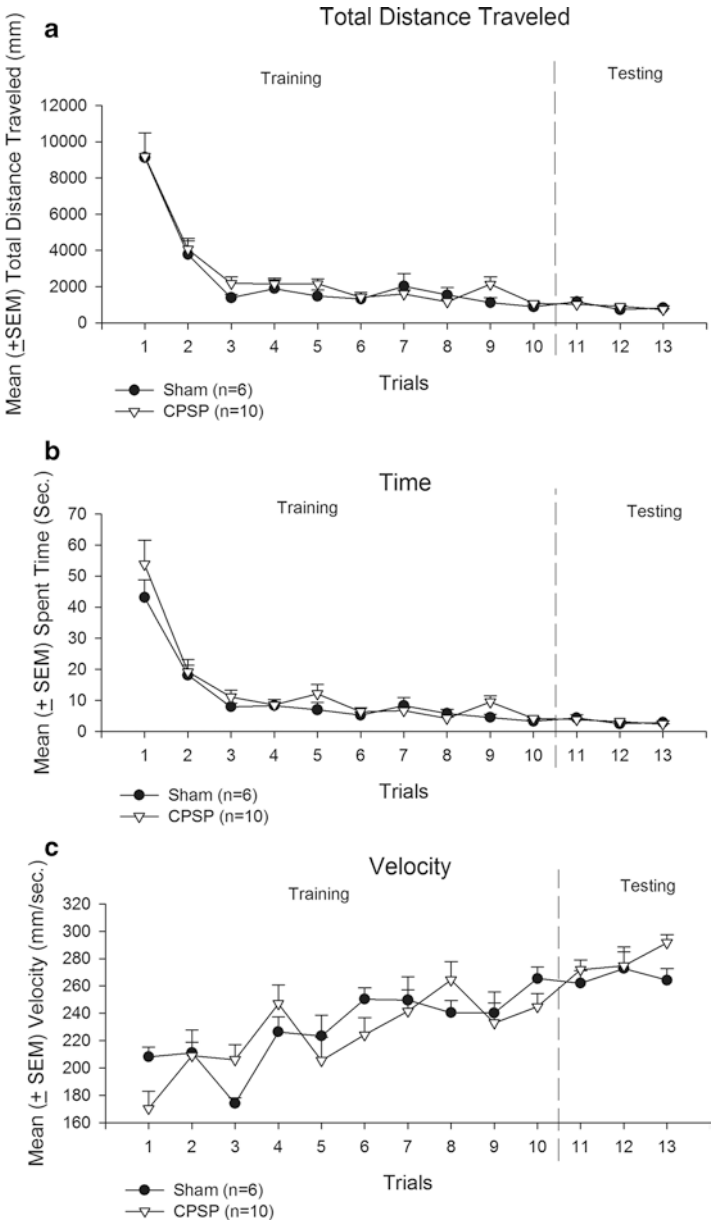

Fig. 20.2 Mean ± SEM (**a**) total distance traveled (in millimeters), (**b**) time spent (in seconds), and (**c**) velocity (mm/s) in the sham and CPSP groups in the acquisition and testing phases. CPSP, central poststroke pain

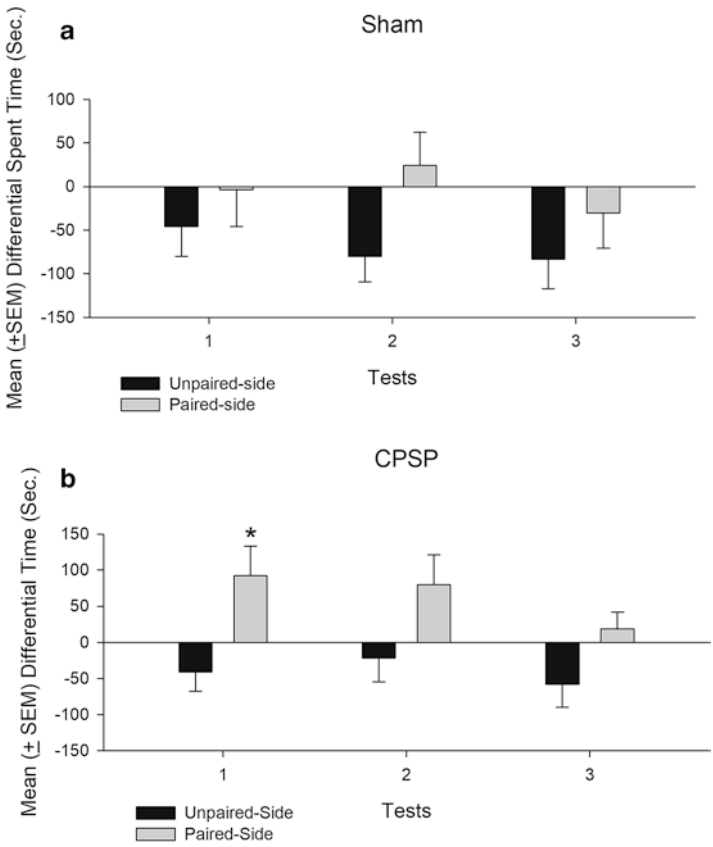

Fig. 20.3 Mean ± SEM differential time spent (in seconds) between the unpaired and paired sides in each test in (**a**) the sham group and (**b**) the CPSP group during the retrieval phase. CPSP, central poststroke pain. *$p < 0.05$, compared with unpaired side

hyperalgesia was induced during the acquisition or the retrieval phase. Moreover, the CPSP rats exhibited morphine-induced preference conditioning over extinction sessions compared to the sham rats. However, spatial learning was not affected for the CPSP rats. The findings indicate that CPSP patients could experience hyperalgesia. These rats may have experienced a persistent effect of morphine-induced conditioned place preference, but there was no change in performance of spatial learning (Table 20.3).

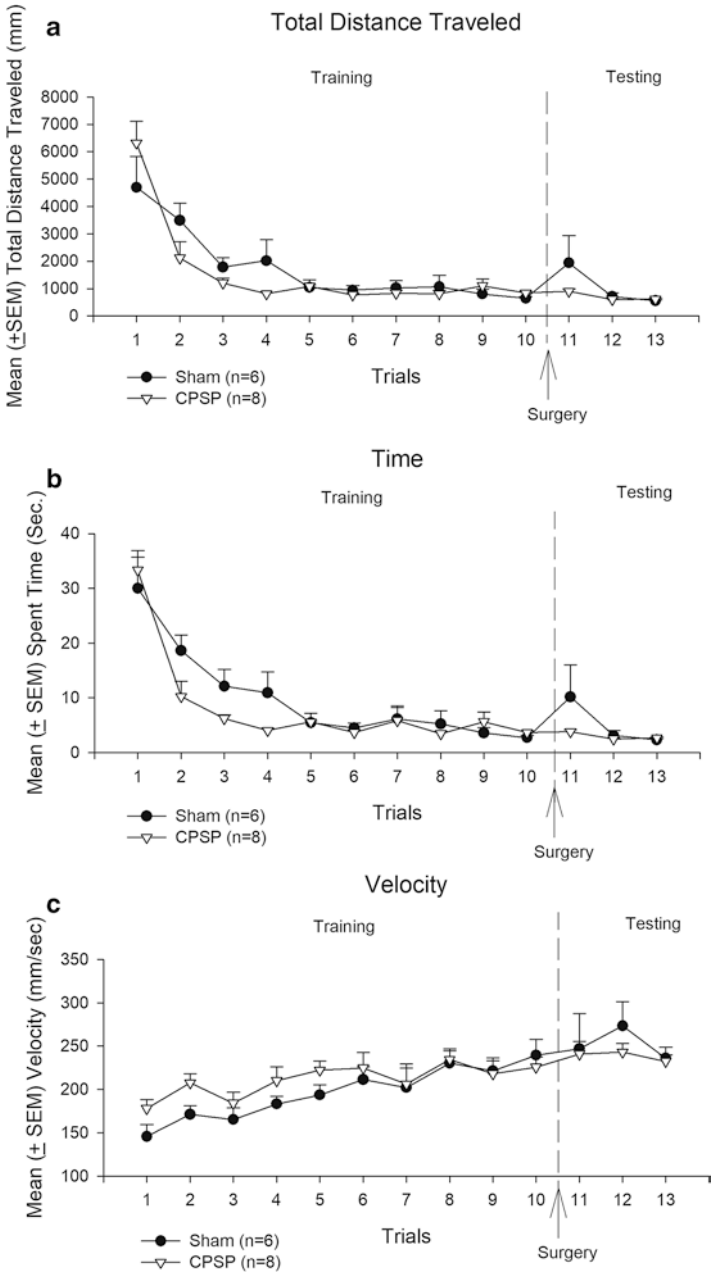

Fig. 20.4 Mean ± SEM (**a**) total distance traveled (in millimeters), (**b**) time spent (in seconds), and (**c**) velocity (mm/s) in the sham and CPSP groups in the acquisition and testing phases. CPSP, central poststroke pain

Table 20.3 Summary of the effects of VBC lesion-induced CPSP on CPP and spatial learning before and after the VBC lesions

	Withdrawal response test			Spatial learning test		
	Left paw	Right paw	CPP test	Total distance traveled	Time spent	Velocity
Acquisition phase	+	+	++	−	−	−
Retrieval phase	+	+	+	−	−	−

+ indicates a significant effect; − indicates a nonsignificant effect; ++ indicates a highly significant effect (i.e., a persistent effect)
The acquisition phase occurred before the VBC lesion, and the retrieval phase occurred after the VBC lesion
CPSP central poststroke pain, *CPP* conditioned place preference, *VBC* ventral basal complex of the thalamus

20.2.3 Issues Emerged: Further Studies in CPSP Learning and Memory

In conclusion, the findings of our animal models of CPSP are not consistent with the previous clinical data. Therefore, the issue of whether lesions of the thalamus, particularly in the ventrobasal complex nuclei, not only induced central neuropathic pain but also produced an alteration of learning and memory in implicit and explicit formats induced. In addition, the neural mechanisms of the brain regarding CPSP and its comorbidities should be investigated further.

20.3 CPSP Comorbidity: Depression and Anxiety

20.3.1 Human Models in CPSP Comorbid Depression and Anxiety

A growing body of clinical data in CPSP has shown that the thalamic stroke hemorrhage could elicit central neuropathic pain, and CPSP is associated with comorbidities such as sleep disturbance, depression, and anxiety [3, 4]. Moreover, neuropathic pain and associated comorbidities including depression and anxiety can be reduced using pregabalin and lamotrigine [5]. Another clinical study found that selective serotonin reuptake inhibitor fluvoxamine could decrease depression symptoms as well as pain symptoms for CPSP patients [16]. Nevertheless, there were conflicting data to demonstrate that CPSP patients may not exhibit comorbid depression and anxiety symptoms. For example, a clinical study indicated that regarding the scores of the Beck Depression Inventory, there were not significant differences between the CPSP and control groups, although thalamic stroke could induce central neuropathic pain [15]. This similar result was also demonstrated in another clinical study;

thus, these authors suggested that CPSP symptoms may exclude comorbid depression and anxiety [7]. Therefore, the findings of the human models of CPSP are not consistent regarding whether CPSP involves depression and anxiety symptoms in addition to the pain symptom.

20.3.2 Issues Emerged: Further Studies in CPSP Comorbid Depression and Anxiety

The following crucial issues should be examined. First, does the CPSP of human models induce comorbid depression and anxiety as well as the neuropathic pain symptom? This issue needs to be scrutinized in the future. Second, further studies should be considered developing animal models of CPSP associated with depression and anxiety symptoms. Furthermore, the CPSP comorbid depression and anxiety of animal models should be evaluated regarding the neural mechanism of the brain, and thereby novel pharmacology and non-pharmacology interventions should be developed to reduce the pain symptom and comorbidities of depression and anxiety.

20.4 CPSP Comorbidity: Motor Function

20.4.1 Human Models in CPSP Motor Function

Previously, a few research studies have been conducted to examine or report whether CPSP patients are associated with any motor symptoms in addition to the neuropathic pain symptom. As we know, a clinical review paper has suggested that CPSP patients may experience mild motor dysfunctions in terms of joint position and vibration sensation; however, the joint position and vibration dysfunctions very seldom occurred for CPSP patients [9]. In conclusion, motor dysfunction is probably not a typical symptom for CPSP; thus, fewer studies reported evidence that CPSP not only induces the pain symptom but also motor deficits.

20.4.2 Animal Models in CPSP Motor Function

Regarding the examinations of the motor function for the animal studies of CPSP, the findings were shown to be diverse [8, 18]. For example, based on our data of the CPSP animal models related to motor function [18], we have demonstrated that rats with lesions of the ventrobasal complex of the thalamus were seemingly unaffected in terms of the various motor indices of motor function, including the latency to fall

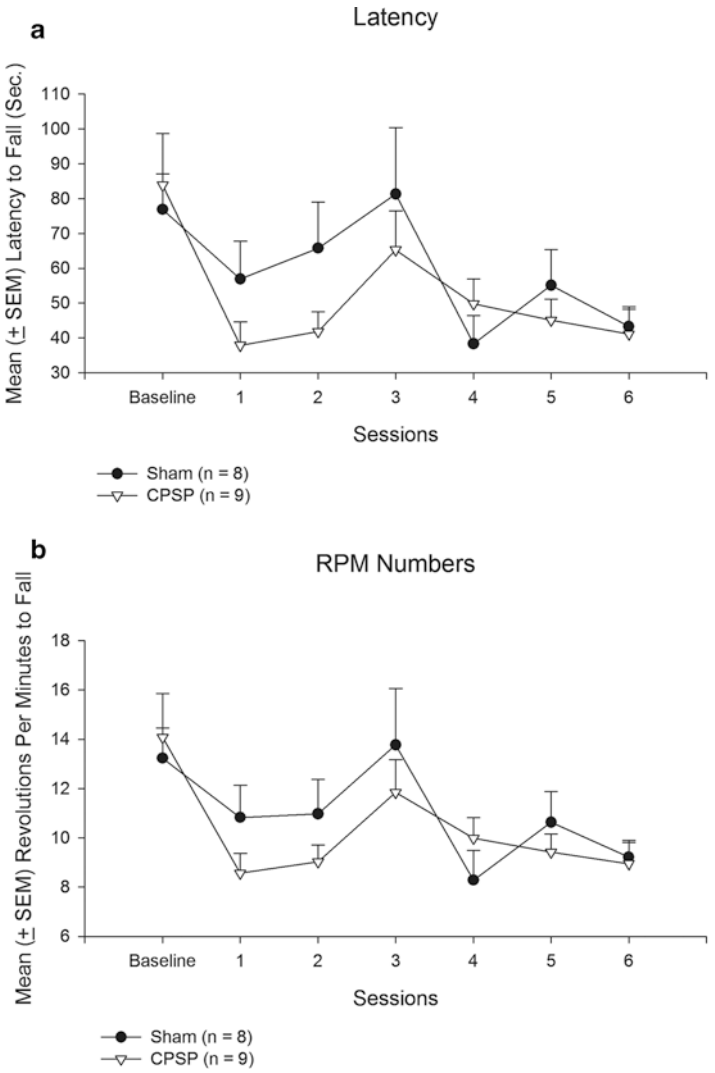

Fig. 20.5 Mean ± SEM (**a**) fall latency (in seconds) and (**b**) rotation speed on various days in the sham and CPSP groups in the rotarod test. CPSP, central poststroke pain

and revolution to fall on the rotarod task (Fig. 20.5). The present data suggest that CPSP might not affect the motor functions of animals. Instead, CPSP animals can still maintain their normal motor activity and function.

Alternatively, another study involving CPSP animal models showed similar results that the locomotion performance was not significantly different between the CPSP and control groups on the rotarod test. However, this study demonstrated that the speed of the CPSP group was significantly slower than that of the control group

in the open-field test. Therefore, the velocity data are not consistent with the viewpoint that lesions of the ventrobasal complex of the thalamus did not change the motor function.

20.4.3 Issues Emerged: Further Studies in CPSP Patients' Motor Function

Based on the data presented above, the animal CPSP data of motor function were likely not very congruent. Moreover, the CPSP animal data regarding motor symptoms were not consistent with the clinical data of CPSP in motor function. That is probably due to the following reasons. The motor dysfunction of clinical patients might involve higher variance; thus, CPSP patients who encountered thalamic stroke were shown to less frequently experience the joint position and vibration sensation. Obviously, the diversity of motor symptoms is restricted in the stroke brain areas. The second question is why the previous findings showed different results. It might be due to the different sensitivities of these motor behavioral tasks. To test motor function, the open-field task might be more sensitive than the rotarod task. Therefore, the motor symptoms or deficit of CPSP symptoms is easily shown through the open-field task. How to determine a suitable animal model to imitate the motor symptoms of CPSP is an essential issue. The research of the neural mechanisms of the brain for CPSP should concern this issue in further studies.

20.5 Conclusions

The present article comprehensively reviews the findings of animal and human models related to the lesion or dysfunction of thalamus-induced CPSP symptoms including central neuropathic pain and its comorbidities of depression, anxiety, and dysfunctions of learning and memory. Although some evidence was not consistent regarding the data of animal and human models, the review provides some implications to examine how the neural mechanism of the brain is involved in CPSP symptoms. Accordingly, the pharmacology and non-pharmacology interventions could be based on the neural mechanism findings to develop a novel treatment for reduction of CPSP symptoms and its comorbidities.

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