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THE ROLE OF SUBSTANCE P AND ITS SIGNALING PATHWAYS IN CENTRAL SENSITIZATION

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THE ROLE OF SUBSTANCE P AND ITS SIGNALING PATHWAYS IN CENTRAL SENSITIZATION

by
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To

My dearest parents

JIALIANG YAN AND MEIBIN SONG

My beloved husband

HUI CHEN

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Central sensitization of nociceptive dorsal horn neurons is an underlying mechanism of behavioral phenotype, such as secondary hyperalgesia. Substance P (SP) is regarded as an important neuropeptide and mainly exists in primary afferent fibers in the superficial dorsal horn. Release of SP in the dorsal horn can be triggered in response to peripheral noxious stimuli. After release, substance P binds with neurokinin-1 (NK-1) receptors, which are abundantly expressed in the superficial layers of the dorsal horn. The first part of this study investigates the change in release of SP in the dorsal horn following intradermal injection of capsaicin and evaluates the role of NK-1 activation in a capsaicin-induced exaggerated pain state in rats. A significant increase of SP in rat dorsal horn was observed after capsaicin injection. Electrophysiological and behavioral results showed that NK-1 activation is required for the development of central sensitization.

Recent evidence has also demonstrated that several important protein kinases are involved in central sensitization evoked by SP. The second part of this study is to investigate the contribution of secondary messengers, such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), to spinal cord pain mechanisms, following NK-1 activation. Spinothalamic tract (STT) cells in lumbar segments of the rat spinal cord were retrogradely labeled with fluorogold (FG). Colocalization of NK-1 receptors with phosphorylated CaMKIIα (pCaMKIIα), or with

PKCγ, was observed in STT neurons, as well as in unidentified dorsal horn neurons. Moreover, intrathecal injection of SP induced increased expression of pCaMKIIα and PKCγ in the superficial dorsal horn. Also, it was demonstrated that activation of CaMKII and PKC plays an important role in SP-mediated pain transduction.

This study helps elucidate the involvement of the NK-1 receptor and its signaling pathways in the nociceptive processing following peripheral inflammation. Hopefully, the results will lead to further understanding of central sensitization, and this will contribute to the improvement in pain therapy.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-t-methylisoxasole-propionic acid
CaMKII	calcium/calmodulin dependent protein kinase II
CGRP	calcitonin-gene releated peptide
CREB	cAMP-responsive element binding protein
CTB	cholera toxin subunit b
DAG	diacylglycerol
DRG	dorsal root ganglion
EAA	excitatory amino acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FG	fluorogold
GFP	green fluorescent protein
HRP	horseradish peroxidase
HT	high threshold
IP_3	inositol-1, 4, 5-triphosphate
LT	low threshold
LTP	long-term potentiation
NK-1	neurokinin-1
NMDA	N-methyl-N-aspartate
NO	nitric oxide
PAG	periaqueductal gray
pCaMKII	phosphorylated CaMKII
pCREB	phosphorylated cAMP-responsive element binding protein
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC_β	phosphoinositide-specific phospholipase Cβ
pPKC	phosphorylated PKC
PWR	paw withdrawal response
SAP	saporin
SMT	spinomesencephalic tract
SP	substance P
SRT	spinoreticular tract
STT	spinothalamic tract
VPL	ventroposterolateral
VR1	vanilloid receptor
WDR	wide dynamic range

CHAPTER 1: BACKGROUND

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with the actual or potential tissue damage, or described in terms of such damage or both". This definition has revealed that besides a sensory-discriminative component, pain also has a motivational-affective aspect, which makes it different from other kinds of sensations, for example, touch or vision (Hardy et al., 1952; Melzack and Casey, 1968; Price 1999). Pain has been recognized as a critical health problem in the community and has high prevalence among the population (Hart, 1974, Crook et al., 1984; Elliott et al., 1999; Brower, 2000). Nearly one third of Americans suffer from chronic or recurring pain (Gershon, 1986). Pain is also the most common and frequent cause of suffering and disability that impairs quality of life. It accounts for about 4 billion work days and $65 billion loss in work productivity in the United States annually (Brower, 2000).

TRANSDUCTION OF PAIN

Based on the time course, the source, or the origin, pain can be classified as acute or chronic, cutaneous, somatic or visceral, peripheral or central. No matter in which form, nociceptive signals are detected in the periphery by free nerve endings of Aδ and C fibers, many of which are important nociceptors (Sherrington, 1906). Upon peripheral stimulation, these fibers with their cell bodies located in dorsal root ganglia (DRG) are activated and may become sensitized and transmit pain signals centrally to the spinal cord or brain stem. As second-order neurons in the pain transmission pathway, specific dorsal horn neurons, such as spinothalamic tract (STT) cells, are responsible for the signal relayed to the thalamus and then to the somatosensory cortex and other cortical areas, upon which the perception of pain depends. In addition, other ascending tracts, including the spinomesencephalic tract (SMT) and spinoreticular tract (SRT), also contribute to conveying pain information from the periphery to the thalamus. These signals are

delivered to brain structures that are responsible for the triggering of the emotional aspect of pain. Furthermore, besides forward neural pathways, feedback control by a descending modulatory system from higher centers is an important feature of pain mechanisms (reviewed in Willis, 1982, 1988).

PERIPHERAL SENSITIZATION

Thinly myelinated Aδ fibers with medium-sized cell bodies in the DRG mediate fast pain, which is described as sharp, acute pain. In contrast, unmyelinated C fibers with small-sized cell bodies in the DRG mediate slow pain, which is reported as dull, throbbing pain (reviewed in Julius and Basbaum, 2001). Besides conduction velocity, another difference between these two kinds of nociceptors is response specificity to different modalities of stimuli. Generally, Aδ fibers are activated by noxious intensities of mechanical and heat stimuli, whereas C fibers often respond to noxious mechanical, thermal or chemical stimuli (Bessou and Perl, 1969).

In addition to these strong noxious stimuli, the nociceptors also show enhanced activity in response to a low pH environment (Steen et al., 1992, 1996) or local release of chemical factors, including bradykinin (Rueff and Dray, 1993a; Liang et al., 2001), catecholamines (Hayley et al., 2002), prostaglandins (Martin et al., 1987; Rueff and Dray, 1993b), neurokinins (Nakamura-Craig and Gill, 1991), 5-hydroxytryptamine (5-HT, Rueff and Dray, 1993b) and cytokines (Sorkin et al., 1997). Such sensitization is termed peripheral sensitization and gives rise to primary mechanical and thermal hyperalgesia and allodynia (Meyer and Campbell, 1981; LaMotte et al., 1982; Merskey and Bogduk, 1994). Hyperalgesia means exaggerated pain in response to noxious stimuli and allodynia refers to pain cuased by originally innocuous stimuli. Primary hyperalgesia and allodynia occur around the injured site and disappear gradually as the injury heals. The molecular basis of peripheral sensitization is the activation of ion channels on the surface membranes of nociceptors, such as transient receptor potential vanilloid 1 (TRPV1) receptors. TRPV1 receptors are found on many polymodal C and mechano-heat Aδ fibers and are activated by capsaicin, other vanilloid chemicals, protons and noxious heat

stimulation (Bevan, 1990; reviewed in Willis and Coggeshall, 2004). Sodium and calcium influx through the channel is initiated following ligand binding, which leads to the excitation and sensitization of the nociceptive neurons (Marsh et al., 1987; Wood et al., 1988).

CENTRAL SENSITIZATION AND CAPSAICIN-INDUCED PAIN MODELS

Following the excitation of nociceptors during peripheral inflammation, the neurotransmission between the primary afferents and dorsal horn neurons is facilitated, which leads to increased activity and prolonged hyperexcitability of spinal cord neurons in response to innocuous and noxious stimuli applied to the inflamed and even adjacent normal tissue. This phenomenon is termed central sensitization. Central sensitization of spinal cord neurons is the mechanism underlying the behavioral phenotypes of secondary mechanical hyperalgesia and allodynia. In contrast to primary hyperalgesia and allodynia, secondary hyperalgesia and allodynia is evoked by stimulation of an adjacent uninjured site. There is no change in the thresholds of primary afferents supplying the area of secondary allodynia and hyperalgesia. It has been proposed that following a relatively strong noxious stimulus, not only the dorsal horn neurons that receive the input from the injured area are activated, but central neurons with their receptive fields in the adjacent normal area are also sensitized. Subsequently, behavioral hyperexcitability in the adjacent normal area develops as a result of the chronic plastic changes in the central nervous system. A lot of evidence has shown that intradermal injection of capsaicin induces both increased sensitivity of nociceptive DRG neurons and primary hyperalgesia in response to heat and mechanical stimuli, as well as secondary mechanical hyperalgesia and allodynia (Gamse et al., 1979; Jancso et al., 1981; Bergstrom et al., 1983; Nagy and Van Der Kooy, 1983; Aimone and Yaksh, 1989; LaMotte et al., 1991, 1992; Simone et al., 1991; Torebjörk et al., 1992; Zhao et al., 1992; Dougherty et al., 1994; Sluka et al., 1997a; Afrah et al., 2002; Zou et al., 2002, 2004; Marivizon et al., 2003; Sun et al., 2004a, b). However, capsaicin injection fails to evoke secondary hyperalgesia in response to thermal stimulation (Willis and Coggeshall, 2004). Similar results were obtained in

both human subjects (Simone et al., 1989; LaMotte et al., 1991, 1992; Torebjörk et al., 1992) and nonhuman primates (Dougherty and Willis, 1991; Dougherty et al., 1992; Palecek et al., 1994a, b) following intradermal injection of capsaicin, which makes capsaicin an important tool in the study of inflammatory pain.

NOCICEPTIVE DORSAL HORN NEURONS IN CENTRAL SENSITIZATION

STT cells in the spinal cord dorsal horn are responsible for nociceptive transmission, especially for the sensory-discriminative aspect of pain, from the spinal cord to the thalamus. (White and Sweet, 1955; Willis et al., 1974; Noordenbos and Wall, 1976; Vierck and Luck, 1979; Chung et al., 1979; Willcockson et al., 1984; Dougherty et al., 1992; Sorkin and Wallace, 1999; Tsuda et al., 1999; reviewed in Willis & Coggeshall, 2004). STT cells are found in humans and mammals, such as monkey, rat and cat (reviewed in Willis and Coggeshall, 2004). The amount and distribution pattern of STT cells vary among the different species. In the rat spinal cord, the number of STT cells is estimated to be over 9,500 (Burstein et al, 1990). The origin and distribution of STT cells can be mapped using retrograde injection of tracers into the lateral and medial thalamus, such as horse-radish peroxidase (HRP, Giesler et al., 1981a, b; Carlton et al., 1990), FG (Zou et al., 2000, 2002, 2004), and cholera toxin subunit b (CTb, Yu et al., 2005). The majority of STT cells in rat spinal cord are found in the cervical and lumbar enlargements. Most STT cells are located in the superficial laminae, the neck of dorsal and the medial intermediate gray matter and are on the side contralateral to the thalamic injection site (reviewed in Kobayashi Y, 1998 and Willis and Coggeshall, 2004). Only a small percentage of STT cells are located on the side ipsilateral to the thalamic injection site (Apkarian and Hodge, 1990).

After decussating in the anterior white commissure, the axons of STT cells ascend through the lateral and ventral funiculi of the spinal cord to the lateral and medial thalamus, respectively (Giesler et al., 1981; Willis et al., 1979; Apkarian and Hodge, 1990). In rats, the axons of most STT cells end in the VPL nucleus, the intralaminar complex, and the posterior complex (Lund and Webster, 1967; Mehler, 1969; reviewed in

Willis and Coggeshall, 2004). Other termination sites of STT neurons include collaterals to the PAG and the brain stem reticular formation (Kevetter and Willis, 1982, 1983; Zhang et al., 1990).

Using antidromic activation from the thalamus, the STT cells in the spinal cord can be identified electrophysiologically. Most STT cells show spontaneous activity without any stimulation. As they chiefly receive excitatory input from primary afferent fibers, STT cells can be activated by electrical stimulation of peripheral nerves. There are two discharge patterns of STT cells in response to volleys evoked by electrical stimulation of peripheral nerves: an early discharge due to A-fiber volleys and a late discharge initiated by C-fiber volleys (Chung et al., 1979; Ferrington et al., 1987). Another important electrophysiological feature of STT cells is that they also respond to natural stimuli of peripheral tissues, including mechanical and thermal stimulation of the skin, mechanical and chemical stimulation of muscle afferent fibers, joint afferents and visceral afferent fibers (reviewed in Willis and Coggeshall, 2004). Based on the responses to a standard series of mechanical stimuli, such as brush (an innocuous stimulus), press (close to the pain threshold), pinch (a noxious stimulus), and squeeze (a strong noxious stimulus), STT cells can be categorized into three classes (Chung et al., 1979; Owen et al., 1992). One class of STT cells, termed wide dynamic range (WDR) neurons, can be activated by noxious stimuli, as well as by innocuous stimuli. Other types of STT cells include high threshold (HT) cells, which respond almost exclusively to noxious stimuli but rarely to innocuous stimuli, and low threshold (LT) cells, which have a higher preference for innocuous stimuli rather than noxious stimuli (Willis et al., 1974, 2001, Giesler et al., 1976; Price et al., 1978; Apkarian and Hodge, 1989). A lot of rat STT cells in lamina I are HT cells with relatively small receptive fields. In contrast, the majority of STT cells in lamina IV-VI belong to the class of WDR cells; these have bigger receptive fields which are limited to a portion of the ipsilateral extremity (Giesler et al., 1981b). Recently, three groups of STT cells in the marginal zone of rat dorsal horn with their axons projecting to VPL nucleus were identified, according to different responses to thermal stimuli (Zhang et al., 2006).

Previous electrophysiological studies have indicated that the responsiveness of STT cells to innocuous mechanical stimuli or heat stimulation can be facilitated following peripheral tissue injury (Kenshalo et al., 1982; Ferrington et al., 1987). Such plastic changes of STT cells have been further investigated in the capsaicin-induced inflammatory pain model. It has been found that both the background activity and the responses to different mechanical or thermal stimuli of STT cells can be enhanced following intradermal injection of capsaicin, leading to primary mechanical and heat hyperalgesia (Simmone et al., 1989; LaMotte et al., 1992; Dougherty and Willis, 1992; Dougherty et al., 1994., Lin et al., 1996a, b, 1997, 1999a, b, 2002; Sun et al., 2004a).

THE ROLE OF SP IN CENTRAL SENSITIZATION

Sensitization of STT cells is closely related to increased spinal release of excitatory amino acids (EAAs) and neurotransmitters, such as SP and calcitonin-gene related peptide (CGRP). Iontophoretically released glutamate and coadministration of SP and EAA in spinal cord dorsal horn induces enhanced responses of STT cells to both innocuous and noxious stimuli (Willcockson et al., 1984; Simone et al., 1991; Dougherty and Willis, 1991, 1992; Dougherty et al., 1995). The role of SP and EAA in central sensitization has been further demonstrated by the fact that application of inhibitors of EAAs and SP receptors could block the sensitization STT cells following capsaicin injection (Dougherty et al., 1992, 1994).

The essential role of SP as an important neuropeptide in neurogenic inflammation and nociceptive neurotransmission has been extensively studied. In 1931, it was first discovered that SP had hypotensive and spasmogenic effects in the mammalian brain and gut (Von Euler and Gaddum, 1931). With the clarification of its sequence, distribution and physiological functions, it was consequently classified as a member of the tachykinin family and is now thought of as a major neurotransmitter in both the central and peripheral nervous systems (Lembeck, 1953; Chang and Leeman, 1970; Erspamer and Melchiorri, 1973; Hökfelt et al., 1975a, b; Nicoll, 1980; reviewed in Erspamer, 1981; Jessel, 1982; Pernow, 1983; Ostuka and Yoshioka, 1993; reviewed in Maggi, 1995; Fürst,

1999; reviewed in Zubrzycka and Janecka, 2000; reviewed in Willis, 2001; reviewed in Willis and Coggeshall, 2004).

SP has high expression in the brain stem and spinal cord dorsal horn, especially in the superficial layers, including laminae I and II, as well as in lamina V (Hökfelt et al., 1975; 1977; Takahashi and Otsuka, 1975; Pickel, 1977; Cuello and Kanazawa, 1977; Gibson et al., 1981; Jancso et al., 1981; de Lanerolle and LaMottte, 1982; Charnay et al., 1983; Bresnahan et al., 1984; Atkinson and Shehab, 1986; Micevych et al., 1986; Vacca et al., 1986; Warden and Young, 1988; Arvidsson et al., 1990; Bonfanti et al., 1991; Marlier et al., 1992; reviewed in Maggi, 1995; Delander et al., 1997; Ribeiro-da-Silva and Hökfelt, 2000; reviewed in Zubryzycka and Janecka, 2000; Ishigooka et al., 2002). Immunostaining evidence has revealed that most SP-immunoreactive fibers in the superficial layers of dorsal horn are small myelinated or unmyelinated Aδ- or C-fibers, with lesser expression in intrinsic neurons in the deeper layers (Hylden and Wilcox, 1981; Nagy and Van Der Kooy, 1983; Lynn and Hunt, 1984). Direct evidence of the expression of SP in nociceptive DRG neurons is provided by the electrophysiological studies of S.N. Lawson et al. (1997). Using intracellular recording from rat DRG somata with a fluorescent dye-filled electrode, individual DRG neurons can be recorded and the expression pattern of neuropeptides in these neurons can be studied by subsequent immunohistochemical staining. It was reported that 20% of DRG neurons are positive for SP-LI (Lawson et al., 1992) and these neurons were all nociceptive, with the majority being Aδ- and C-fibers (Lawson et al., 1997). Unique electrophysiological properties of nociceptive fibers were recorded in these neurons and most of them also contained CGRP, another important neuropeptide for nociceptive neurotransmission (McCarthy and Lawson, 1997).

The nociceptive DRG neurons are the first-order neurons in the pain transduction pathways: they receive input from peripheral tissues and then transmit the signal into the central nervous system by forming synapses with second-order neurons in the superficial layers of dorsal horn. SP can also be synthesized in dorsal horn interneurons. In all these nociceptive neurons, SP is stored in dense-core synaptic vesicles and is usually

colocalized with other neurotransmitters, such as glutamate and CGRP (Pelletier et al., 1977; Pickel et al., 1977; Chan-Palay et al., 1978; Barber et al., 1979; DiFiglia et al., 1982; de Lanerolle and LaMotte, 1983; Bresnahan et al., 1984; Merighi et al., 1989; Ribeiro-da-Silva and Hökfelt, 2000).

The release of SP from the dense-core vesicles into the dorsal horn can be induced in response to a number of forms of noxious stimulation of the primary afferent fibers, including capsaicin treatment (Angelucci, 1956; Otsuka and Konishi, 1976; Gamse et al., 1979; Bergstrom et al., 1983; Duggan and Hendry, 1986; Brodin et al., 1987; Go and Yaksh, 1987; Holzer, 1988; Dougherty and Willis, 1991; McCarson and Goldstein, 1991; Afrah et al., 2001, 2004). Both spinal infusion of capsaicin through a microdialysis fiber (Afrah et al., 2001, 2004) and superfusion of a spinal cord slice with capsaicin (Theriault et al., 1979; Marvizón et al., 2003) could generate increased SP release from the dorsal horn. In addition, it has been suggested that blockade of N-methyl-D-aspartatic acid (NMDA) receptors could inhibit capsaicin-induced SP release (Afrah et al., 2001). SP content in the lumbar spinal cord dorsal horn was also up-regulated in response to noxious mechanical stimulation following formalin-induced peripheral inflammation (McCarson and Goldstein 1991). Using the antibody microprobe technique, increased SP release was also detected in the superficial layers of dorsal horn tissue following either electrical stimulation of a peripheral nerve (Morton et al., 1990) or noxious cutaneous stimuli (Duggan et al., 1988a; Morton et al., 1990). In neuropathic pain models, partial lesions of the sciatic nerve (Wallin and Schött, 2002) or a spinal nerve (Malcangio et al., 2000) induced SP release from the dorsal horn. Nevertheless, SP content in the dorsal horn was decreased following axotomy (Barbut et al., 1981; Jessell et al., 1979).

SPINAL NK-1 RECEPTORS

After release, SP contributes to hyperalgesia and central sensitization by activating NK-1 receptors (Hökfelt et al, 1975a, b; Hylden and Wilcox, 1981; DeKoninck and Henry, 1991; Dougherty et al., 1994, 1995). The NK-1 receptor is a seven transmembrane domain protein, coupled with Gq proteins. The majority of NK-1

receptors are expressed in lamina I of the dorsal horn, the lateral spinal nucleus and the area around the central canal, but very few NK-1 receptors are found in lamina II. Although some are in the deeper layers, a population of neurons with cell bodies in laminae III or IV sends out NK-1 receptor-containing dendrites into the superficial laminae, forming synapses with SP-immunoreactive boutons (Naim et al., 1997). In the superficial layers of the dorsal horn, NK-1 receptors are found mainly in dendrites. In the deep layers of dorsal horn, NK-1 receptors are primarily associated with cell bodies (Moussaoui et al., 1992). It was reported that NK-1 receptors are found in about 35% of STT cells in rat lumbar spinal cord, most of which are distributed in lamina I, the lateral spinal nucleus, laminae III-V, and the area around the central canal (Marshall et al., 1996). For the STT cells in the deep layers, SP interacts with the NK-1 receptors on the dorsal dendrites of these cells, which extend in the superficial layers.

Expression of NK-1 receptors in the dorsal horn is upregulated in a variety of models of pain, such as chronic inflammatory pain, neuropathic pain (Abbadie et al., 1996), or visceral pain (Palecék et al., 2003). Similarly, SP metabolite treatment in the spinal cord also enhanced the immunoreactivity of NK-1 receptors in the dorsal horn (Velázquez et al., 2002). Spinal NK-1 receptor internalization from the plasma membrane to endosomes was observed after local SP treatment, which was blocked by an NK-1 receptor antagonist (Mantyh et al., 1995). Such internalization could be enhanced in response to noxious stimulation of the primary afferent fibers following peripheral inflammation or nerve transaction (Allen et al., 1997). Based on these findings, SP-saporin (SAP), a targeted neurotoxin which selectively eliminates NK-1 receptor containing neurons, was developed and is widely used in pain research. After binding with NK-1 receptors, the SP-SAP conjugate is internalized into the neuron and translocated to endosomes. As a highly active ribosome inactivating protein, saporin interrupts normal protein synthesis and subsequently destroys the NK-1 receptor containing neuron through an interaction with ribosomes. Therefore, this technique is termed “molecular neurosurgery” (reviewed in Wiley and Kline IV, 2000). A significant reduction in expression of NK-1 receptor containing neurons in the superficial dorsal

horn, as well as some in the deep layers, was observed after intrathecal application of SP-SAP (Mantyh et al., 1997; Suzuki et al., 2002). Animals with this lesion showed attenuation of behavioral hypersensitivity induced by a variety of noxious stimuli, such as thermal stimuli (Suzuki et al., 2002; Vierck et al., 2003), intradermal injection of capsaicin, formalin, Freund's adjuvant (Mantyh et al., 1997; Khasabov et al., 2002; Castro et al., 2006), and nerve injury (Suzuki et al., 2005). These results have demonstrated the essential contribution of NK-1 receptors to persistent pain states. The important role of spinal NK-1 receptors in the development of central sensitization was also confirmed in the study of NK-1 receptor knockout mice, by the fact that repeated C-fiber input failed to evoke increased responses of nociceptive dorsal horn neurons in the absence of NK-1 receptors (Dougherty et al., 2001).

Spinal superfusion of SP induces increased neuronal responses to noxious stimuli, which could be blocked by an NK-1 receptor antagonist (Wiesenfeld-Hallin et al., 1984, 1990; Cridland and Henry, 1986; Salter and Henry, 1991; Xu et al., 1990, 1992a, b; Yashpal et al., 1991; Dougherty et al, 1994). Parallel behavioral evidence also suggests that an NK-1 receptor antagonist could attenuate the increased nociceptive responses caused by intrathecal application of SP (Takahasi et al., 1987; Eide and Hole, 1992; Iyengar et al., 1997). Therefore, it is reasonably argued that the effect of SP in central sensitization is mediated through the activation of NK-1 receptors and subsequent down-stream molecules (reviewed in Willis, 2001; reviewed in Willis and Coggeshall, 2004).

POTENTIAL NK-1 SIGNALING PATHWAYS IN THE DORSAL HORN

Central sensitization usually persists for hours, much longer than the peak release and duration of action of EAA or SP in the dorsal horn. It is the subsequent activation of the intracellular signaling machinery that contributes to the prolonged phase of central sensitization (reviewed in Willis 2001, 2002). Our previous studies have identified the involvement of several important signaling pathways in capsaicin-evoked central sensitization, including the PKC, protein kinase A (PKA), nitric oxide (NO)-protein kinase G (PKG), and CaMKII pathways (Lin et al., 1996a, 1999a, b, 2002; Sluka 1997;

Sluka and Willis, 1997; Sluka et al. 1997b; Fang et al. 2002, 2005; Sun et al. 2004b). Activation of the PKC and PKA pathways mediates the phosphorylation of the NR1 subunits of NMDA receptors and therefore enhances their activity in central sensitization and hyperalgesia (Zou et al. 2000, 2002, 2004). Furthermore, it has been suggested that dephosphorylation of some protein kinases could block capsaicin-induced behavioral hyperactivity (Zhang et al., 2003).

In this study, we focus on the investigation of the role of some important protein kinases in central sensitization following spinal NK-1 receptor activation. Local delivery of SP evokes a slow depolarization in dorsal horn neurons, probably by facilitating a voltage-dependent inward Ca^{2+} current (Henry, 1976; Nowak and MacDonald, 1982; Fleetwood-Walker, 1990; Li and Zhuo, 2001). Increased cytoplasmic Ca^{2+} concentration has been observed in dorsal horn neurons after SP application (Womack et al., 1988). Therefore, the signaling pathway following SP binding with NK-1 receptors is supposed to be initiated by the activation of Gq protein and subsequent hydrolysis of membrane phospholipids by activating phosphoinositide-specific phospholipase C_β (PLC_β). One of the hydrolysis products, inositol trisphosphate (IP_3), enhances calcium mobilization from the endoplasmic reticulum (ER). Then both the increased intracellular calcium concentration and diacylglycerol (DAG), another membrane phospholipid hydrolysis product, activate PKC. Protein kinase C is expressed in dorsal horn neurons and has been suggested to be actively involved in the hyperalgesia and central sensitization process. For instance, a PKC inhibitor could not only reduce the nociceptive behavior and attenuate the dose-dependent hyperalgesia in rats following formalin- or capsaicin-induced tissue injury, but can also selectively block the thermal hyperalgesia induced by intrathecal application of SP (Coderre et al., 1992; Sluka and Willis, 1997; Sluka et al., 1997a; Wajima et al., 2000). In electrophysiological experiments, the activation of PKC regulates A-type K^+ current and induces the excitation of STT cells (Palecek et al., 1994; Lin et al., 1994; Peng et al., 1997; Hu et al., 2003a, b). Moreover, capsaicin- or mustard oil-induced central sensitization of dorsal horn neurons was prevented by PKC inhibitors (Munro et al., 1994; Lin et al., 1996a; Sluka et al., 1997b). Involvement of PKC

activation has also been shown in CGRP receptor signaling pathways (Sun et al., 2004b). Subsequently, the activation of PKC leads to the phosphorylation of extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB) in sensitized dorsal horn neurons (Kawasaki et al., 2004).

Increased intracellular calcium concentration is an important down-stream event in the SP signaling pathways. The mobilization of calcium current by SP was observed in murine colonic myocytes, spiral ganglion neurons and human astrocytoma cells. All of these increased calcium currents could be blocked by an intracellular calcium chelator (Ito et al., 2002; Lallemend et al., 2003; Bayguinov et al., 2003; Galiano et al., 2004). Induced influx of Ca^{2+} into these cells is not only a prerequisite for the activation of PKC, but is also actively involved in a variety of biological responses, such as cell growth, differentiation, and migration.

Another important result of Ca^{2+} influx in post-synaptic neurons is the activation of CaMKII, which is a widely distributed serine/threonine kinase in many tissues. There are four isoforms of CaMKII (α, β, γ and δ). CaMKIIα and CaMKIIβ are mainly expressed in the nervous system and can be phosphorylated by Ca^{2+}/calmodulin at different sites. It is suggested that CaMKIIα is the key enzyme in the regulation of synaptic plasticity in learning, memory and LTP in hippocampus. The binding of Ca^{2+}/calmodulin to CaMKIIα leads to conformational changes of the enzyme, which transform the enzyme from an originally inactive form into an active autophosphorylated form at Thr286, subsequently resulting in a significantly increased affinity of the kinase for Ca^{2+} and calmodulin. More importantly, the autophosphorylated kinase shows prolonged activity even at low calcium concentration or after dissociation of calmodulin (reviewed in Colbran, 2004; reviewed in Colbran and Brown, 2004). The change in subcellular localization of CaMKIIα in before and after autophosphorylation has also been studied, using CaMKIIα fused to the GFP in cultured neurons. Translocation of GFP-CaMKIIα to postsynaptic sites was observed after glutamate treatment, which suggests that Thr286-autophosphorylation promotes the interaction of CaMKIIα with synapses (Shen and Meyer, 1999). Furthermore, the requirement of autophosphorylation

of CaMKIIα at Thr286 in the modulation of neuronal function was supported by evidence from CaMKIIαT286A mutant mice (Hardingham et al., 2003).

After autophosphorylation and translocation, activated CaMKII regulates synaptic function by interacting with many receptors, signaling molecules, and transcription factors. For example, the effect of CaMKII on α-amino-5-hydroxy-3- methyl-4-isoxazole propionic acid (AMPA) receptor activity has been well demonstrated in the early phase of LTP (Derkach et al., 1999; Liao et al., 2001; Shi et al., 2001). By phosphorylating the GluR1 subunit of AMPA receptors at Ser831, activated CaMKII facilitates the conductance of AMPA receptors (Derkach et al., 1999). CaMKII may regulate the redistribution of AMPA receptors and enhance the recruitment of AMPA receptors to postsynaptic silent synapses, which originally lack AMPA receptors (Liao et al., 2001; Shi et al., 2001). Activated CaMKII could also trigger CREB-dependent transcription pathways and contribute to the late phase of LTP.

Recently, CaMKII has been demonstrated to be a key molecule in central sensitization with a mechanism similar to that in LTP (Fang et al., 2002). It is reported that phosphorylated CaMKII is constitutively expressed at primary nociceptive afferent synapses in naive rat dorsal horn, which suggests a regulatory role of the kinase in synaptic plasticity (Larsson and Broman, 2005). The expression of phosphorylated CaMKII can be remarkably increased in the superficial layers of the dorsal horn after peripheral injection of capsaicin (Fang et al., 2002) or formalin (Liang et al., 2004). Microdialysis administration of a selective CaMKII inhibitor, KN93, significantly decreased the capsaicin-induced central sensitization of WDR neurons, as well as the enhanced nociceptive behavior in rats (Fang et al., 2002). The role of CaMKII activation in neuropathic pain models has also been illustrated. Up-regulation of both CaMKII and phosphorylated CaMKII in the dorsal horn (Dai et al., 2005) and the trigeminal subnucleus caudalis (Ogawa et al., 2005) was observed following peripheral nerve injury, respectively. KN-93 inhibited the mechanical allodynia induced by nerve transaction (Ogawa et al., 2005). The signaling event after CaMKII activation has also been investigated in central sensitization. It has been demonstrated that CaMKII activation

promotes phosphorylation of both GluR1 subunits of AMPA receptors (Fang et al., 2002) and CREB (Fang et al., 2005) in the rat dorsal horn following capsaicin injection. However, although these results have advocated the involvement of CaMKII activation in central sensitization, limited data are known for its role in SP-mediated nociception in dorsal horn neurons. Choi et al., (2005) found that the expression of phosphorylated CaMKII in the superficial layers of the dorsal horn in mice was significantly increased following intrathcal application of SP, and KN-93 could reverse SP-induced nociceptive behaviors. Therefore, we suggest that CaMKII activation is potentially a crucial part of the NK-1 signaling pathways in dorsal horn neurons.

PROPOSED EXPERIMENTS AND SIGNIFICANCE

In this study, it is hypothesized that intradermal injection of capsaicin causes increased release of SP from rat dorsal horn. SP then activates spinal NK-1 receptors, which contributes to plastic changes in the dorsal horn neuron in response to capsaicin injection. Meanwhile, several signaling pathways in the dorsal horn neurons could be activated following activation of NK-1 receptors.

Aim I: To demonstrate in vivo SP release and the role of NK-1 receptor activation in capsaicin-induced central sensitization

Although increased release of SP in the dorsal horn following spinal capsaicin treatment has been demonstrated in the previous reports, the effect of intradermal injection of capsaicin on spinal release of SP has not been demonstrated. In this study, the effect of intradermal injection of capsaicin on the release of SP from the rat dorsal horn, as well as the changes in SP content in rat dorsal horn tissues, is investigated. Next, in both electrophysiological and behavioral experiments, an NK-1 antagonist is used to determine if spinal NK-1 receptor activation is required for the exaggerated pain state caused by intradermal injection of capsaicin.

Aim II: To determine the signaling pathways following the activation of NK-1 receptor in dorsal horn neurons

So far, limited data are known about the role of second messengers following the activation of NK-1 receptors in dorsal horn neurons, especially in STT cells. It is suggested that activation of spinal CaMKII and PKC is involved in NK-1 signaling pathways in dorsal horn neurons. The distribution of these two kinases and their colocalization with NK-1 receptors in the dorsal horn is investigated in immunohistochemical experiments. The changes of their expression in the dorsal horn in response to spinal SP treatment with or without pretreatment with an NK-1 antagonist are also studied. Furthermore, the functional role of activation of CaMKII and PKC in SP-mediated central sensitization is discussed.

We believe that these findings will not only help in the elucidation of molecular mechanisms underlying inflammatory pain, but will also contribute to the further development of specific antiallodynic and antihyperalgesic drugs.

CHAPTER 2: GENERAL METHODS

EXPERIMENTAL ANIMALS

Adult male Sprague-Dawley rats weighing 260-350g were used in the experiments. All the experimental procedures were approved by the Institutional Animal Care and Use Committee and were consistent with the ethical guidelines of the National Institutes of Health and of the International Association for the Study of Pain.

The animals were housed and maintained in accordance with the guidelines of the University of Texas Medical Branch (UTMB) Animal Care and Use Committee (ACUC). UTMB is a registered research facility under the Animal Welfare Act, with a valid letter of Assurance on file with the Office for Protection from Research Risks, in accordance with NIH policy. UTMB operates to comply with the USDA Animal Welfare Act (Public Law 89-544), amended by PL 91-597 (197), PL 94-279 (1976) and 45CPR37618 (6-30-80), Health Research Extension Act of 1985 (PL 99-158), Public Health Service Policy on Human Care and Use of Laboratory Animals and the Guide the care and Use of Laboratory Animals DHEW (NIH) 85-23, and MLAC approval. The UTMB Animal Care Center is led by a Doctor of Veterinary Medicine and is equipped with an experienced veterinarian expert in laboratory animal care, medicine, diagnostic pathology and surgery.

DRUGS AND SOLUTIONS

Substance P acetate salt hydrate, L-703,606 oxalate salt (an antagonist of NK-1 receptor), BAPTA-AM (a calcium chelator), KN-93 (an inhibitor of CaMKII), NPC15437 (an inhibitor of PKC) are all purchased from Sigma (St. Louis, MO). Substance P, BAPTA-AM, KN-93, and NPC15437 are all dissolved in saline. L-703,606 is dissolved in saline with 10% dimethyl sulfoxide (DMSO).

Artificial cerebrospinal fluid (ACSF): 151.1 mM Na^+, 2.6 mM K^+, 1.3 mM Ca^{2+}, 0.9 mM Mg^{2+}, 122.7 mM Cl^-, 21.0 mM HCO_3^-, 2.5 mM HPO_4^{2-}, 3.87 mM glucose, bubbled with 95% O_2, 5% CO_2, adjusted to pH 7.2-7.4.

Capsaicin: Capsaicin (Sigma, St. Louis) is dissolved in Tween 80 (7%), alcohol (20%), and saline. 0.1% capsaicin is used in the behavioral experiments. 1% capsaicin is used in the electrophysiological studies and 3% capsaicin is used in the ELISA studies.

SPINAL SP CONTENT QUANTIFICATION STUDY

Dorsal quadrant tissue collection

All the rats used in the experiments are anesthetized by sodium pentobarbital (50 mg/kg, i.p.). A surgical laminectomy is performed to expose the lumbar segments of the spinal cord. Dorsal quadrant tissues from the lumbar spinal cord (L4-L6) are collected from four groups of animals: control animals and animals at 5, 30, 60 min after intradermal injection of capsaicin (3%, 50 μl), respectively. Capsaicin is injected into the plantar surface of the glabrous skin of the left hindpaw. All the tissues are then divided into ipsilateral and contralateral groups with respect to the side of capsaicin injection. Immediately after removal, the tissues are placed into liquid nitrogen, followed by homogenation in 50 mol/l Tris buffer, pH 7.4 (0.1 mol/l EGTA, 0.14 μl/ml β-mercaptoethanol, 100 mol/l PMSF, and 0.2 mg/ml trypsin inhibitor). After centrifugation of the homogenate at 13, 000× g for 15 min at 4°c, the supernatant is decanted from the pellet. A BCA kit (Pierce, Rockford, IL) is used to measure the protein concentration in the homogenate, which is used for ELISA analysis at a 1:100 dilution.

ELISA studies

By following the instructions in the manual, the samples are analyzed by using a Substance P ELISA Kit (Cayman Chemical Industry, Ann Arbor, MI). The optical densities (OD) are read on a plate reader (BioRad Laboratories, Hercules, CA) at 450 nm and processed by Dynex Revelation 3.2 software. A standard curve is drawn and the

concentration of SP in each sample can be calculated from the curve, based on its OD value.

IN VIVO SP RELEASE STUDY

Tubing construction

Polyimide tubes (SWPT-008: 0.0089 in ID, 0.00075 wall thickness, Small Parts Inc. Miami Lakes, FL) are used in the sample collection experiments. Under the light microscope, a single 1mm diameter hole is made on a 10 cm-length of polyimide tubing by a surgical scalpel. A tiny drop of superglue is applied in the center of the hole to separate the hole into two compartments. Therefore, the infusion fluid goes into one compartment to exchange with the tissue and is pulled out from the other compartment (Figure 1). A 1.5-2 cm length of PE 20 tubing is wrapped and sealed around the polyimide tubing, 1mm away from the border of the hole, which marks the edge of the vertebrae. One end of the polyimide tubing is glued to a stainless steel dissecting pin (0.1 mm in diameter, 0.5 mm in length), which will guide the polyimide tubing so it can be pulled through the spinal cord. The other end of the tubing is enclosed in a 20 cm length of PE 50 tubing which is connected with the push end of a push/pull infusion pump (Harvard Apparatus, Holliston, MA) after implantation.

Animal preparation and surgical procedures

All the rats used in the *in vivo* SP release experiments are anesthetized by sodium pentobarbital (50 mg/kg, i.p.). A catheter is placed in the jugular vein for continuous intravenous anesthesia (sodium pentobarbital, 5 mg/ml/hr) during the experiments. Laminectomy surgery is performed to expose the spinal lumbar enlargement over a spinal cord length of 3-4 cm. A controlled heating pad with feedback from a rectal thermometer probe is used to keep body temperature at 37°C during the experiment. A polyimide tube with a single hole pore is implanted into the L5 segment by the following steps: the tip of a dissecting pin attached to the fiber is grasped in jewelers forceps and inserted into the

spinal cord at a point approximately one-third the distance between the dorsolateral sulcus and the dentate ligament and out through the contralateral side of the spinal cord at the same horizontal level. The 1 mm length hole is placed within the dorsal horns. The pin is then cut off and the tubing end is glued to premeasured PE 20 tubing, which is connected to the inlet end of a push/pull infusion pump. The other end of the polyimide tubing is enclosed in a PE 50 tubing (inner diameter: 0.58 mm) and connected with the outlet end of the push/pull infusion pump, therefore forming a circuit between the push/pull infusion pump and the polyimide tubing to allow the infusion of ACSF through the spinal cord (Figure 2). The effluent ACSF dialysate samples are collected continuously from the outlet end of the tubing in ice. In order to measure the release of SP in the dorsal spinal cord, ACSF is infused through the polyimide tubing. The ACSF infusion begins one hour before the sample collection so that the substances released due to implantation damage can be flushed out. The total volume of the polyimide tubing and the PE50 tubing is about 10-12 μl and the infusion rate is kept at 2 μl/min. Therefore, it takes about 5-6 min for the effluent samples to go through before collection. The placement of the fiber is confirmed in each animal after euthanization of the rat by an overdose of sodium pentobarbital. The spinal cord tissue is removed and postfixed in 10% formalin. The tissues are then sectioned on a sliding microtome and mounted on a glass slide, followed by staining with neutral red to visualize the fiber placement site. All the sites were found to be in laminae I-V of the dorsal horn in the L5 segment.

Sample collection

Rats are divided into two groups: one group is treated with intradermal capsaicin (3%, 50 μl) injected into the plantar surface of the glabrous skin of left hindpaw and the other group is injected with vehicle of the same volume (control group). Extracellular fluid samples are collected in 500 μl Eppendorf tubes on ice from the outlet end of the tubing, before and after the injection. The samples are then analyzed using the SP ELISA kit. In both groups, the baseline levels of SP concentration are regarded as the concentration of SP samples collected before the injection. The concentrations of SP after the injection at different time periods (5, 10, 20, 30 and 40 min) are expressed as a

percentage of the baseline level and presented as the mean±SEM. Statistical analysis is done using analysis of variance with repeated measures and two sample t-tests for differences from the saline group.

ELECTROPHYSIOLOGICAL STUDY

Construction of microdialysis fibers

Cuprophan hollow fibers (150 μm inner diameter, 9 μm thick wall, 9 kDa molecular cutoff, Spectrum Industry) are used in the electrophysiological experiments. Two 1mm marks, separated by a 2 mm space, are made on a 30cm length fiber. On each side of the marks, a polyethylene sleeve (PE 20 tubing) is placed around the Cuprophan fiber. A few drops of silicon rubber (Dow Chemical 3140 RTN coating) are applied to the fiber. By moving the polyethylene sleeve back and forth, the fiber is coated with a thin layer of silicon rubber, except for the 2 mm long dialysis region. After drying at room temperature, the dull end of the fiber is glued to a pin and the other end of the fiber is connected with a 5 ml syringe on the infusion pump (Harvard Apparatus, Holliston, MA). The dose chosen for the drugs is more than 100 fold higher than the optimal concentration in the spinal cord tissue, which could compensate for the decrease across the microdialysis fiber, and degradation and diffusion within the tissue.

Anesthesia and surgery

The rats used in the electrophysiological experiments are anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for the induction and maintained with continuous intravenous administration of sodium pentobarbital (5 mg/ml/hr) through a jugular catheter. A tracheotomy is carried out and a cannula is placed into the trachea for unobstructed ventilation. A controlled heating pad, as described above, is also put underneath the animal. The level of anesthesia is checked during the experiments by examining corneal and withdrawal reflexes and observation of end tidal CO_2 level. Once a stable level of surgical anesthesia is achieved, the animals are paralyzed with an initial dose of pancuronium (1 mg/kg) and connected with an artificial ventilator to maintain an

end tidal CO_2 of 4.0 ± 0.5%. Laminectomy surgery is performed and a microdialysis fiber is implanted as described above, with the 2 mm collection zone of the microdialysis fiber placed in the dorsal horns bilaterally. The exposed spinal cord is then bathed in a pool of warm (37°C) mineral oil. The fiber is glued to a premeasured length of PE 20 tubing, which is connected with a 1ml syringe on an infusion pump. The infusion rate is maintained at 5 μl/min. Saline is infused when drugs are not given.

Extracellular recording

After the laminectomy surgery, the rat is put in a stereotaxic frame and fixed with two ear bars and a nose clamp. The vertebral column is held stable with vertebral clamps attached to the frame. Under the microscope, the dura of the exposed spinal cord is opened carefully. A pool of warm mineral oil is created over the exposed spinal cord.

A low-impedance carbon fiber microelectrode (3-5 MΩ, Kation Scientific, Minneapolis, MN) is inserted into the spinal cord. Extracellular recordings from dorsal horn neurons in the L_5 segment are made in response to mechanical stimuli (BRUSH and PRESS). The spike activity is amplified and displayed on an oscilloscope and fed into a window discriminator, and then to a computer analysis system (CED 1401), which constructs histograms for data analysis using spike-2 software.

WDR neurons are recognized by their gradually increasing responses to graded intensities of innocuous and noxious stimuli. The search is made in the vicinity of the inserted microdialysis fiber (within 750 μm) to minimize the latency of the onset of drug action and the duration of the subsequent drug washout. Data are analyzed only from recordings of the activity of single neurons whose spike amplitude could be easily distinguished from spikes of neighboring neurons. The experimental trial on each cell begins with the determination of the control background activity and the activity evoked in response to mechanic stimuli. The receptive fields are mapped using a brush stimulus before the application of graded mechanic stimuli. The innocuous BRUSH stimulus is applied by repeated brushing in a stereotyped manner with a camel's hair brush. The PRESS stimulus, which is close to the pain threshold, is delivered with an arterial clip

that exerts a pressure of 144 g/mm^2. The background activity is recorded for 2 min before the mechanical stimulus is applied, The stimulus begins with an additional recording of background activity for 10 sec, and then the BRUSH and PRESS stimuli are applied to the receptive field for 10 sec respectively. Each stimulus is applied to the same marked location.

Data analysis

The stored digital record of unit activity is retrieved and analyzed offline from peristimulus time histograms. Responses to mechanical stimuli applied to the receptive field for 10 seconds are expressed by subtracting the background activity to give a net increase in discharge rate. A repeated measures ANOVA with post hoc Newman-Keuls test is used to compare the difference at each time point tested. All values are expressed as mean ± SEM. Statistical significance is accepted when $p<0.05$.

BEHAVIORAL STUDY

Implantation of catheter

Animals used in behavioral tests are first anesthetized with sodium pentobarbital (50 mg/kg i.p.), for the implantation of an externally accessible intrathecal catheter based on the method described by Storkson et al. (1996). A 2–3 cm long skin incision is made along the dorsal midline over the lower lumbar region. The intrathecal part of a catheter (PE10 tubing, original OD 0.61 mm) is pre-pulled to a diameter half of its original size. Then the catheter is gently inserted into the subarachnoid space through a guide cannula connected to a 20-gauge needle, which pierces the dura mater at the level of the cauda equina. The catheter is carefully threaded rostrally, aiming its tip to reach the dorsal surface of the spinal cord between the L4 and L5 segments. The remaining length of the catheter is sutured to the adjacent muscles and tunneled subcutaneously to the cervical region for intrathecal administration of drugs during the experiment. The catheter (10 μl volume) is filled with ACSF and sealed by heating. The animals are allowed to recover for 5-7 days after the surgery. On the day of the experiment, the animals are checked for

any motor or sensory deficits due to the catheter implantation. Animals with any deficits are discarded. The position of the catheter is checked postmortem.

Test of paw withdrawal responses

The responses of rats to the application of von Frey filaments on the hindpaw are recorded after one-week's recovery from intrathecal implantation surgery. Animals are placed in plastic cages on an elevated screen and allowed to acclimate to the testing environment for 30 min. After adaptation, animals are tested three times at 10 min intervals as baseline. Filaments with three bending forces (20, 60, and 200 mN) are used and each von Frey filament is delivered with enough force to cause slight bending. A positive response is recorded if the hindpaw is sharply withdrawn. Ten applications per filament are made in each testing trial and the number of withdrawals is recorded as a positive response. The observer is blinded to the treatment of the rats. The force of 20 mN is considered innocuous and increased responses to 20 mN indicated mechanical allodynia. The forces of 60 mN and 200 mN are considered noxious stimuli since they activate cutaneous nociceptors (Leem et al., 1993). Therefore increased responses to these two forces are regarded as a sign of mechanical hyperalgesia (Sun et al., 2003; Zhang et al., 2003; Tu et al., 2004).

After the rats are briefly anesthetized with halothane, capsaicin (0.1%, 15 μl) is injected into the plantar surface of the paw about 2 cm away from the testing site. Paw withdrawal response (PWR) is tested at 10, 20, 30, 60, 90, 120, 150 and 180 min after the capsaicin injection. In the experimental groups, L-703,606 at 0.01, 0.1 and 1 mM (15 μl each) is injected intrathecally 30 min after the capsaicin injection, respectively. The same volume of saline is given instead of L-703,606 in the control group.

Data analysis

The responses to repeated applications of von Frey filaments do not have a normal distribution. Two way repeated measures ANOVA with post hoc Student-Newman-Keuls test is used to test the differences between the groups at different time points. A value of $p< 0.05$ is considered significant. All values are given as the mean ± SEM.

IMMUNOBLOTTING STUDY

Antibodies and chemicals

pCaMKIIα (Thr286) and PKCγ rabbit polyclonal antibody are purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NK-1 receptor rabbit polyclonal antibody and beta-actin mouse monoclonal antibody are purchased from Sigma (St. Louis, MO). Biotin-labeled goat anti-rabbit IgG is obtained from PerkinElmer (Boston, MA). Horseradish peroxidase-linked goat anti-rabbit IgG and goat anti-mouse IgG are purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents are obtained from Amersham Bioscience (Piscataway, NJ).

Sample collection and blotting procedures

An intrathecal catheter is implanted in animals used for drug delivery 5-7 days before the experiments. On the day of experiments, the rats are deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Spinal cord tissue in L4-L5 segments is removed and immediately put into liquid nitrogen at 30 min after intrathecal injection of substance P (1 mM, 15 μl) with or without L-703,606 at three doses (0.01, 0.1, 1 mM, 15 μl). Then the tissues are homogenized in ice-cold homogenization buffer (HB) containing phosphatase and protease inhibitors (200 mM calyculin, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 μM microcystin-LR). The homogenate is centrifuged at 13,000 g for 15 min at 4°C. The supernatant is decanted from the pellet and used for all blot analyses. The concentration of protein in the homogenate is measured using the BCA kit (Pierce, Rockford, IL). Equal amounts of protein (60 μg) are fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on size and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The blots are placed in blocking buffer for 1 hr and then incubated in washing buffer. Subsequently, the blots are incubated with primary monoclonal antibody to pCaMKIIα overnight at 4°C. On the second day, the blots are incubated with horseradish peroxidase conjugated IgG (Bio-Rad, Hercules, CA) diluted in 5% non-fat milk in washing buffer. After that, the blots are washed three times for 30

min and incubated with secondary antibody (horseradish peroxidase conjugated with goat anti-rabbit IgG) for 2 hrs at room temperature. Next, the blots are enhanced with a chemiluminescence reagent (ECL kit, Amersham, Arlington Height, IL), followed by exposure to autoradiographic film (Kodak, Rochester, NY).

Data analysis

As an internal control, the expression of β-actin (Sigma, St. Louis, MO) is evaluated in all groups. The intensity of specific immunoreactive bands is quantified using densitometric scanning analyses and the densitometric units of specific bands are expressed relative to the values of background units using AlphaEase software. One-way ANOVA with post hoc t-test is used to compare the differences of the relative density among all the groups. Significance is accepted as $p<0.05$. All data are expressed as mean±SEM.

IMMUNOHISTOCHEMICAL STUDY

Double labeling

Intrathecal implantation for drug delivery is performed on rats one week before the experiments. On the day of experiments, the animals are deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Thirty min after intrathecal injection of substance P (1 mM, 15 μl) or saline (15 μl), the animals are perfused for about 20 min. The perfusion fluid, pumped through the rat aorta after 200 ml of warm heparinized saline, is 4% paraformaldehyde at 4°C. The lumbar spinal cord is removed and immediately placed in fresh fixative for 1.5-2 hrs at 4°C. Then the tissues are incubated in 30% sucrose overnight and frozen-sectioned on a cryostat or a sliding microtome, with a typical thickness of 14 μm. The Tyramide Signal Amplification kit (PerkinElmer, Boston, MA) is used to perform double staining with two polyclonal rabbit antibodies (pCaMKIIα, 1:5,000+NK-1 receptor, 1:5,000, or PKCγ, 1:10,000+NK-1 receptor, 1:5000) on the sections. Absorption control of each antibody is done in respective slides (Figure 3).

Furthermore, in another group of control slides, NK-1 receptor antibody is omitted for the second immunostaining to confirm that second secondary anti-rabbit antibody could not detect pCaMKIIα or PKCγ. Streptavidin Alexa Fluor®488 goat anti-rabbit, Alexa Fluor®568 goat anti-rabbit secondary antibody are obtained from Molecular Probes (Eugene, OR).

Retrograde labeling of the STT cells

Retrograde labeling of the STT cells is done in anesthetized rats (sodium pentobarbital, 50mg/kg i.p.). Fourteen injections of 0.2 μl of 2% fluorogold (FG, Fluorochrome, Denvor, CO) into the VPL nucleus of the right thalamus, using a 5 μl Hamilton microsyringe, are performed after craniotomy surgery. The coordinates are obtained from a stereotaxic atlas of the rat brain (Paxinos and Watson, 1986). All the rats are allowed to recover for about one week. On the day of experiment, the rats are re-anesthetized with sodium pentobarbital (50mg/kg i.p.) and are perfused transcardially with 200 ml of warm heparinized saline, followed by 4% paraformaldehyde (pH 7.4, 4°C) after drug treatment. The brain and spinal cord tissues of rats are collected after anesthesia and perfusion. The brain and spinal cord tissues are cut transversely on a freezing microtome at 40 and 14 μm, respectively. Immunofluorescence staining for NK-1 receptors and protein kinases are performed as described above.

Visualization of the sections

A Nikon microscope linked through a video camera to an NIH Image Computer-Assisted image processing system is employed to visualize and quantify the immunoreactivity of the sections. The sections are examined at 200× and 600× magnification. The entire superficial dorsal horn from the lumber segments is outlined to quantify the density of pCaMKIIα-LI or PKCγ -LI. The density of pCaMKIIα-LI or PKCγ -LI within the superficial laminae on 15-20 sections per animal is calculated and averaged. The profiles of retrogradely labeled STT cells are counted. Counts are obtained for cell bodies in the dorsal horn that contained the FG retrograde tracer. Twenty-five sections that are separated by at least 50 μm between sections are selected from each

animal for counting and averaging cell numbers. The percentages of all labeled STT cells that are positive for pCaMKIIα or PKCγ in each group of animals are counted.

Data analysis

The differences of the average density of pCaMKIIα-LI or PKCγ -LI in the superficial dorsal horn between the saline and SP groups are compared using a two sample t-test. T-tests are also used to examine the differences of percentage of pCaMKIIα-LI STT cells or PKCγ -LI STT between the two groups. Significance is accepted as $p<0.05$. All data are expressed as mean±SEM.

Figure 1: The single pore fiber used in sample collection experiments. A single 1mm diameter hole is made on a 10 cm-length of polyimide tubing with a tiny drop of superglue in the center of the hole to create two separated compartments.

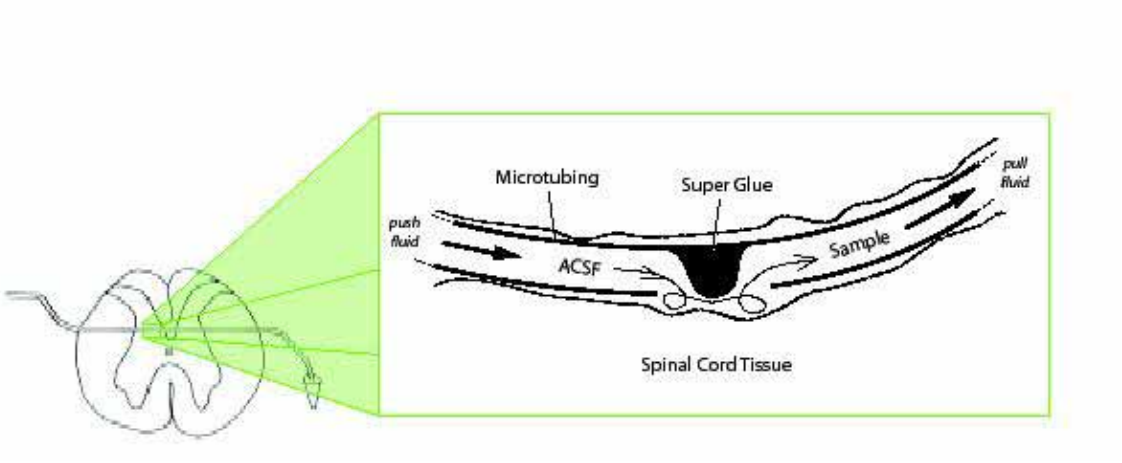


Figure 2. Illustration of the sample collection experiment in the rat spinal cord dorsal horn. Rat spinal cord is exposed at the lumbar segmental level and a polyimide tubing with a single pore is implanted into the L_5 segment. One of the tubing end is connected with the push end of a push/pull infusion pump and the other end of the tubing is connected with the pull end of the push/pull infusion pump. As a result, the infusion fluid enters one compartment and exits through the other compartment after fluid exchange in the spinal cord tissue.

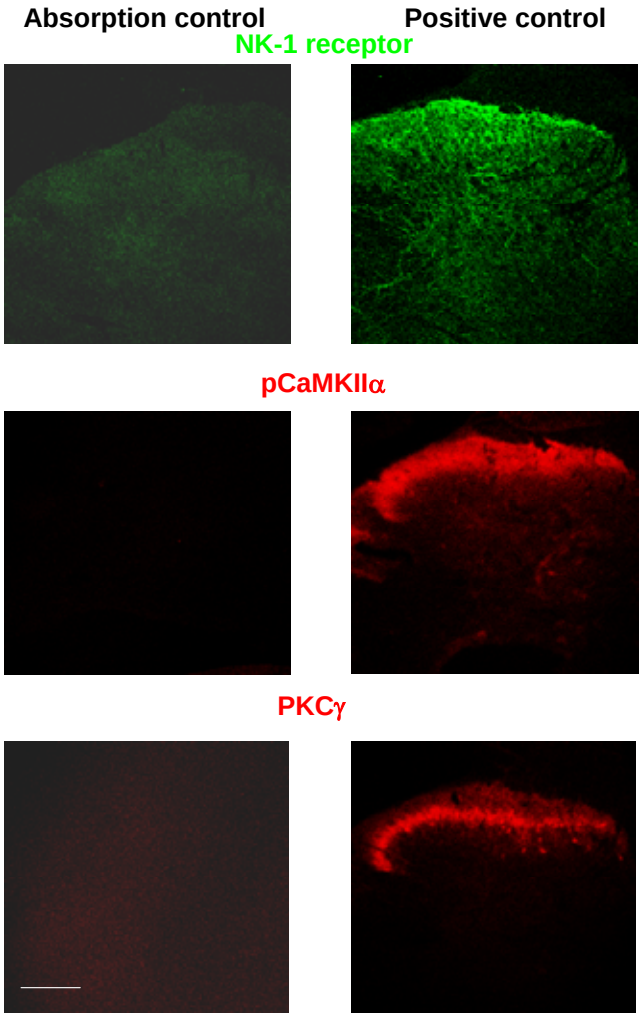


Figure 3. Results from the absorption control experiments show the specificity of anti-NK-1, anti-pCaMKIIα and anti-PKCγ. First panel: anti-NK-1 receptor; Second panel: anti-pCaMKIIα; Third panel: anti-PKCγ. Left column: absorption control; Right column: positive control. Bar scale: 150 μm.

CHAPTER 3: RESULTS

RELEASE OF SP AND ACTIVATION OF NK-1 RECEPTORS IN THE DORSAL HORN FOLLOWING INTRADERMAL INJECTION OF CAPSAICIN

In this study, the correlation between SP release and capsaicin-induced central sensitization is discussed. We hypothesize that peripheral capsaicin injection induces SP release from the primary afferents in the dorsal horn. Spinal NK-1 receptors are activated by the increased SP, which subsequently contributes to an exaggerated pain state. First, *in vivo* SP release following intradermal injection of capsaicin was determined, using a novel single pore fiber introduced into the rat dorsal horn. The SP content in the dorsal horn tissues was also analyzed before and after peripheral capsaicin injection. Next, extracellular recordings were made from rat nociceptive dorsal horn neurons and the effect of a new NK-1 receptor antagonist, L-703,606, on capsaicin-induced central sensitization was tested. Finally, the involvement of NK-1 receptors in secondary mechanical allodynia and hyperalgesia was investigated in a behavioral study.

In vivo release of SP from rat spinal cord dorsal horn

In both vehicle and capsaicin-treated groups, extracellular fluid samples were collected at 10 min intervals and SP concentration in the samples was analyzed using ELISA. A peak of SP release was observed 10 min after capsaicin injection, compared with that in the vehicle group (n=8 in each group, Figure 4). Thirty minutes after the capsaicin injection, the increased SP release declined and recovered to baseline level. Figure 5 demonstrates that there was a significant increase in SP release in the capsaicin-injection group, when the values of mean area under the curve (MAUC) from 0 to 20 min after capsaicin- or vehicle-injection are compared.

Content of SP in dorsal horn tissues

Twenty-eight rats were used for analysis of SP content analysis in dorsal horn tissue. In the experimental groups, the tissues were collected at 5, 30, and 60 min after intradermal injection of capsaicin, respectively. The SP content in the tissues was

analyzed using ELISA. Figure 6 compares the difference of SP content in the ipsilateral side and contralateral side at different time points. The results suggest there is no significant difference in SP content between ipsilateral and contralateral sides of the dorsal quadrant tissue after capsaicin injection ($p>0.05$, one-way ANOVA).

Electrophysiology

The activity of a total of 17 WDR neurons in the L5 segment with afferent input from the plantar surface of the hindpaw was recorded. Extracellular recordings were made from 290-780 μM below the surface of the spinal cord. Most of the neurons were estimated to be located in laminae V and VI (500-800 μM). All the recorded neurons exhibited spontaneous background activity and responded to both BRUSH and PRESS.

Figure 7 shows rate histograms of the activity of one representative WDR neuron in response to capsaicin injection (1%, 0.02 ml). The top row illustrates the baseline background activity and responses to BRUSH and PRESS stimuli. After the baseline level was recorded, capsaicin was injected, and 15 min later, saline infusion in the spinal cord was started. The second row shows a significant increase in the background activity immediately following the capsaicin injection; this increase was termed the "capsaicin-peak". This capsaicin-induced activity then slowly decreased, but was still much higher than the baseline level and persisted even 15 min after the injection. The responses to PRESS 15 min (the third row) and 45 min (the fourth row) after capsaicin injection were also increased, suggesting that saline infusion had no effect on the increased activity induced by capsaicin. Grouped data from a total of eight WDR neurons are summarized in Figure 8, which shows that intradermal injection of capsaicin elicited a significant increase in the background activity and responses to PRESS. The mean background activity was increased to 216.5 ± 48.5% at 15 min and 199.0 ± 30.6% at 45 min after the induced inflammation, respectively. The average response to PRESS was increased to 169.2 ± 34.0% at 15 min and 193.2 ± 47.5% at 45 min after the injection, respectively. However, the responses to BRUSH remained the same before and after capsaicin.

In another group of animals, a specific NK-1 antagonist, L-703,606 (100 μM), was infused 15 min after the capsaicin injection, instead of saline. The activity of a representative WDR neuron is illustrated in Figure 9. There was a capsaicin peak immediately following the injection (the second row) and increases in the background activity and responses to PRESS 15 min after injection (the third row). However, 30 min after L-703,606, the capsaicin-induced WDR neuron activity was attenuated (fourth row). The statistical analysis of grouped data from a total of nine neurons in Figure 10 demonstrates that the background activity and responses to PRESS were increased 15 min after capsaicin injection, compared with the baseline level. However, the increased activity was significantly reduced by the superfusion of L-703,606 for 30 min, compared with the values at 15 min after capsaicin injection. The mean background activity was decreased from 272.0 ± 50.1% to 124.3 ± 43.4% and the average response to PRESS was reduced from 153.6 ± 16.1% to 120.5 ± 20.0%. These results suggest that the activation of NK-1 receptors plays an important role in capsaicin-induced central sensitization.

Behavioral study

Three von Frey filaments with bending forces of 20, 60 and 200 mN were used to test PWR in rats before and after intradermal injection of capsaicin. Before capsaicin, almost no withdrawal responses were evoked by the mechanical stimuli, which were regarded as baseline level. After the capsaicin injection, there were significant increases in PWR on the side ipsilateral to the injection, starting from 10 min and reaching a peak level at about 30 min after the injection, compared with the contralateral side (Figure 11, $p<0.05$, one-way ANOVA). For the three mechanical forces applied, the PWR increased in proportion to the stimulus intensity. In the saline group, such increases persisted for over 180 min after the capsaicin injection, suggesting that saline infusion had no effect on capsaicin-induced hypersensitivity.

However, in the experimental group, intrathecal injection of the NK-1 antagonist significantly attenuated nociceptive behaviors induced by intraplantar capsaicin. In the ipsilateral paw, L-703,606 decreased the responses to von Frey filaments of three bending forces from 30 to 180 min after the capsaicin injection, compared with the saline group

(Figure 12A, 13A, 14A, *$p<0.05$, one-way ANOVA with post hoc Student-Newman-Keuls test). The time courses for these behaviors are shown in Figures 12B, 13B and 14B, suggesting an inhibitory effect of L-703,606 on the capsaicin-evoked responses ($p<0.05$, two-way repeated mesuares ANOVA with post hoc Student-Newman-Keuls test). The most obvious effect of L-703,606 was seen using the 200 mN von Frey filament, compared with the parallel data in the control group. Both the duration and magnitude of the capsaicin-induced behaviors were reduced by the application of L-703,606 at concentrations of 0.1 and 1 mM (Figure 14 B). However, treatment with L-703,606 at a concentration of 0.01 mM had no effect on the increased response.

CHANGES OF CaMKIIα IN THE RAT DORSAL HORN FOLLOWING SPINAL NK-1 ACTIVATION

In this study, phosphorylation of CaMKIIα after intrathecal injection of SP was examined in the rat spinal cord, using an antibody that recognizes pCaMKIIα at site Thr286 for Western blots and immunofluorescent labeling. STT cells were identified by retrograde transport of FG from the VPL nucleus of the thalamus. Double labeling experiments for pCaMKIIα and NK-1 receptors were performed on STT cells, as well as on unidentified dorsal horn neurons. The effect of an inhibitor of CaMKII on SP-induced sensitization of nociceptive dorsal horn neurons was also investigated using extracellular recording, respectively.

Immunohistochemistry and retrograde labeling

The distribution of pCaMKIIα in rat spinal cord is illustrated in Figure 15. A dense band of pCaMKIIα-IL was found in the superficial layers of the dorsal horn with some expression in the deep layers of the dorsal horn, the area around the central canal, and the ventral horn (Figure 15A). Figure 15B shows the immunofluorescent images of STT cells identified with FG and labeled for pCaMKIIα in the dorsal horn after saline injection. The FG injection sites in the right thalamus are shown in Figure 16A. Consistent with previous studies (Burstein et al., 1990; Zou, et al., 2000, 2002, 2004; Yu et al., 2005), retrogradely labeled STT cells in the lumbar segments were observed

predominantly in lamina I, lamina III-VII, and the area around the central canal of the left dorsal horn, with few in the right dorsal horn (Figure 16B).

The colocalization of pCaMKIIα and NK-1 receptors was characterized using double immunostaining. The photomicrographs in Figure 17A1-A3 illustrate the distribution of pCaMKIIα and NK-1 receptors in the superficial dorsal horn. Extensive co-expression of pCaMKIIα and NK-1 receptors was found in laminae I of the dorsal horn (Figure 17A3). Examples of double-labeled neurons in lamina I and in the deep layers are represented in Figure 17B1-B3 and Figure 17C1-C3, respectively. We also investigated the two patterns of staining in FG-labeled STT cells. Figure 18 illustrates some representative STT cells that contain NK-1 receptors in both the superficial and deep laminae of the dorsal horn in the control rat. The immunoreactivity for NK-1 receptors was found in the cell bodies, as well as the dendrites of the STT cells. Co-existence of pCaMKIIα and NK-1 receptors in STT cells was mainly found in the superficial layers (Figure 19), as well as some in the deep layers of the dorsal horn.

The change of pCaMKIIα expression in the dorsal horn in response to SP treatment was examined. Thirty minutes after intrathecal SP (1 mM, 15 μl) injection, pCaMKIIα-LI was greatly increased in the superficial dorsal horn, compared with the control group (Figure 20A). The average density of pCaMKIIα-LI per section in the superficial layers was 132.2 ± 20.38 in the SP-injected group and 66.82 ± 13.20 in the saline injected group. The difference was significant ($n=6$, $p<0.05$, t-test, Figure 20B).

The distribution of STT cells in rat spinal cord is described in Table 1, which shows that there was no significant differences of the number of STT cells in different laminae between the saline and SP injected groups. The percentage of pCaMKIIα-LI STT neuronal profiles in the dorsal horn was counted in these two groups (Figure 21). The proportions of pCaMKIIα-LI STT neuronal profiles in laminae I-III or in laminae IV-VII in the saline group were 13.33±7.67% or 40.37±10.37%, respectively. There were significant increases in the proportion of STT cells containing pCaMKIIα-LI both in the superficial laminae (96.67±2.16%) and the deep laminae (91.35±3.17%) after intrathecal

SP injection. There was no difference in the total number of STT cells in the dorsal horn between the two groups.

Western blots

To investigate the effect of spinal NK-1 receptor activation on pCaMKIIα expression in rat dorsal horn, the relative density of immunoblots of pCaMKIIα was analyzed. Saline (15 μl) was used as control of SP and 10% DMSO (15 μl) was used as control of L-703,606. A significant increase of pCaMKIIα expression was observed 30 min after intrathecal SP injection (1 mM, 15 μl), compared with the control group. However, this SP-induced increase was blocked by pretreatment with an NK-1 receptor antagonist, L-703,606 (1 mM, 15 μl), 30 min before SP injection. Lower doses of L-703,606 (0.1 or 0.01 mM, 15μl) had no inhibitory effect on pCaMKIIα expression induced by SP (Figure 22A). Grouped data are summarized in Figure 22B (n=6-7, $p<0.05$, one-way ANOVA).

Electrophysiology

The effects of an inhibitor of CaMKII on the central sensitization of dorsal horn neurons following spinal SP infusion were tested. The background activity and responses to mechanical stimuli were recorded before the drug treatment as baseline. In the experimental groups, all the responses were recorded 30 min after infusion of KN-93 (100 μM), through a microdialysis fiber inserted in the dorsal horn. In the control group, saline infusion was used as control for KN-93. In both groups, SP (100 μM) was infused for 30 min following the drug or saline and the test was repeated at the end of SP infusion.

Extracellular recordings were made from WDR neurons located in the L5 segment of the spinal cord at depths of 250 to 760 μm. The activity of a representative nociceptive neuron in the dorsal horn in response to the application of SP is illustrated in Figure 23. The first row of the rate histogram represents the baseline activity of a typical WDR neuron to mechanical stimuli. Saline infusion for 30 min had no effect on the neuronal activity, as shown in the second row of Figure 23. However, the responses of this WDR

neuron to BRUSH and PRESS stimuli were significantly enhanced after SP infusion for 30 min (100 μm in the microdialysis fiber), compared with the baseline level (the third row, Figure 23). There was no obvious increase in the background activity after SP treatment.

Figure 24 illustrates a typical experiment on one WDR neuron after KN-93 (100 μM) infusion. KN-93 infusion for 30 min had no obvious effect on the baseline level (the second row). The responses to mechanical stimuli were not obviously increased following SP infusion (the third row). The grouped data from a total of 7 WDR neurons are presented in Figure 25, which shows a significant reduction of SP-induced responses to mechanical stimuli after KN-93 pretreatment, compared with the saline group ($n=7$, $p<0.05$, one-way ANOVA). The responses were greatly reduced in the KN-93/SP group (post-SP BRUSH: 105.5± 16.18%; post-SP PRESS: 68.53 ± 15.37%).

ACTIVATION OF PKC IN DORSAL HORN NEURONS FOLLOWING SPINAL SP TREATMENT

Immunohistochemistry and retrograde labeling

Figure 26 shows the distribution of PKCγ and NK-1 receptors in the dorsal horn of L4-L5 segments in saline-treated rats. In transverse sections of spinal cord, colocalization of PKCγ and NK-1 receptors was mainly observed in the superficial part of the dorsal horn. The two labels were also distributed in the soma and dendrites of FG-labelled STT cells in the dorsal horn (Figure 27). The average density of PKCγ-LI in the superficial layers was significantly increased after intrathecal SP injection (174.5 ± 13.9), compared with the saline group (83.8 ± 24.2) ($n=6$, $p<0.05$, t-test, Figure 28).

Western blots

Increased expression of PKCγ in the rat dorsal horn tissues was detected 30 min after intrathecal SP injection (1 mM, 15 μl), compared with the saline group. Similarly, L-703,606 prevented this SP-induced increase in a dose-dependent manner (Figure 29A). Statisitical results from grouped data indicates the inhibitory effect of L-703,606 at a relatively high dose (1 mM, 15 μl) ($n=8$, $p<0.05$, one-way ANOVA, Figure 29B).

Electrophysiology

These experiments tested if activation of PKC contributes to SP induced sensitization of nociceptive dorsal horn neurons. The spinal cord was pretreated with a PKC inhibitor, NPC15437 (10 mM), for 30 min before the administration of SP. Figure 30 shows rate histograms of typical WDR neurons that illustrated inhibitory effects of NPC15437 on SP-induced enhancement of responses to mechanical stimuli. No changes caused by the drug itself were observed in background activity or responses to mechanical stimuli (the second row, Figure 30). Nevertheless, SP-induced enhancement of responses to mechanical stimuli was clearly prevented following the drug pretreatment (the third row, Figure 30). The statistical analyses of grouped data are summarized in Figure 31, which illustrate that pretreatment of NPC15437 has blocked SP-induced sensitization, compared with the saline group ($n=8$, $p<0.05$, one-way ANOVA).

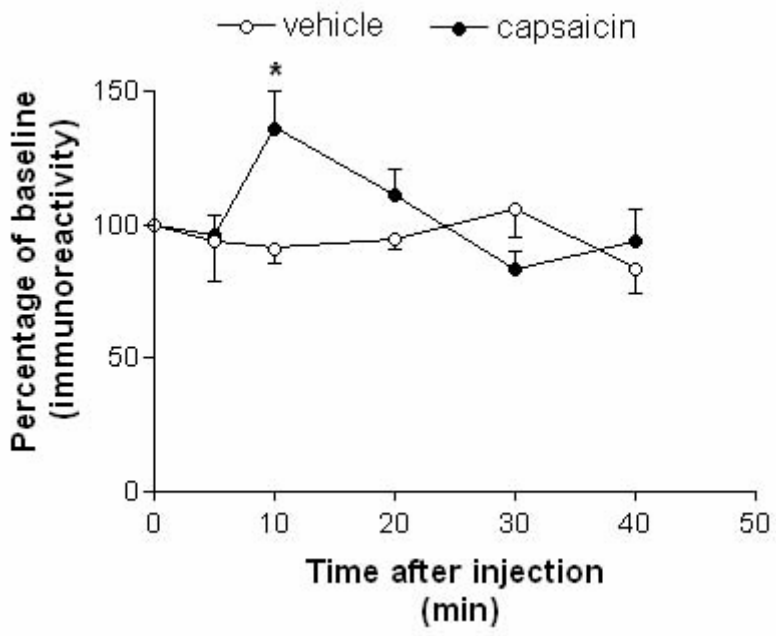


Figure 4. *In vivo* release of SP from rat dorsal horn before and after capsaicin or vehicle injection. The baseline SP concentration was regarded as that in the samples collected before the injection in each group. The concentration of SP after saline- or capsaicin-injection during each time period was expressed as percentage of the baseline level. Statistical analysis was done using analysis of variance with repeated measures and two sample t-tests to compare differences between these two groups during each time period (n=8). *$p<0.05$, compared with the vehicle group.

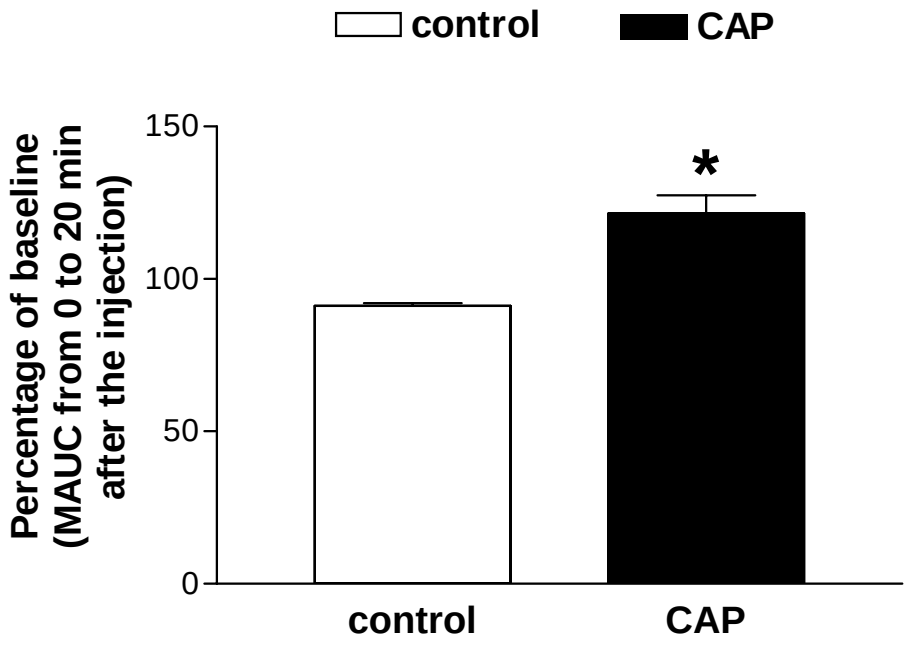


Figure 5. Bar graph summarizes the grouped data for the values of mean area under the curve (MAUC) from 0 to 20 min after the intradermal injection. A two sample t-test was used to evaluate the difference between the two groups (n=8). *$p<0.05$, compared with the control group.

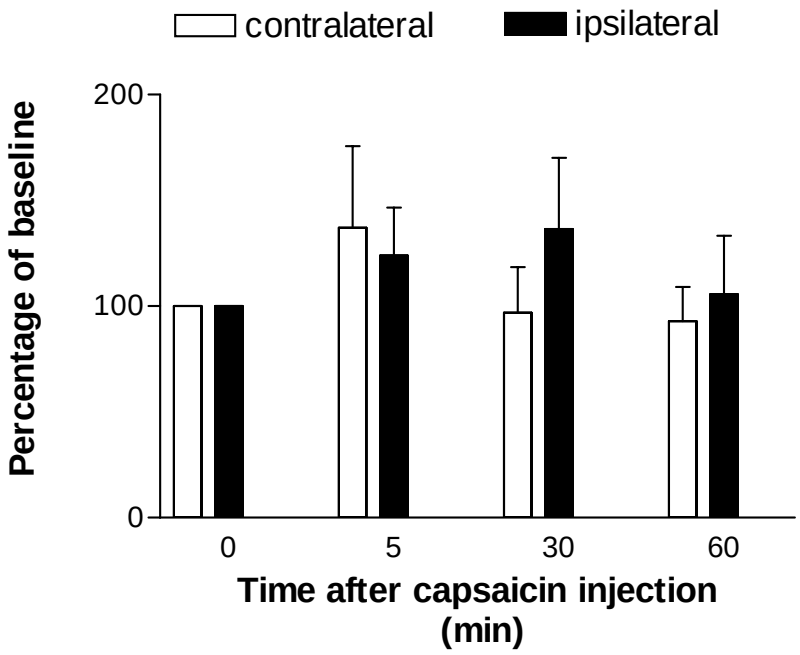


Figure 6. Comparison of SP content in the spinal cord tissue following intradermal injection of capsaicin at different time points. Differences between the ipsilateral and contralateral side of the dorsal quadrant tissue are compared using one-way ANOVA (n=7 in each time point, $p>0.05$). The SP content in the ipsilateral and contralateral side of the dorsal quadrant tissue in the control group are regarded as baseline.

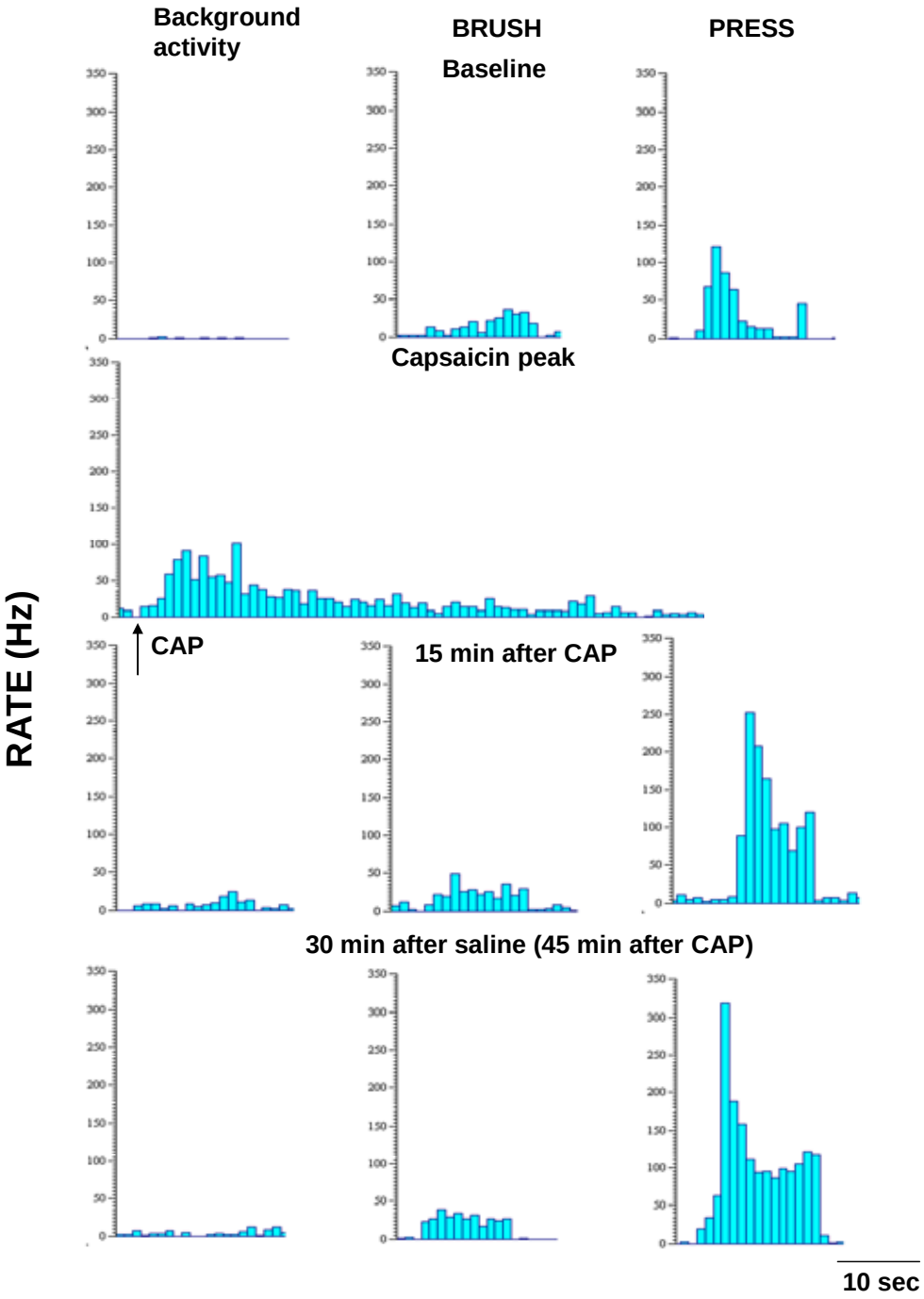

Background activity
BRUSH
Baseline
PRESS
Capsaicin peak
CAP
15 min after CAP
30 min after saline (45 min after CAP)
RATE (Hz)
10 sec

Figure 7. Central sensitization in the rat dorsal horn caused by intradermal capsaicin injection. Rate histograms represent the activity of a representative WDR neuron before and after capsaicin injection. *Top row*, Background activity and responses to mechanical stimuli (BRUSH and PRESS) before capsaicin injection. *Second row*, Capsaicin-induced increase of background activity. *Third to fourth row*, the activity of the WDR neuron at different time points after capsaicin injection.

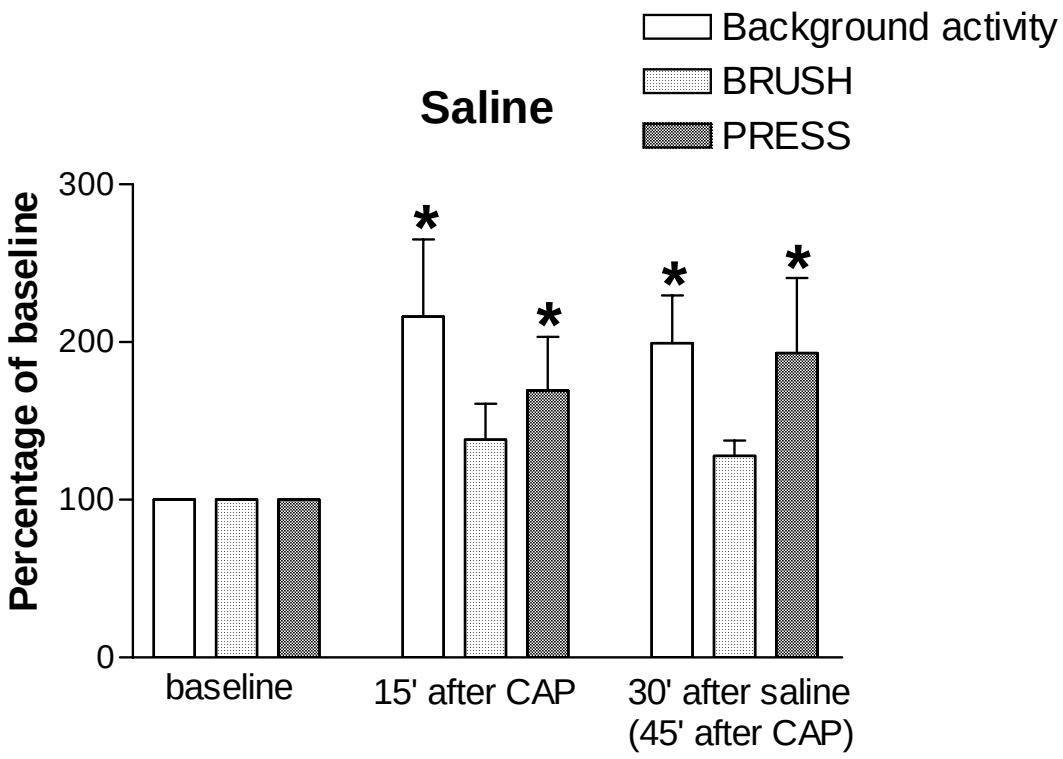


Figure 8: Bar graph summarizes the grouped data for the responses of nociceptive dorsal horn neurons to capsaicin from a total of eight animals. The values were expressed as percentage of baseline. *$p<0.05$, compared with the baseline level (n=8, a repeated measures ANOVA with post hoc Newman-Keuls test).

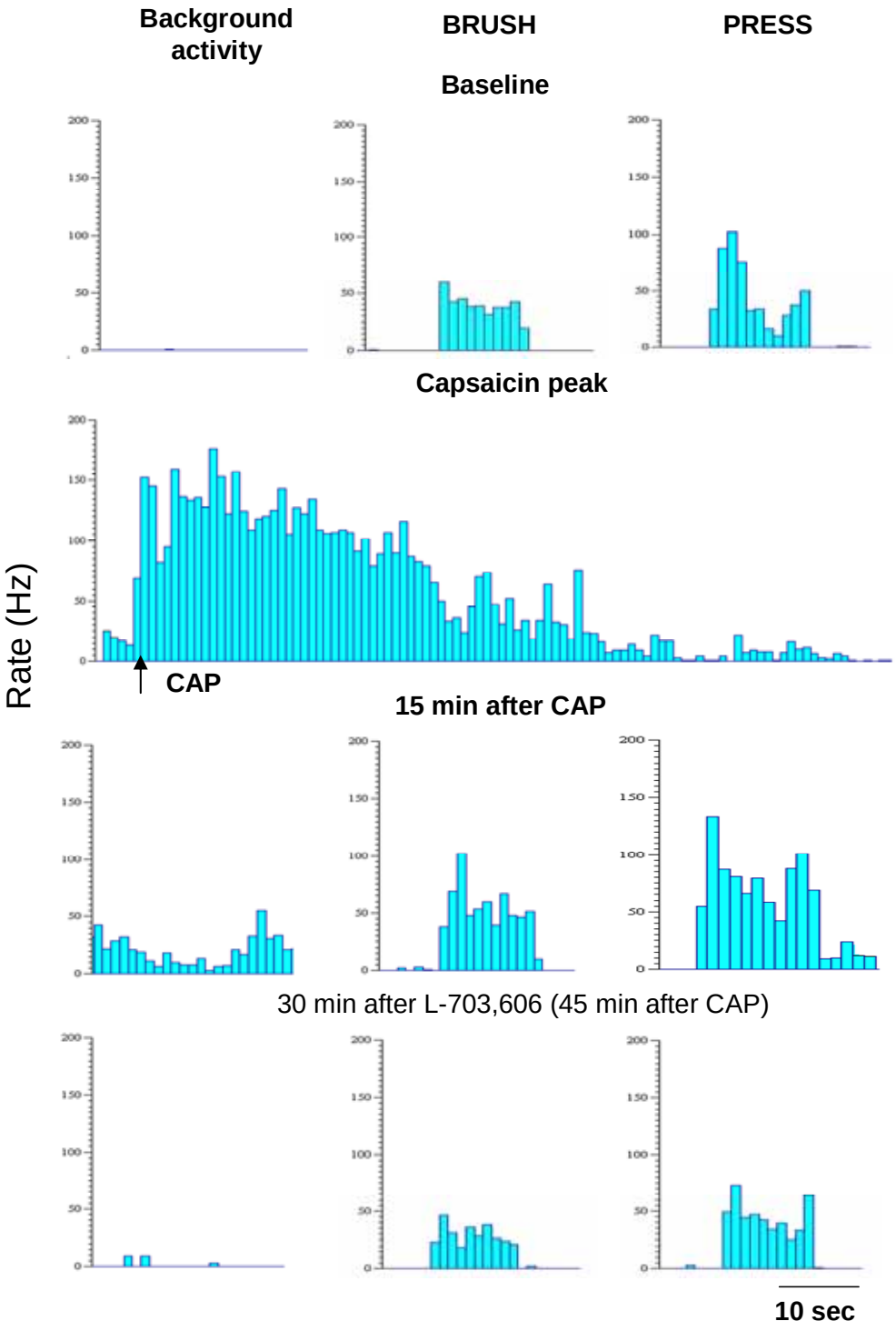

Background activity
BRUSH
PRESS
Baseline
Capsaicin peak
Rate (Hz)
CAP
15 min after CAP
30 min after L-703,606 (45 min after CAP)
10 sec

Figure 9. An NK-1 antagonist (L-703,606) reversed capsaicin-induced central sensitization of WDR neurons. *Top row*, Background activity and responses to BRUSH and PRESS. *Second row*, Capsaicin-induced increase of background activity. *Third row*, Background activity and responses to mechanical stimuli at 15 min after capsaicin injection. After the testing, L-703,606 (100 μM in the microdialysis tube) is infused into the spinal cord for 30 min. *Fourth row*, Background activity and responses to mechanical stimuli after 30 min infusion of L-703,606 (45 min after capsaicin injection).

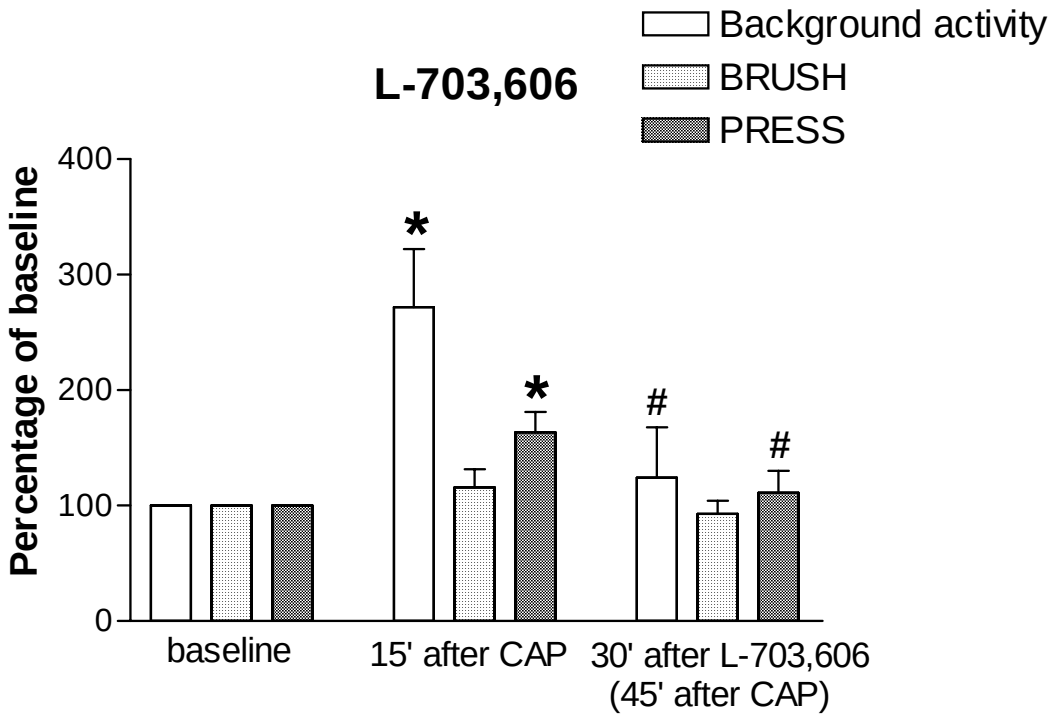


Figure 10. Bar graph summarizing the effect of L-703,606 on the capsaicin-induced activity of dorsal horn neurons. The values were expressed as percentage of baseline. *$p<0.05$, compared with the baseline level. #$p<0.05$, compared with the values at 15 min after capsaicin injection (before L-703,606 treatment) (n=9, a repeated measures ANOVA with post hoc Newman-Keuls test).

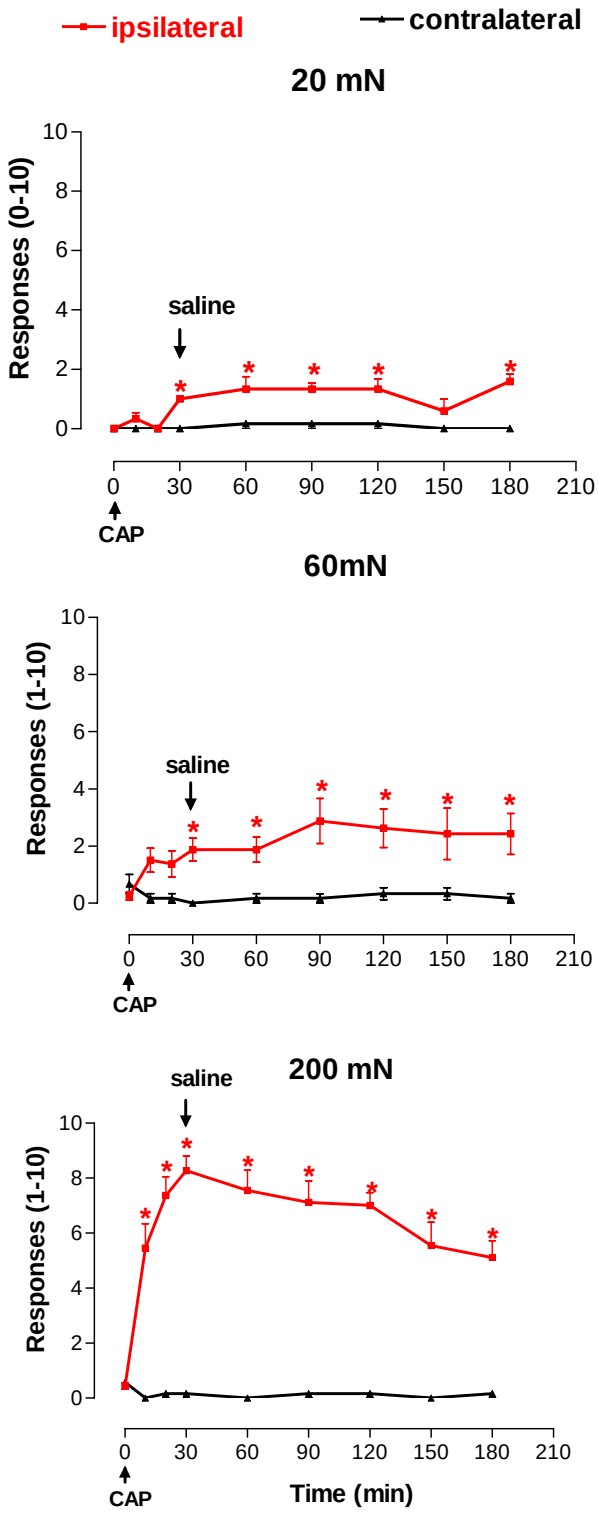

ipsilateral
contralateral
20 mN
Responses (0-10)
saline
CAP
60mN
Responses (1-10)
saline
CAP
200 mN
saline
Responses (1-10)
CAP
Time (min)

Figure 11. The number of responses to 10 applications of von Frey filaments with 20, 60 and 200 mN bending forces to the plantar surface of rat hindpaws on both ipsilateral and contralateral sides before and after intradermal capsaicin injection. Saline was administered through the intrathecal fiber at 30 min after capsaicin injection. *P<0.05, significant difference from baseline (n=10, one-way ANOVA)

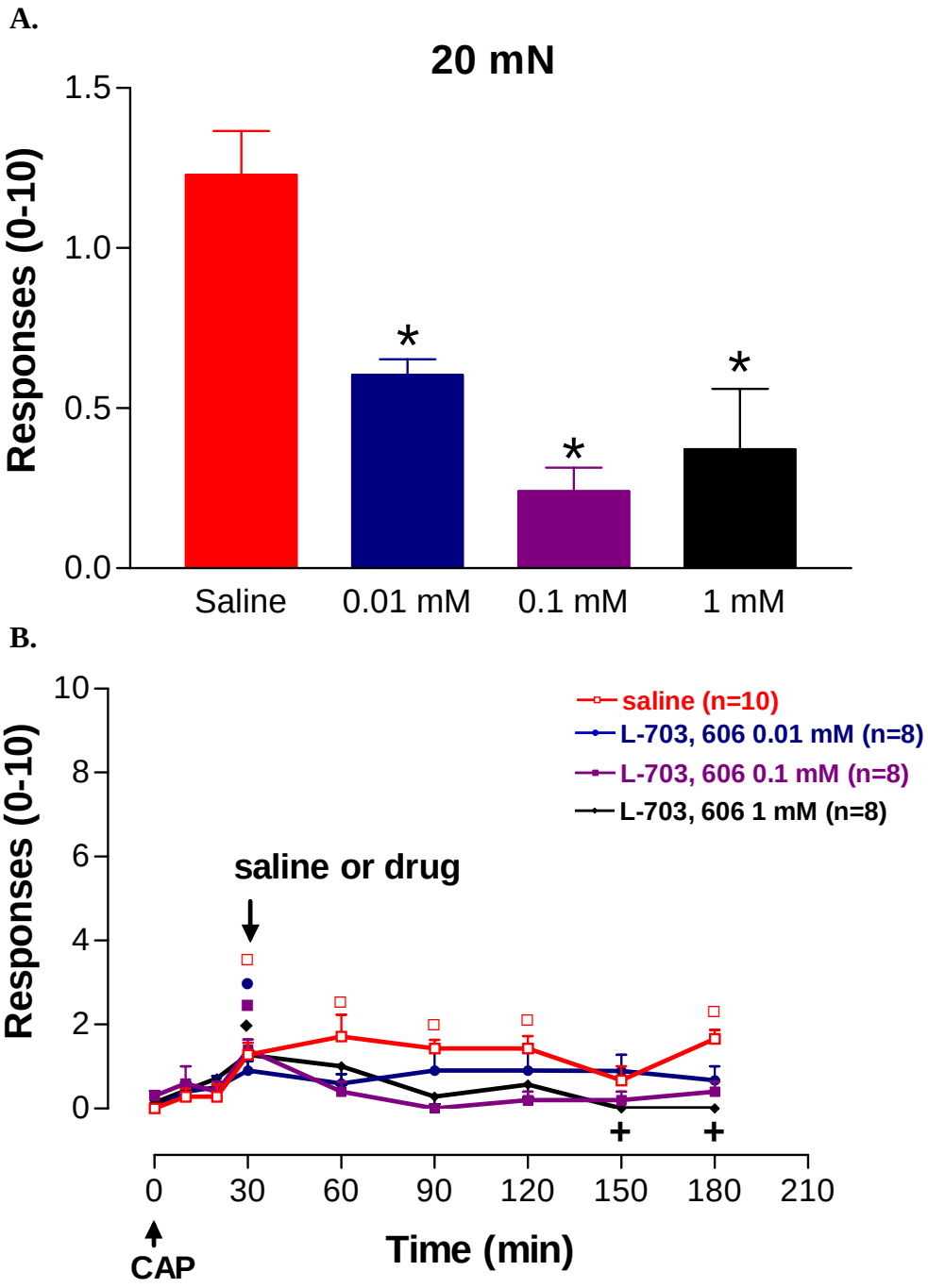

A.
20 mN
Responses (0-10)
1.5
1.0
0.5
0.0
*
*
*
Saline
0.01 mM
0.1 mM
1 mM
B.
Responses (0-10)
10
8
6
4
2
0
saline (n=10)
L-703, 606 0.01 mM (n=8)
L-703, 606 0.1 mM (n=8)
L-703, 606 1 mM (n=8)
saline or drug
+
+
0
30
60
90
120
150
180
210
CAP
Time (min)

Figure 12. Effect of intrathecal L-703,606 injection on CAP-induced secondary mechanical allodynia. *A.* Bar graphs show the total ipsilateral PWRs to a von Frey filament with a 20 mN bending force from 30 to 180 min after capsaicin injection in each group (*p<0.05, compared to saline group, one-way ANOVA with post hoc student-Newman-Keuls test). Saline (red) or L-703,606 at 0.01 (blue), 0.1 (purple) or 1 mM (black) concentration was administered through the intrathecal fiber at 30 min after capsaicin, respectively. B. The dose-response curves of the capsaicin-induced nociceptive behaviors. Statistical analysis was carried out by two way repeated measures ANOVA with post hoc Student-Newman-Keuls test. □ ● ■ ♦P<0.05, significant difference from baseline in each line. +P<0.05, significant difference from the 30 min time point in the 1 mM group.

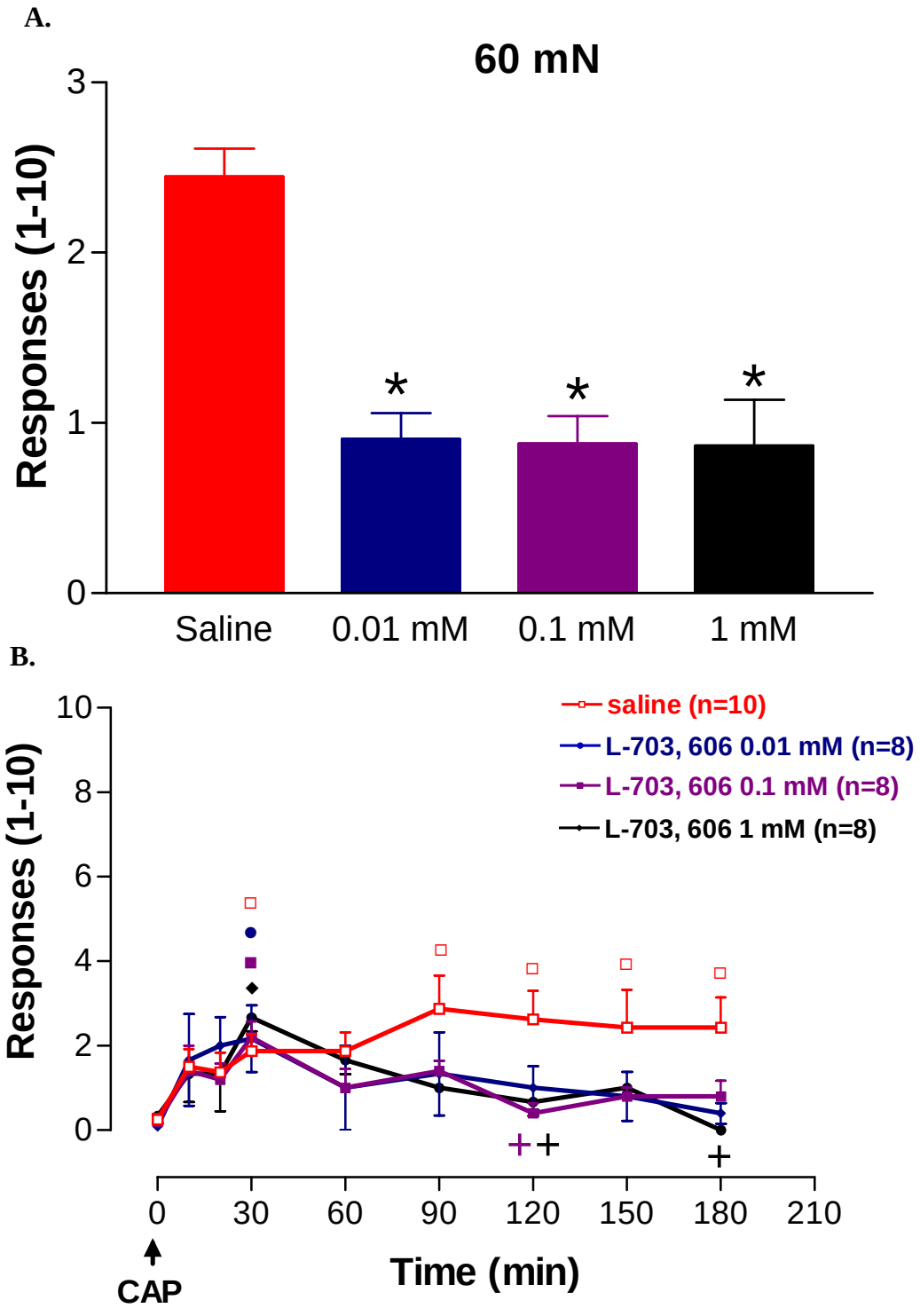

A.
60 mN
Responses (1-10)
3
2
1
0
*
*
*
Saline
0.01 mM
0.1 mM
1 mM
B.
saline (n=10)
L-703, 606 0.01 mM (n=8)
L-703, 606 0.1 mM (n=8)
L-703, 606 1 mM (n=8)
Responses (1-10)
10
8
6
4
2
0
0
30
60
90
120
150
180
210
CAP
Time (min)

Figure 13. L-703, 606 blocked CAP-induced changes of rat PWR to von Frey filament stimulation with a 60 mN bending force. *A.* Bar graphs show the total ipsilateral PWRs to a von Frey filament with a 60 mN bending force from 30 to 180 min after capsaicin injection in each group (*p<0.05, compared to saline group, one-way ANOVA with post hoc student-Newman-Keuls test). Saline or L-703,606 at 0.01 (blue), 0.1 (purple) and 1 mM (black) concentration was administered through the intrathecal fiber at 30 min after capsaicin, respectively. B. The time course indicates that both duration and magnitude of CAP-induced PWR are reduced by L-703,606. Statistical analysis was carried out by two way repeated measures ANOVA with post hoc Student-Newman-Keuls test. □ • ■ ♦P<0.05, significant difference from baseline in each line. +P<0.05, significant difference from the 30 min time point in the 1 mM group. +P<0.05, significant difference from the 30 min time point in the 0.1 mM group.

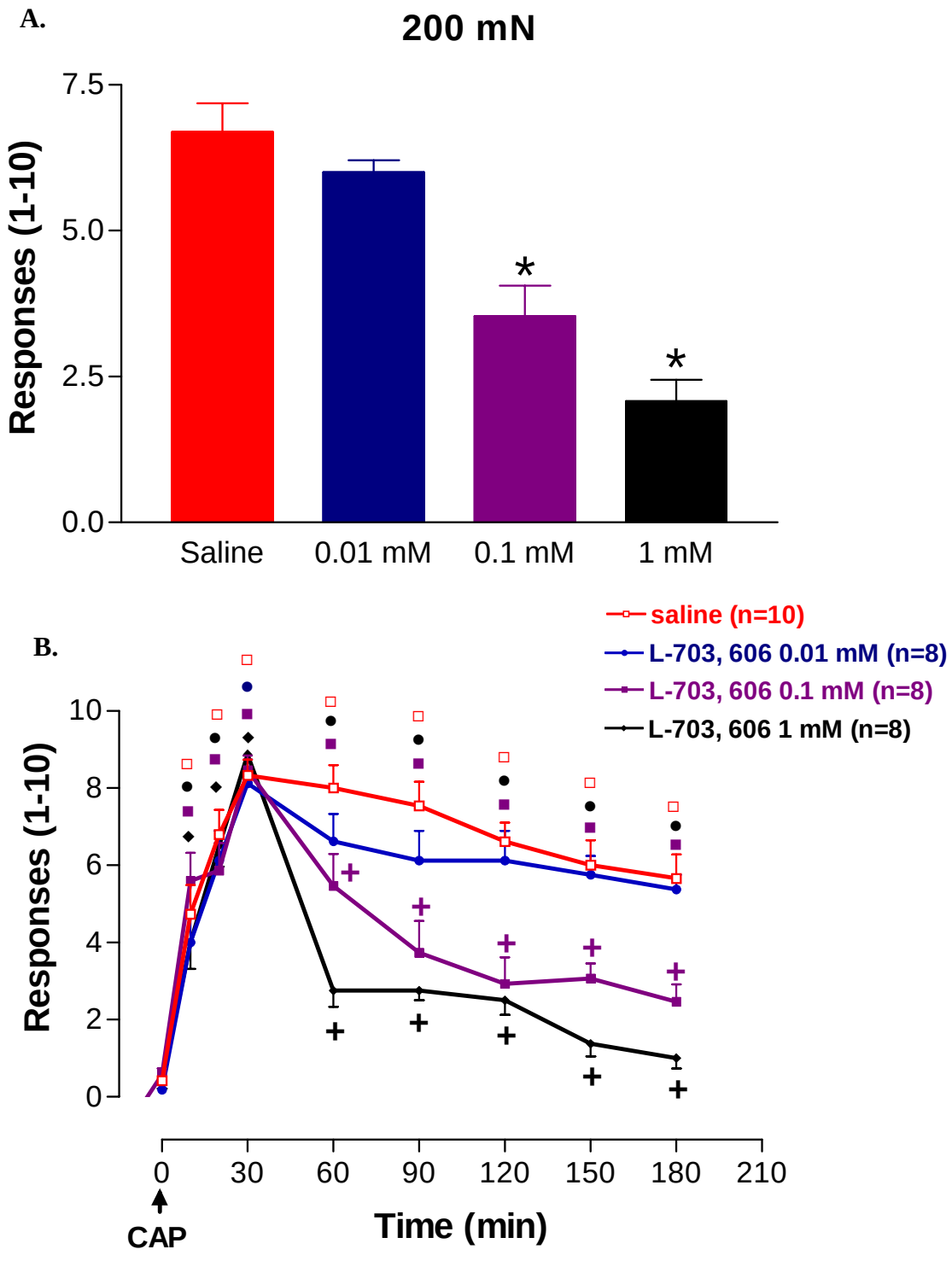

A.
200 mN
Responses (1-10)
7.5
5.0
2.5
0.0
*
*
Saline
0.01 mM
0.1 mM
1 mM
B.
saline (n=10)
L-703, 606 0.01 mM (n=8)
L-703, 606 0.1 mM (n=8)
L-703, 606 1 mM (n=8)
Responses (1-10)
10
8
6
4
2
0
0
30
60
90
120
150
180
210
CAP
Time (min)

Figure 14. L-703, 606 blocked CAP-induced changes of rat PWR to von Frey filament stimulation with 200 mN bending force. *A.* Bar graphs show the total ipsilateral PWRs to a von Frey filament with a 200 mN bending force from 30 to 180 min after capsaicin injection in each group (*p<0.05, compared to saline group, one-way ANOVA with post hoc student-Newman-Keuls test). Saline or L-703,606 at 0.01 (blue), 0.1 (purple) and 1 mM (black) concentration was administered through the intrathecal fiber at 30 min after capsaicin, respectively. B. The time course indicates that both duration and magnitude of CAP-induced PWR are reduced by L-703,606. Statistical analysis was carried out by two way repeated measures ANOVA with post hoc Student-Newman-Keuls test. □ • ■ ♦P<0.05, significant difference from baseline in each line. +P<0.05, significant difference from the 30 min time point in the 1 mM group. +P<0.05, significant difference from the 30 min time point in the 0.1 mM group.

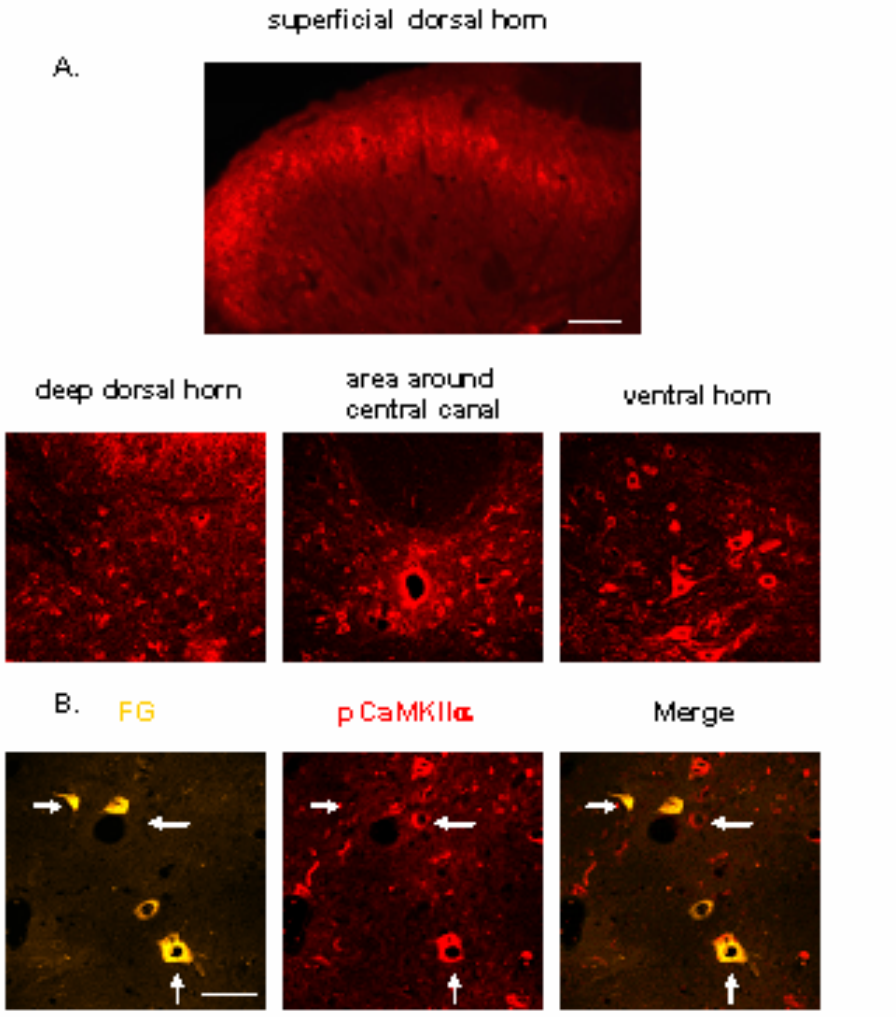
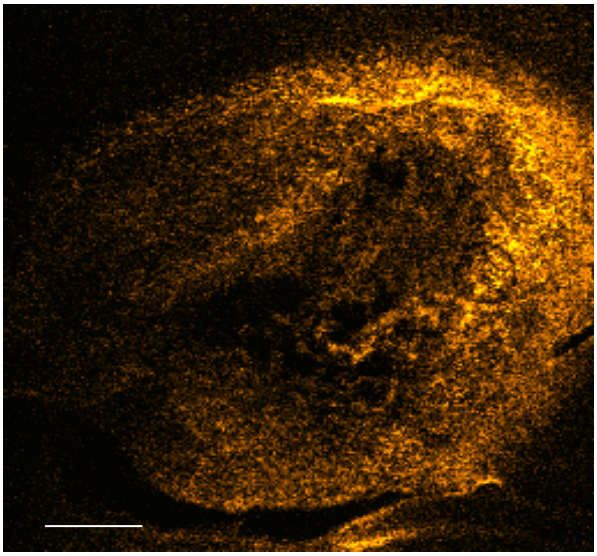


Figure 15. Immunofluorescence images illustrate the distribution of pCaMKIIα-LI neuronal profiles in the lumbar segments of spinal cord. Tissues were collected 30 min after the animals were given an intrathecal injection of saline (15 μl). (A) Expression of pCaMKIIα-LI neuronal profiles in different areas of the spinal cord. Red fluorescence: pCaMKIIα. Scale bar: 100 μm. (B) STT cells in the dorsal horn double labeled for pCaMKIIα. Yellow fluorescence, retrograde tracer FG; red fluorescence, pCaMKIIα. Two STT cells labeled by FG (↑, →). One was positive for pCaMKIIα (↑), whereas another STT cell did not express pCaMKIIα (→). One pCaMKII-LI positive neuron, which was not an STT cell, was also shown (red, ←). Scale bar: 50 μm.

A.



B.

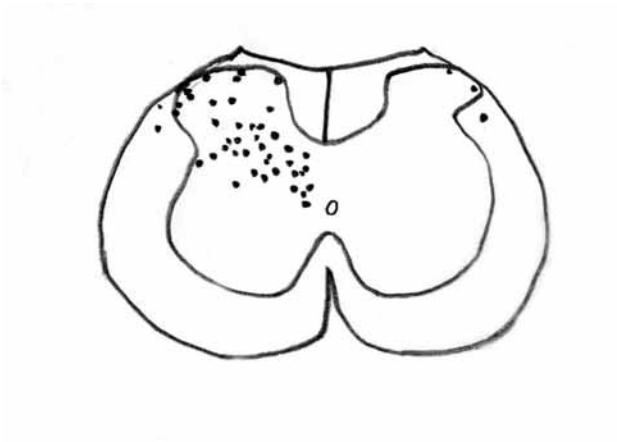


Figure 16. The injection sites of FG in the thalamus and retrogradely labeled STT cells in the lumbar spinal cord. (A) The location of FG injections in the right thalamus, including the VPL nucleus. The coordinates (distance from bregma, distance from the right of the midline, distance from the surface of the skull) were: -3.0 mm, 2.8 mm, 5.7/5.4 mm; -3.5 mm, 2.2 mm, 6.0/5.6 mm; -3.5 mm, 2.6 mm, 6.0/5.6 mm; -3.5 mm, 3.3 mm, 5.5/5.2 mm; -4.0 mm, 2.2 mm, 6.0/5.6 mm; -4.0 mm, 2.6 mm, 6.0/5.6 mm; -4.0 mm, 3.3 mm, 5.5/5.2 mm. Scale bar: 300 μM. (B) Distribution of labeled STT cells in the L4-L5 segments of the spinal cord.

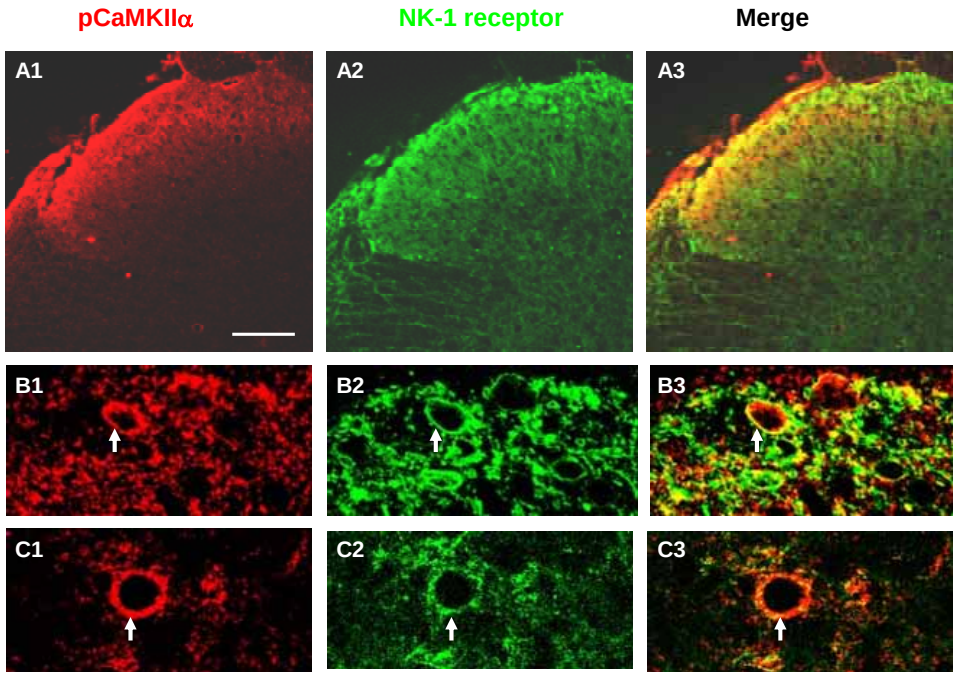


Figure 17. Immunofluorescence imaging showing the distribution and colocalization of pCaMKIIα and NK-1 receptors in the dorsal horn of rats with saline injection. Red fluorescence, pCaMKIIα; green fluorescence, NK-1 receptors. (A1-A3) Dense colocalization of pCaMKIIα and NK-1 receptors is mainly found in the superficial layers of the dorsal horn, with little expression in the deep layers. (B1-B3) Higher magnification of the immunofluorescence confocal images demonstrates a morphologically identified neuron that was both positive for pCaMKIIα (B1, ↑) and NK-1 receptors (B2, ↑) in lamina I of the dorsal horn. Coexistence of the two immunostains could be seen in the same neurons (B3, ↑). (C1-C3) A representative neuron in a deep layer of the dorsal horn with the coexpression of pCaMKIIα (C1, ↑) and NK-1 receptors (C2, ↑). Scale Bar: A1-A3, 150 μm; B1-C3, 50 μm.

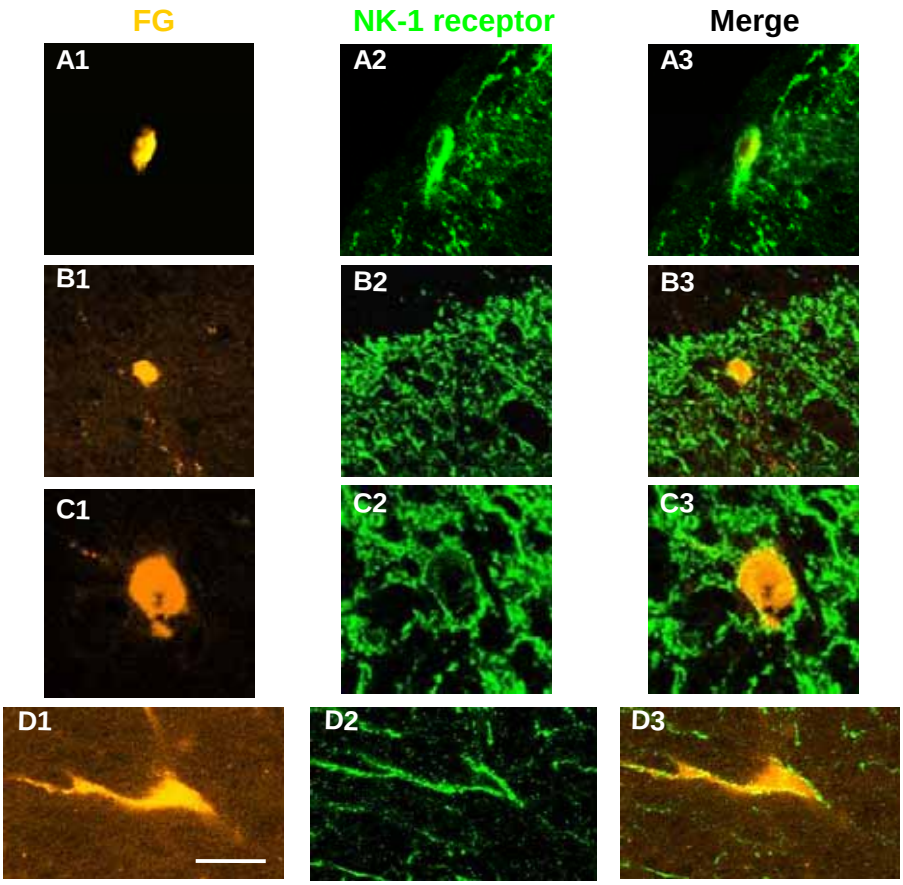


Figure 18. Distribution of NK-1 receptors in retrogradelly labeled STT cells in the dorsal horn of control animals. Yellow fluorescence, FG; green fluorescence, NK-1 receptors. (A1-B3) Two respresentative STT neurons containing NK-1 receptors in lamina I. (C1-D3) Expression of NK-1 receptors were also found in STT cells in lamina V. Bar scale: 50 μm.

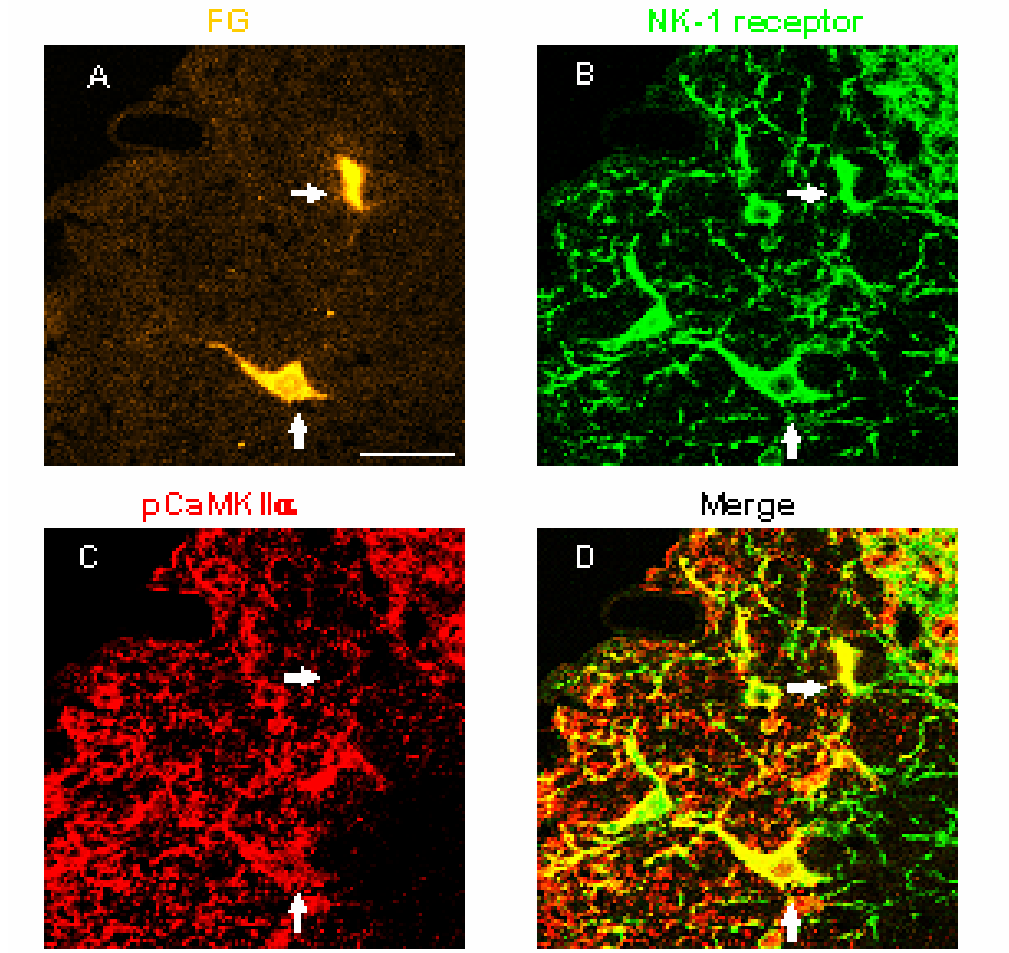


Figure 19. Immunohistochemical colocalization of pCaMKIIα and NK-1 receptors in STT cells retrogradely labeled with FG in the superficial layers of rat dorsal horn following saline treatment. Yellow fluorescence, FG; green fluorescence, NK-1 receptors; red fluorescence, pCaMKIIα. Two STT cells were identified by staining for FG (A, ↑, →). One cell was positive for both NK-1 receptors (B, ↑) and pCaMKIIα (C, ↑). The coexistence of pCaMKIIα and NK-1 receptors in the same STT cell is seen (D, ↑). The other cell was positive for NK-1 receptor staining (B, →), but not pCaMKIIα staining (C, →). Scale bar, 50 μm.

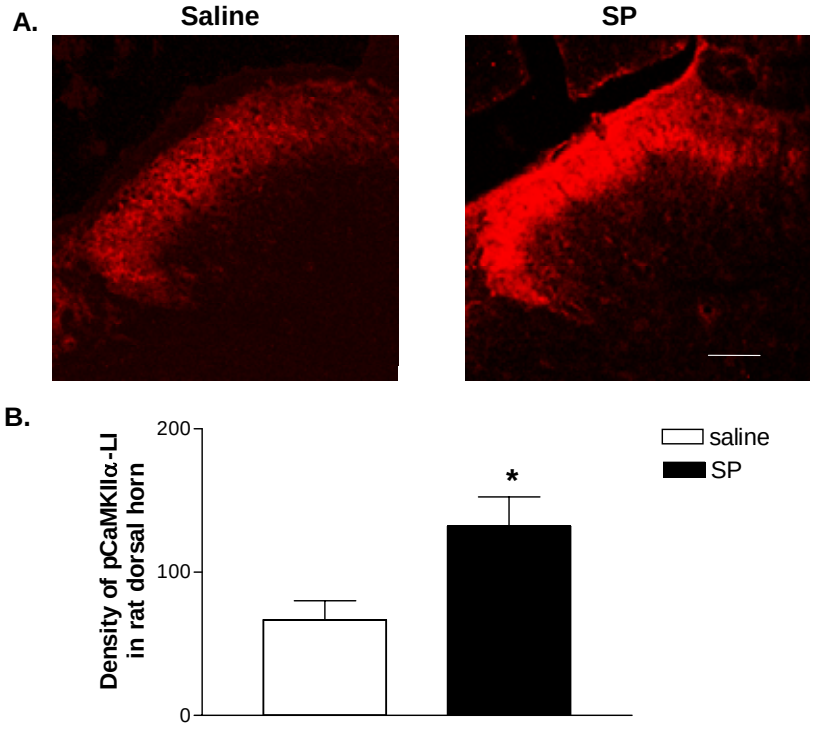


Figure 20. The effect of intrathecal injection of SP on the expression of pCaMKIIα in the superficial dorsal horn. (A) After SP injection, there was a significant increase in the number of pCaMKIIα-LI in the superficial layers, compared with that in the saline group. (B) Bar graph shows the difference in the density of pCaMKIIα-LI in the superficial layers of the dorsal horn between the two groups. *$p<0.05$, compared with the saline group (n=6, t-test). Scale bar. 150 μm.

Table 1. The number of STT cells in different laminae of rat spinal cord.

	saline (n=6)	SP (n=6)
STT cells (I-III)	15	14
STT cells (IV-VII)	54	56
Total STT cells	69	70

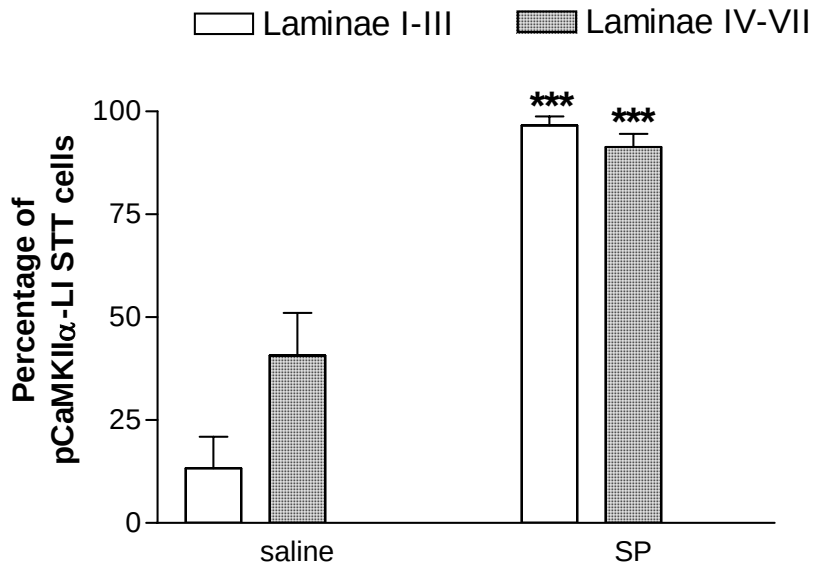


Figure 21. Bar graphs showing the proportion of pCaMKIIα-LI STT cells in laminae I-VII in the L4-L5 segments in both the saline and SP groups. There were significant differences in the proportion of STT cells with pCaMKIIα-LI between the two groups. ***$p<0.001$, compared with the saline group (n=6, t-test).

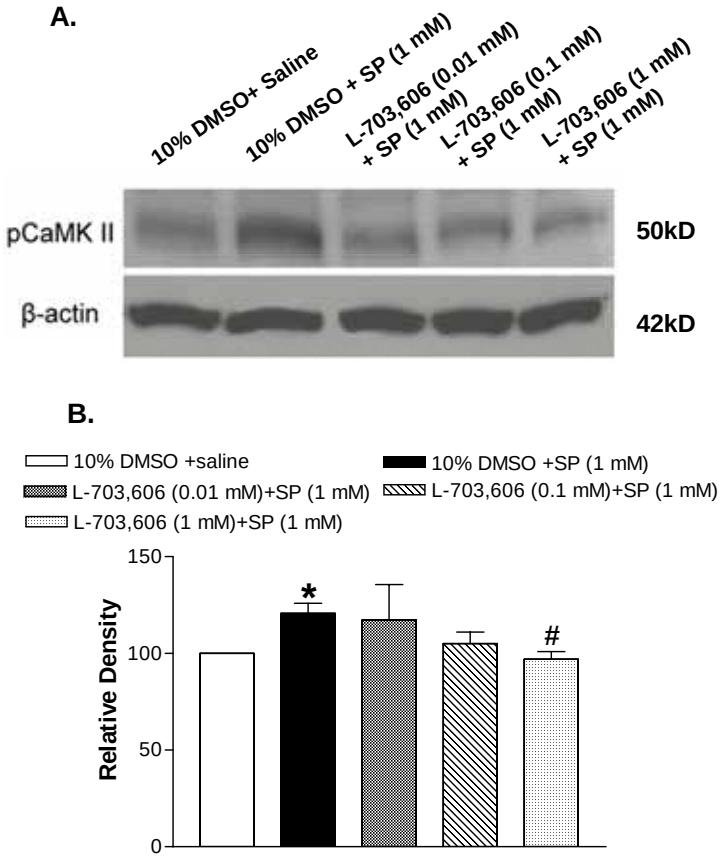


Figure 22. Expression of pCaMKIIα in the dorsal horn after SP injection, with or without pretreatment with the NK-1 antagonist. (A) Western blots in representative experiments. The number on the right indicates the position of a molecular weight marker in kDa. (B) Bar graph summarizes the relative density of immunoblots of rat spinal cord tissues. The amount of pCaMKIIα protein was significantly increased after 10%DMSO+SP treatment. The density of the protein was decreased in animals pretreated with L-703,606 (1 mM), compared with the 10%DMSO+SP group. However, L-703,606 at lower doses (0.01 and 0.1 mM) had no significant effect on SP-induced pCaMKIIα expression. *$p<0.05$, compared with 10%DMSO+saline group; #$p<0.05$, compared with 10%DMSO+SP group (n=7-8, one-way ANOVA with post hoc t-test).

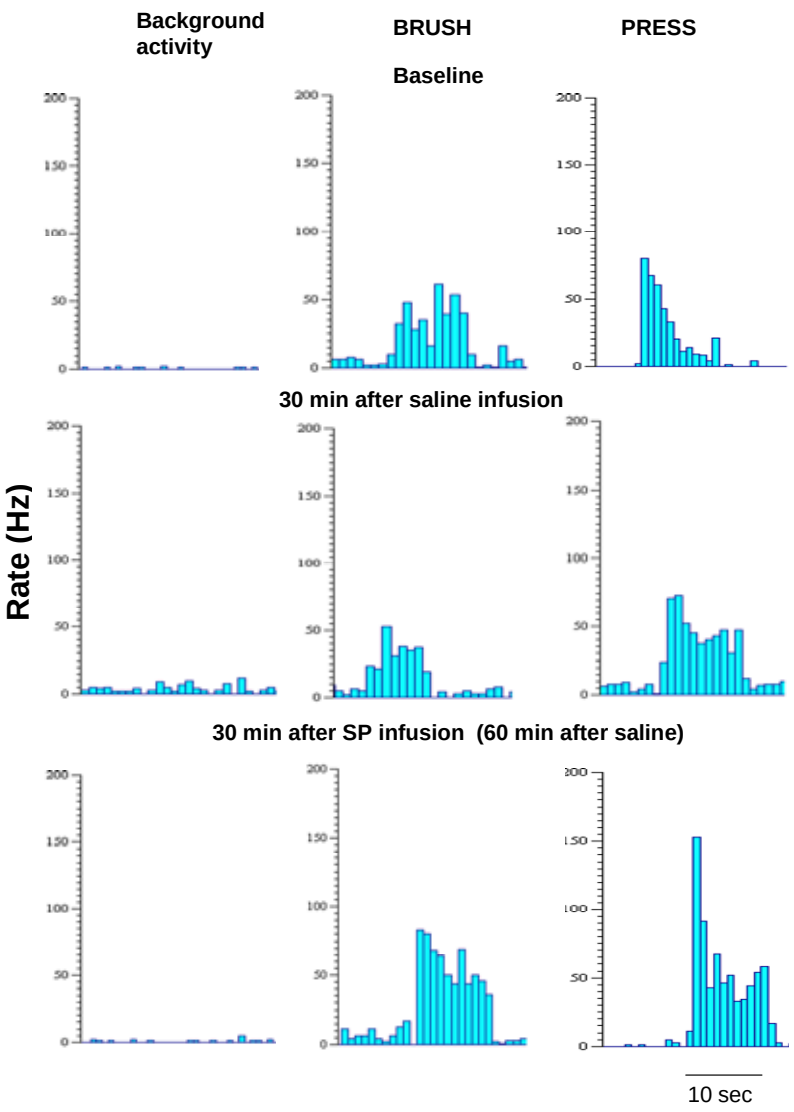


Figure 23. SP contributes to the central sensitization of WDR neurons in the spinal cord dorsal horn. Rate histograms represent the activity of a WDR neuron in response to mechanical stimuli. *First row*, Background activity and responses to mechanical stimuli (BRUSH and PRESS). *Second row*, Responses recorded 30 min after saline infusion. *Third row*, 30 min after SP (100 μM) infusion (60 min after saline).

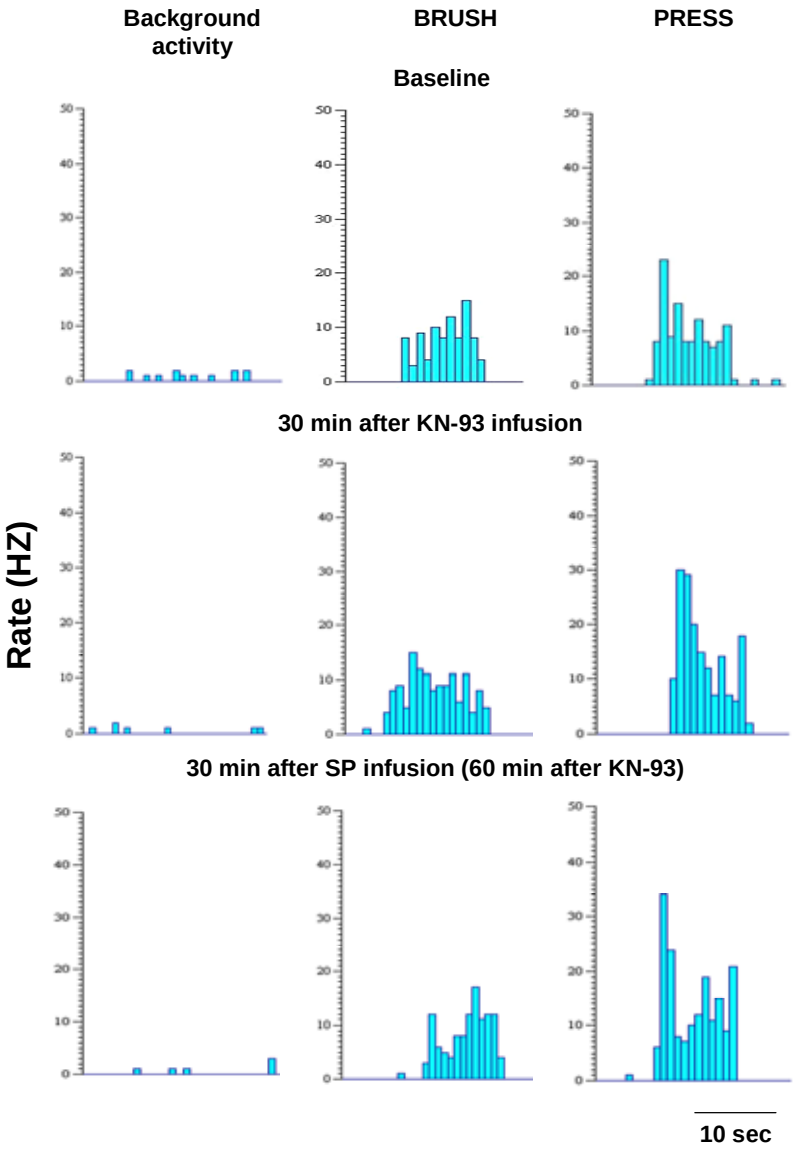


Figure 24. An inhibitor of CaMK II, KN-93, prevented the effect of SP on WDR neurons. *First row*, Baseline level of a WDR neuron. *Second row*, Responses recorded 30 min after KN-93 (100 μM) infusion. Then the KN-93 infusion was stopped and switched to SP infusion. *Third row*, 30 min after SP (100 μM) infusion (60 min after KN-93).

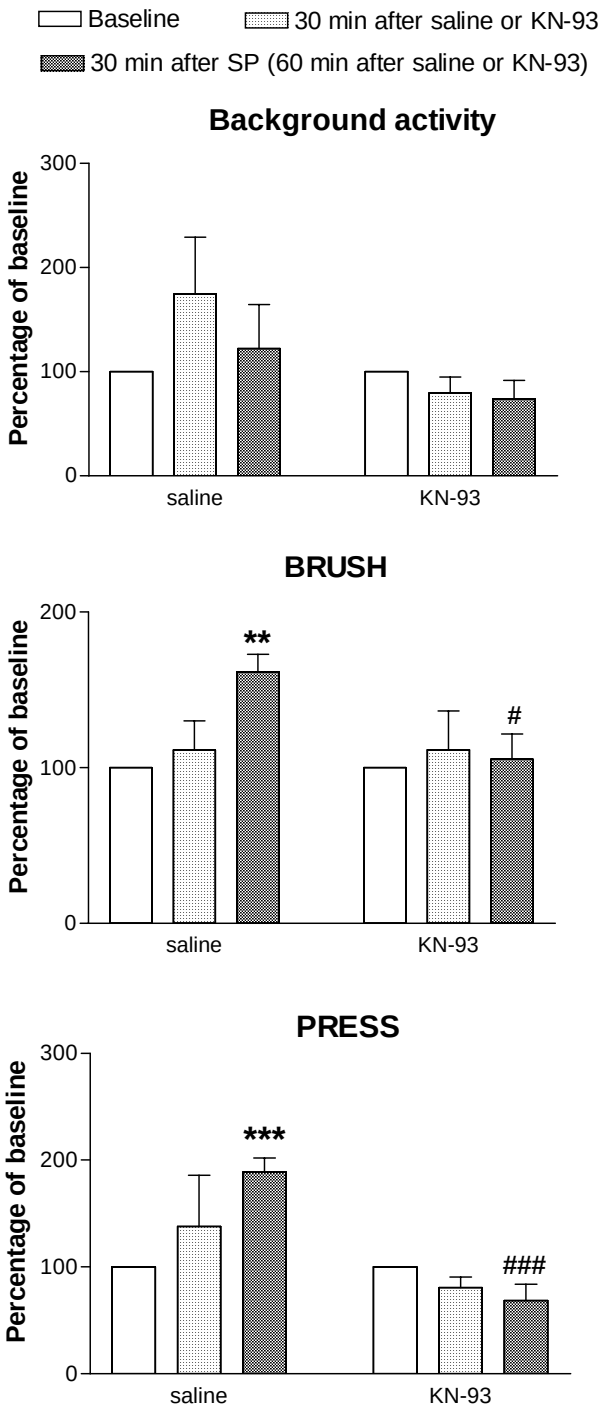

Baseline
30 min after saline or KN-93
30 min after SP (60 min after saline or KN-93)
Background activity
Percentage of baseline
300
200
100
0
saline
KN-93
BRUSH
Percentage of baseline
200
100
0
**
#
saline
KN-93
PRESS
Percentage of baseline
300
200
100
0
***
###
saline
KN-93

Figure 25. Bar graphs summarize the differences in the activity of WDR neurons between the saline/SP and KN-93/SP group. The values are expressed as mean spikes /second. One-way ANOVA, followed by Student Newman-Keuls test, was used to compare the differences. **P<0.01, ***P<0.001 compared with the baseline in the control group; ###P<0.001, compared with the values at the same time point in the control group (n=7).

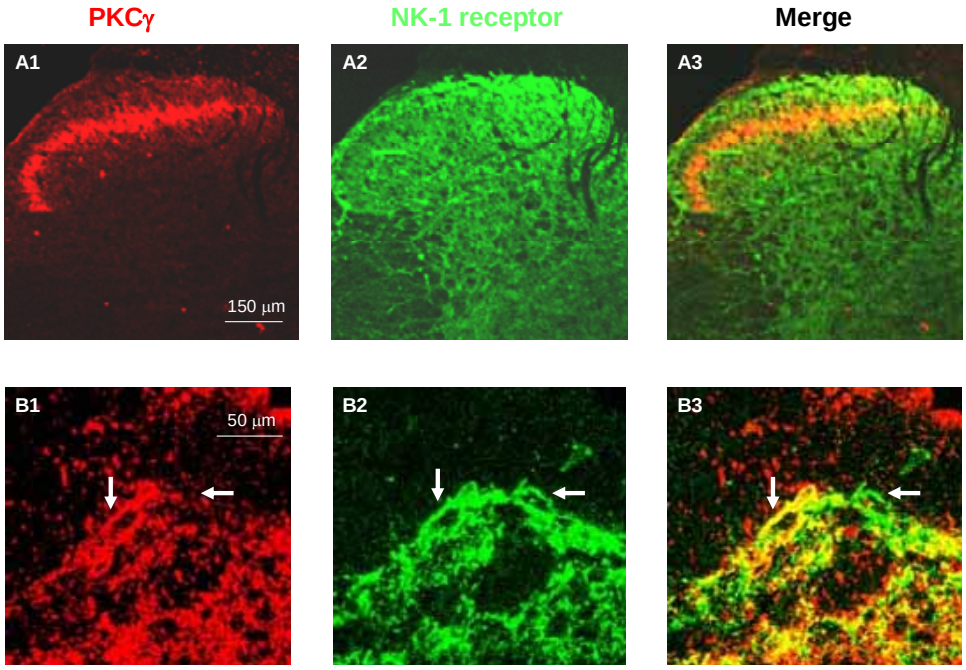


Figure 26. Immunofluorescence imaging showing the distribution and colocalization of PKCγ and NK-1 receptors in the dorsal horn of rats with saline injection. Red fluorescence, PKCγ; green fluorescence, NK-1 receptors. (A1-A3) Distribution of PKCγ and NK-1 receptors is found in the superficial layers of the dorsal horn. (B1-B3) Higher magnification of the immunofluorescence confocal images demonstrates two morphologically identified neurons that were both positive for NK-1 receptors (B2, indicated by ↓ and ←) in the dorsal horn. One neuron was also positive for PKCγ (B1, indicated by ↓), whereas the other one was negative for PKCγ (B1, indicated by ←). Coexistence of the two immunostains could be seen in the same neuron (B3, indicated by ↓). Scale Bar: A1-A3, 150 μm; B1-B3, 50 μm.

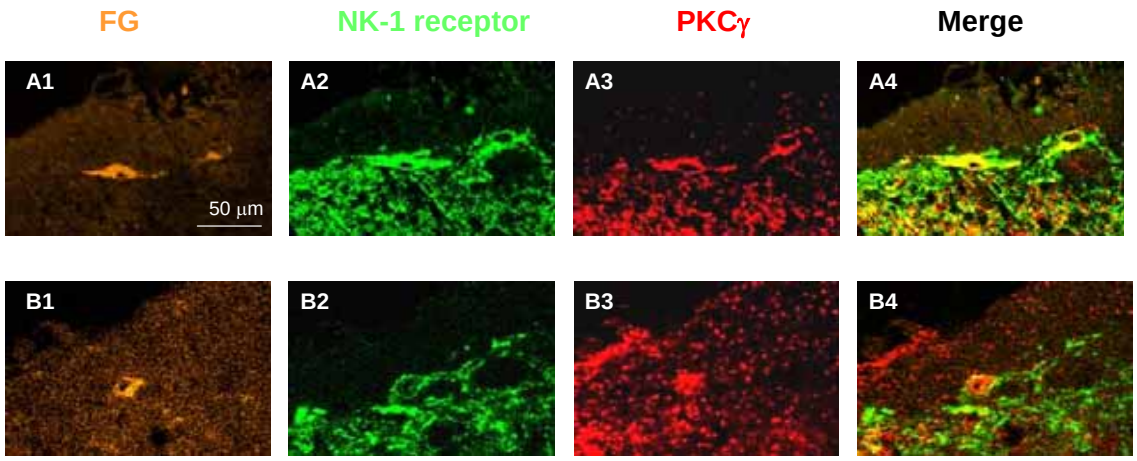


Figure 27. Colocalization of PKCγ and NK-1 receptors in STT cells retrogradely labeled with FG in superficial dorsal horn of a control animal. Yellow fluorescence, FG; green fluorescence, NK-1 receptors; red fluorescence, PKCγ. Three STT cells were identified by staining for FG (A1, B1). All of them were positive for both NK-1 receptors (A2, B2) and PKCγ (A3, B3) stainings. The coexistence of PKCγ and NK-1 receptors in the same STT cells is seen (A4, B4). Scale bar, 50 μm.

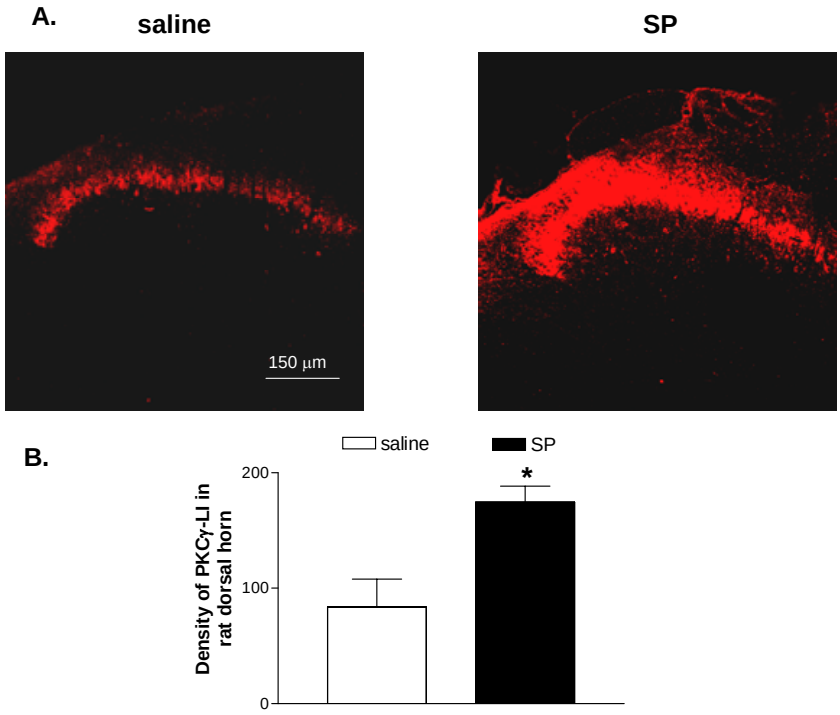


Figure 28. The effect of intrathecal injection of SP on the expression of PKCγ in the superficial dorsal horn. (A) After SP injection, a significant increase in the density of the staining for PKCγ in the superficial layers was seen compared with that in the saline group. (B) Bar graph shows the difference in the density of PKCγ-LI in the superficial layers of the dorsal horn between the two groups. *$p<0.05$, compared with the saline group (n=6, t-test).

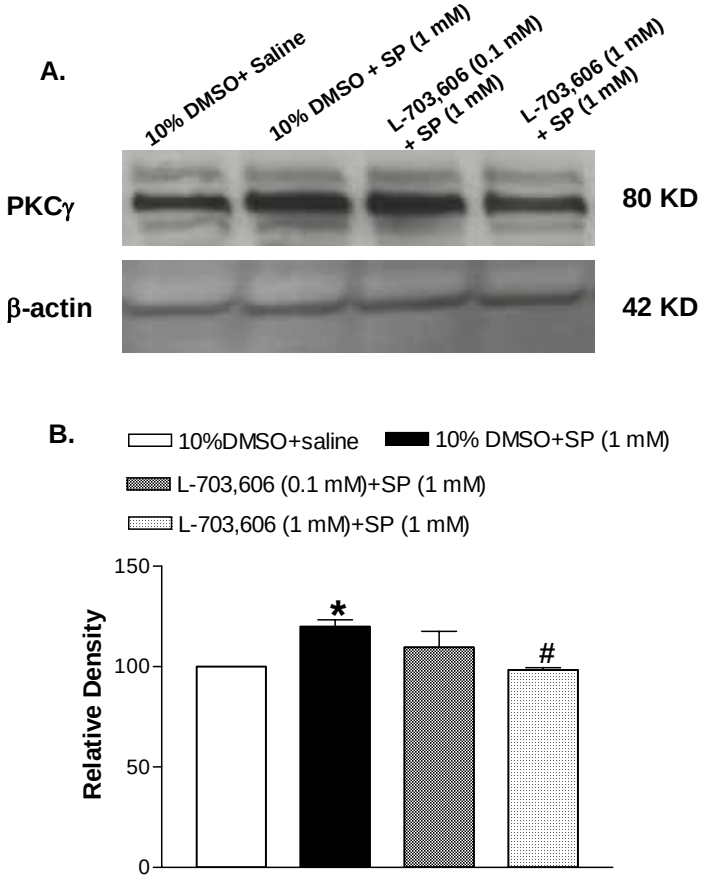


Figure 29. Expression of PKCγ in the dorsal horn after SP injection, with or without pretreatment with an NK-1 antagonist. (A) Western blots in representative experiments. The number on the right indicates the position of a molecular weight marker in kDa. (B) Bar graph summarizes the relative density of immunoblots of rat spinal cord tissue. The amount of PKCγ protein was significantly increased after 10% DMSO+SP treatment, compared with 10%DMSO+saline treatment. The density was reduced in animals pretreated with L-703,606 (1 mM), compared with the 10% DMSO+SP group. However, L-703,606 at a lower dose (0.1 mM) had no significant effect on SP-induced PKCγ expression. *$p<0.05$, compared with 10%DMSO+saline group; #$p<0.05$, compared with 10%DMSO+SP (1 mM) group (n=8, one-way ANOVA with post hoc t-test).

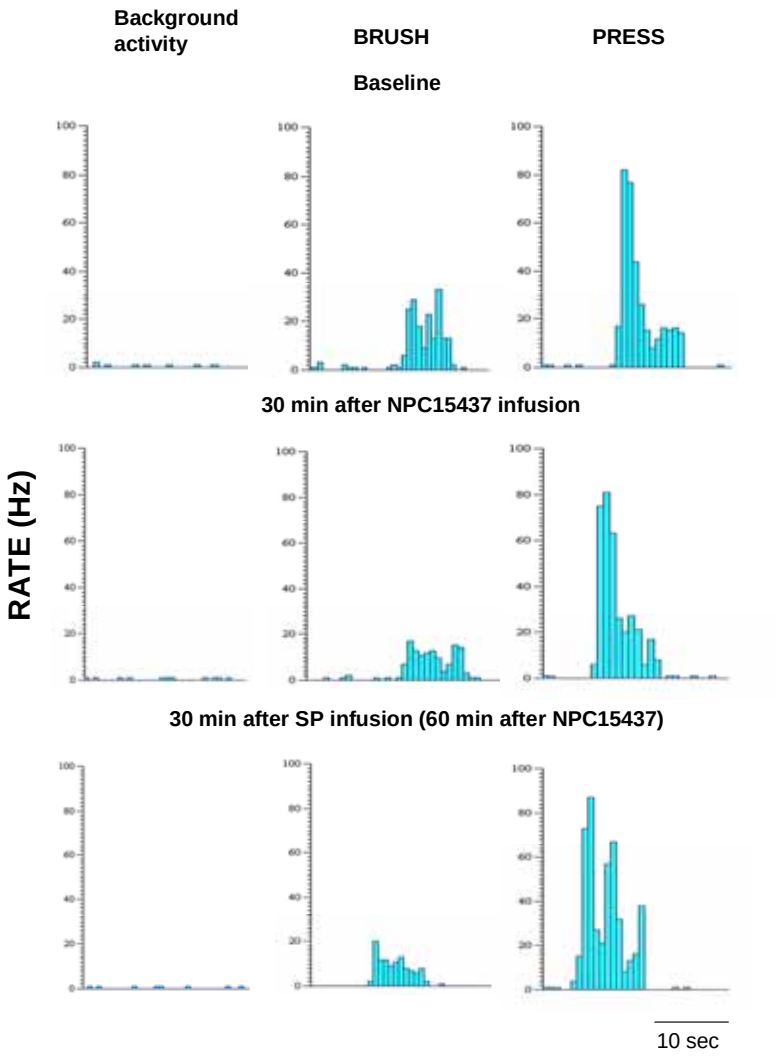


Figure 30. Pretreatment with an inhibitor of PKC, NPC15437, blocked the effect of SP on WDR neurons. *First row*, Baseline level of a WDR neuron. *Second row*, Responses recorded 30 min NPC15437 (10 mM) infusion. Then the NPC15437 infusion was stopped and switched to SP infusion. *Third row*, 30 min after SP (100 μM) infusion (60 min after NPC15437).

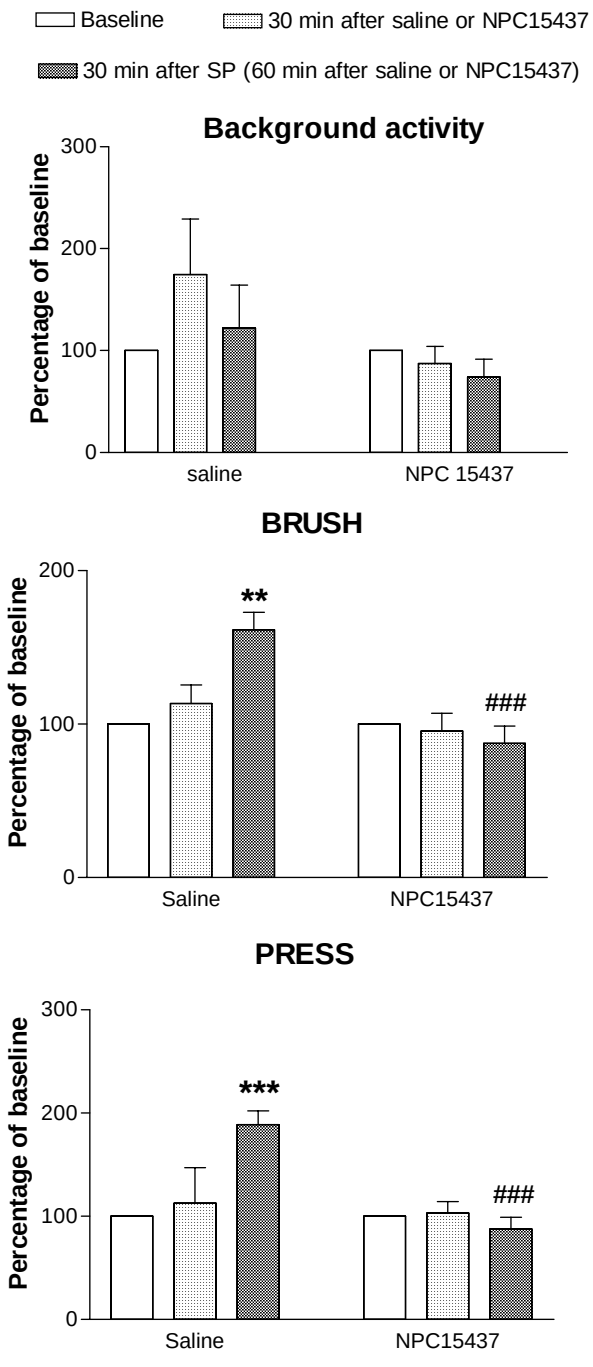
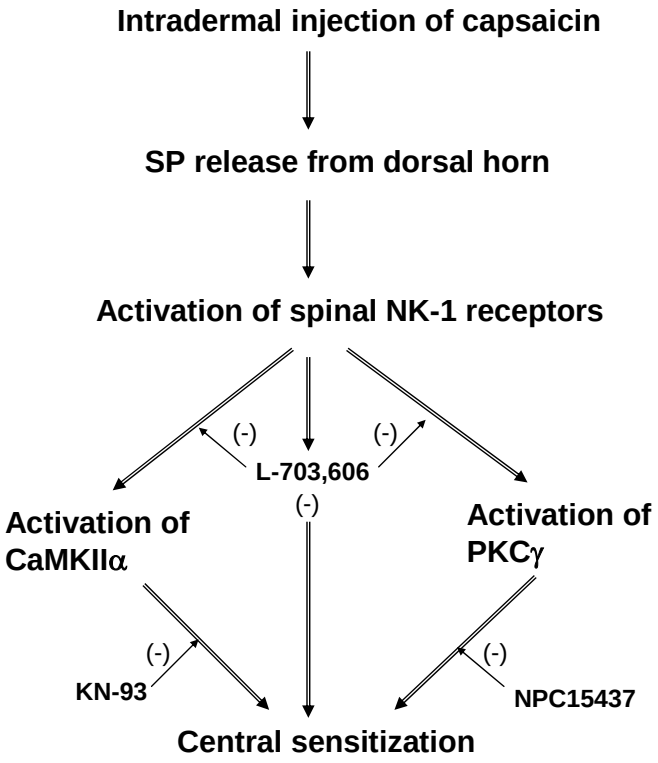

Baseline
30 min after saline or NPC15437
30 min after SP (60 min after saline or NPC15437)
Background activity
Percentage of baseline
300
200
100
0
saline
NPC 15437
BRUSH
Percentage of baseline
200
100
0
**
###
Saline
NPC15437
PRESS
Percentage of baseline
300
200
100
0
***
###
Saline
NPC15437

Figure 31. Bar graphs summarize the differences in the activity of WDR neurons between the saline/SP and NPC15437/SP group. One-way ANOVA, followed by Student Newman-Keuls test, was used to compare the differences. **P<0.01, ***P<0.001 compared with the baseline in the control group; ###P<0.001, compared with the values at the same time point in the control group (n=8 in each group).

CHAPTER 4: DISCUSSION

As summarized in the following illustration, this study showed that intradermal injection of capsaicin into a rat paw induced SP release from rat dorsal horn, which results in subsequent activation of spinal NK-1 receptor and its signaling pathways, including CaMKII and PKC pathways. The activation of NK-1 receptors is important for capsaicin-evoked central sensitization. And the activation of CaMKII and PKC pathways contributes to the nociceptive neurotransmission mediated by SP.



INCREASED RELEASE OF SP AND ACTIVATION OF NK-1 RECEPTORS FOLLOWING INTRADERMAL INJECTION OF CAPSAICIN CONTRIBUTES TO CENTRAL SENSITIZATION

The effect of intradermal injection of capsaicin on spinal SP release

To investigate the involvement of SP in capsaicin-induced central sensitization, we first quantified both SP content in dorsal horn tissues and *in viv*o release of SP from the dorsal horn following capsaicin injection. In the mammalian dorsal horn, SP is concentrated in the central terminals of primary afferents in the superficial layers, as well as in the interneurons in the deep layers (Hökfelt et al., 1975a,b). Coexistence of SP with 5-HT is also observed in the nerve endings of the ventral horn (Arvidsson et al., 1990). Our results show a rapid increase of SP release from the dorsal horn at 10 min after intradermal injection of capsaicin, which gradually decreased to baseline 30 min after capsaicin (Figures 4-5). Therefore, we suggest that by activating TRPV1 receptors on the peripheral terminals of nociceptive primary afferent neurons, intradermal injection of capsaicin facilitates the release of stored SP from synaptic vesicles in the central terminals of primary afferent neurons into the extracellular space in the dorsal horn. Furthermore, due to the direct excitatory effect of capsaicin on the primary afferent neurons, we postulate that the released pool of SP mainly originates from the central terminals of primary afferents. However, we did not rule out the possibility that in the absence of transection of the cord rostrally or block of supraspinal control, there also might be some release of SP from the interneurons or descending pathways.

Our findings are consistent with those of other studies, in which spinal infusion of capsaicin was used to detect SP release either *in vivo* (Aimone and Yaksh, 1989; Afrah et al., 2001, 2004) or *in vitro* (Theriault et al., 1979; Go and Yaksh, 1987; Lever and Malcangio, 2002; Marivizón et al., 2003). A small difference is the timing of the peak release of SP. Marivizón et al., (2003) found that the peak appeared 3-7 min after the initiation of capsaicin infusion, whereas there is no significant increase of SP at 5 min after capsaicin injection in our study. The discrepancy may be due to the time delay of

the dialysate moving from the spinal cord to the collection vial through the polyimide tubing and the PE 50 tubing.

In our study, peripheral capsaicin application had no significant effect on the total amount of SP in the dorsal horn within 60 min after inflammation (Figure 6). We suggest that a lack of change in content of SP in the dorsal horn is attributed to compartment translocation of SP from the vesicles in the presynaptic terminals into the synaptic cleft, which results in an increase in SP level in the extracellular space and a depletion of intracellular SP storage. As we know, the released SP that exists in the synaptic cleft is responsible for neurotransmission. Therefore, in the very early stage of capsaicin-induced inflammation, SP release, rather than the total SP content in the tissues, is a more sensitive and appropriate index to reflect the effect of capsaicin injection on the neurotransmission in the dorsal horn.

Several approaches to study the changes in the release of SP have been developed both *in vitro* and *in vivo*. The traditional way is to analyze SP concentration in a superfusate of isolated spinal cord slices using radioimmunoassay (RIA, Aimone and Yakash, 1989; Malcangio et al., 1997; Afrah et al., 2001; Matsuka et al., 2001; Bowles et al., 2004). An antibody-coated microprobe technique is another option to determine and localize SP release (Duggan and Hendry, 1986; Duggan and Furmidge, 1994). Internalization of NK-1 receptors has also been suggested to be correlated with SP release following noxious stimuli (Marivizon et al., 2003). Furthermore, *in vivo* microdialysis was used to study SP release from the spinal cord following peripheral noxious stimuli (Afrah et al., 2001). However, the major drawback of microdialysis is that samples sometimes do not pass through the porous membrane and there is a dilution effect due to the small amount of sample transported through the microdialysis pores. In the current study, a new kind of "single-hole" fiber was introduced into the rat spinal cord dorsal horn to quantify *in vivo* release of SP, which allows molecules the size of neuropeptides and larger to pass through and be collected. It could also be used to administer neuropeptides and larger molecules into the central nervous system. Using this technique, changes in the release of CGRP from rat dorsal horn were also observed

following intradermal injection of capsaicin (unpublished data). However, more data are needed to verify the efficacy of this technique in the study of neuropeptide release.

SP facilitates nociceptive neurotransmission in the dorsal horn by activating NK-1 receptors

SP terminals end on nociceptive dorsal horn neurons, which have a high content of NK-1 receptors. After release from primary afferent terminals, SP diffuses into the extracellular space and binds with post-synaptic NK-1 receptors. It is suggested that blockage of NK-1 receptors attenuates the responses of nociceptive dorsal horn neurons to peripheral noxious stimuli (De Konick and Henry, 1991; Radhakrishnana and Henry, 1991; Neugebauer et al., 1994, 1995). In this study, the effect of a selective non-peptide NK-1 receptor antagonist, L-703, 606 (IC_{50}=2 nM, Kd=0.3 nM, Cascieri et al., 1992), on capsaicin-induced central sensitization was investigated. Induced background activity and responses to PRESS stimuli of WDR neurons after capsaicin injection was recorded in the electrophysiological experiments. The immediate increase in spontaneous firing of WDR neurons was comparable to the human studies in which an ongoing pain and on going discharge of primary afferent nociceptors was demonstrated (Simone et al., 1989; LaMotte et al., 1991, 1992; Torebjörk et al., 1992). The duration of the capsaicin-evoked activity persisted over 45 min after the injection, which is consistent with previous reports (Fang et al., 2002; Sun et al., 2004a). However, the responses to BRUSH stimulation remained the same before and after capsaicin injection. Similar to our findings, intradermal injection of capsaicin at the same dose had no effect on the discharges of a C-nociceptive fiber to innocuous mechanical stimulation at a site beyond the receptive field of this recorded fiber (Ren et al., 2005). It was found that spinal infusion of L-703,606 dramatically reversed the capsaicin-evoked enhancement of electrophysiological responses of WDR neurons, suggesting that activation of NK-1 receptors is required for the formation of central sensitization (Figures 9-10). Previous reports have identified an inhibitory impact of L-703,606 on arthritic pain (Hong et al., 2002) or SP-induced proinflammation (Mantyh et al., 1994; Kramer et al., 1997), indicating receptor specificity.

In the behavioral studies, a significant increase of ipsilateral responses to mechanical stimuli was observed shortly after intradermal injection of a relatively low dose of capsaicin (0.1%, 15 μl), and the response lasts for more than 3 hours (Figure 11). The von Frey stimuli were applied well outside the site of capsaicin injection (about 2 cm away). A recent electrophysiological study showed that the distance between the edge of the receptive field of the nociceptor sensitized by intradermal injection of capsaicin (1%, 15μl) to the center of the injection was about 5 mm (Ren et al., 2005). Therefore, it is suggested that increased PWR to the application of Von Frey filaments at the site beyond the receptive field of the sensitized nociceptor represents the occurrence of behavioral hypersensitivity, which results from the plastic changes in the dorsal horn neurons (Baumann et al., 1991; Zhang et al., 2003; Willis and Coggeshall, 2004).

When L-703,606 was delivered intrathecally 30 min after the capsaicin injection, it reversed the enhanced PWRs resulting from capsaicin injection (Figures 12-14). These results suggest that activation of NK-1 receptors is necessary for the maintenance of capsaicin-induced mechanical hypersensitivity. Besides abundant expression in dorsal horn neurons, SP and NK-1 receptors are widely distributed in peripheral tissues, such as skin, visceral systems and inflammatory cells (reviewed in Snijdelaar et al., 2000 and in Harrison and Geppetti, 2001). During neurogenic inflammation, such as that produced by intradermal injection of capsaicin, peripheral NK-1 receptors are activated by release of SP from cutaneous sensory nerves. Activation of peripheral NK-1 receptors then induces increased blood flow, vasodilation, plasma extravasation, flare and pain, which are prominent signs of neurogenic inflammation (Pierau et al., 1991; Baluk, 1997; Schmelz et al., 1997; reviewed in Holzer, 1988). However, secondary mechanical hyperalgesia following peripheral capsaicin injection has been demonstrated to be largely dependent on activation of central NK-1 receptors, but not on peripheral NK-1 receptors (Sakurada et al., 1993; Dougherty et al., 1994; Laird et al., 2001). Laird et al., (2001) found that capsaicin had no nociceptive effect on mechanical responses in NK-1 receptor knockout mice. Other support comes from Mantyh's study (1997), which demonstrated that spinal

NK-1 receptors were required for the hyperalgesia caused by peripheral capsaicin injection.

Furthermore, there are several lines of behavioral findings supporting the participation of NK-1 receptors in pain signal transduction. For example, increased nociceptive behaviors in rats due to intrathecal application of SP can be attenuated by NK-1 receptor antagonists (Takahasi et al., 1987; Eide and Hole, 1992; Iyengar et al., 1997). An inhibitory effect of an NK-1 receptor antagonist, CP99994, on primary heat hyperalgesia has also been shown in a previous study (Sluka et al., 1997c). In peripheral nerve injury models, the NK-1 receptor antagonists also displayed an anti-allodynic and anti-hyperalgesic effect (Cumberbatch et al., 1998; Campbell et al., 1998; Gonzalez et al., 2000). Moreover, an NK-1 receptor antagonist attenuated morphine-produced hyperalgesia only in wild type animals, but not in NK-1 receptor knockout mice, which suggests that NK-1 receptors are essential for the formation of this kind of hyperalgesia (King et al., 2005). We hypothesize that NK-1 antagonists may be a promising target to relieve inflammatory pain at appropriate doses. However, this has so far not been verified clinically.

In conclusion, rapid release of SP from the dorsal horn following capsaicin injection has been indicated by the ELISA studies, using a novel technique. After the release, SP activates post-synaptic NK-1 receptors and contributes to enhanced responsiveness of nociceptive dorsal horn neurons, which accounts for the subsequent development of behavioral hypersensitivity.

INVOLVEMENT OF SEVERAL SIGNALING PATHWAYS FOLLOWING NK-1 ACTIVATION IN THE DORSAL HORN

Distribution of NK-1 receptors and their role in sensitization of STT cells

Spinal NK-1 receptors play an important role in mediating nociceptive neurotransmission in the dorsal horn. We found predominant expression of NK-1 receptors in the superficial layers of the dorsal horn, as well as in the deep dorsal horn in a scattered fashion (Figure 16). In lamina I, NK-1 receptors are located in both the cell

bodies and dendrites of neurons which receive synapses from the central terminals of SP-containing primary afferents (Todd et al., 2002). It is estimated that NK-1 receptors are distributed in about 80% of the projection neurons in lamina I (Marshall et al., 1996; Todd et al., 2000), including cells projecting to thalamus, the caudal ventrolateral medulla, lateral parabrachial area, and the periaqueductal grey matter (Ding et al., 1995; Li et al., 1996, 1998; Todd et al., 2002; Spike et al., 2003). For the neurons with their cell bodies located in the deep layers that express NK-1 receptors, as shown in Figs. 15 and 17, it is suggested that these neurons could project NK-1 receptor-containing dendrites into the superficial laminae, forming synapses with SP-immunoreactive boutons (Naim et al., 1997). Immunohistochemical results demonstrate wide expression of NK-1 receptors in STT cells in both the superficial layers and the deep layers of the dorsal horn (Figure 18). It is suggested that most of the NK-1-containing STT cells in lamina I are fusiform and pyramidal, and mainly respond to noxious stimuli (Yu et al., 2005).

The major function of STT cells is to receive nociceptive information from the periphery and to transmit it to the thalamus and sometimes to the brainstem as well. As an important neuropeptide, SP induces prolonged excitation of STT cells by activating spinal NK-1 receptors (Wiesenfeld-Hallin et al., 1984, 1990; Salter and Henry, 1991; reviewed in Dougherty et al, 1993; Dougherty et al., 1994, 1995; reviewed in Willis and Coggeshall, 2004). Consistent with the above observations, increased responses of WDR neurons to mechanical stimuli following spinal SP infusion were recorded in our study (Figure 23).

Role of CaMKII activation in NK-1 signaling pathway in dorsal horn neurons

The involvement of CaMKII in NK-1 signaling pathways and its effect on central sensitization caused by spinal SP treatment was investigated in this study. For the first time, we demonstrate increased phosphorylation of CaMKIIα following NK-1 activation in a specific set of nociceptive dorsal horn neurons, STT cells. We find that activation of CaMKII contributes to the SP-induced sensitization effect in dorsal horn neurons.

Distribution of CaMKIIα and pCaMKIIα in the spinal cord

CaMKII is a widely distributed enzyme in the nervous system, comprising 1-2% of total proteins in the neurons. In pain processing regions, both CaMKIIα- and pCaMKIIα-immunoreactivity have been verified in the superficial laminae of the dorsal horn in naïve animals (Fang et al., 2002; Liang et al., 2004; Dai et al., 2005; Larsson and Broman, 2005; Choi et al., 2005). Phophorylated CaMKII is constitutively expressed at primary nociceptive afferent synapses in naive rat dorsal horn, which suggests a regulatory role of the kinase in synaptic plasticity (Larsson and Broman, 2005). Expression of CaMKII can also be found in the DRG and in the deep laminae of the dorsal horn (Fang et al., 2002). It is reported that 28% of DRG neurons in the L5 segment contain CaMKII (Carlton, 2002). In the present study, in the control animals, pCaMKIIα-immunoreactivity was present in the superficial layers of the dorsal horn, as well as in occasional neurons in the deep layers of the dorsal horn, the area around the central canal and the ventral horn (Figure 15).

CaMKII activation in pain transduction

The critical role of CaMKII in nociceptive neurotransmission has been demonstrated in different pain models (Fang et al., 2002; Dai et al., 2005; Ogawa et al., 2005). The expression of phosphorylated CaMKII was greatly increased in the superficial layers of the dorsal horn after peripheral injection of capsaicin (Fang et al., 2002) or formalin (Liang et al., 2004; Choi et al., 2006). In the superficial dorsal horn, intradermal injection of capsaicin also induced increased postsynaptic expression of both CaMKII and pCaMKII at the synapses formed by peptidergic primary afferents that contain SP and CGRP (Larsson and Broman, 2006). Microdialysis administration of KN93 significantly decreased central sensitization of WDR neurons induced by peripheral inflammation, as well as nociceptive behavior in rats (Fang et al., 2002; Choi et al., 2006). In neuropathic pain models, up-regulation of both CaMKII and pCaMKII in the dorsal horn (Dai et al., 2005) and the trigeminal subnucleus caudalis (Ogawa et al., 2005) was observed, respectively. Moreover, using CaMKIIα gene mutant mice, the

requirement for CaMKII phosphorylation at Thr286 in central sensitization induced by tissue or nerve injury was illustrated (Zeitz et al., 2004).

Changes of CaMKIIα in the rat dorsal horn following spinal NK-1 receptor activation

Though it is well recognized that sensitization of STT cells is dependent on NK-1 activation, the signaling events downstream of NK-1 activation in STT cells remain unknown. Our major concern in this study is to investigate the involvement of CaMKII in NK-1 signaling pathways in the dorsal horn, especially in STT cells. To identify the involvement of CaMKII following NK-1 activation in these dorsal horn neurons, we first investigated the expression of pCaMKII in NK-1 positive neurons. Colocalization of NK-1 receptors and pCaMKIIα was shown in both the superficial and deep layers of the dorsal horn (Figure 16). More importantly, the two labels were present in some STT cells in the superficial dorsal horn (Figure 19), which indicates the potential relationship between the NK-1 receptors and pCaMKII in the dorsal horn neurons.

To verify the relationship, changes of pCaMKIIα expression were investigated in both immunohistochemical and Western blot studies. Up-regulation of pCaMKIIα-LI was found in the superficial dorsal horn 30 min after intrathecal SP injection (Figure 20). Furthermore, increased expression of pCaMKIIα in retrogradely labeled STT cells following SP treatment was observed, compared with the control group (Figure 21). When counts were made of the profiles of STT cells according to their location, significant increases in the percentage of pCaMKIIα-LI STT cells after SP were found in both the superficial layers of the dorsal horn and in deep layers of the spinal cord grey matter (Figure 21). The results from the Western blot studies also showed increased expression of pCaMKIIα in the dorsal horn tissues following intrathecal SP treatment (Figure 22). Administration of the NK-1 receptor antagonist, L-703,606, prevented the increase in expression of pCaMKIIα expected after SP in a dose-dependent manner, suggesting that induced phosphorylation of CaMKIIα is a downstream event of NK-1 receptor activation (Figure 22). These results suggest that CaMKIIα is an important

component of NK-1 signaling pathways in STT cells, as well as in unidentified dorsal horn neurons.

Finally, the functional role of CaMKII activation in the SP-mediated effect was tested in an electrophysiological study. The results indicate that the enhancement of the activity of nociceptive dorsal horn neurons induced by SP was mediated, at least partly, by CaMKII activation (Figures 24-25). Therefore, we suggest that as an important component of NK-1 signaling pathway, CaMKII contributes to the central sensitization of nociceptive dorsal horn neurons.

Molecular events underlying CaMKII phosphorylation following NK-1 activation

The NK-1 receptor is a seven transmembrane domain protein, coupled with Gq proteins. Activation of NK-1 receptors results in hydrolysis of membrane phospholipids by activating phosphinositide-specific PLCβ, leading to enhanced calcium mobilization from the endoplasmic reticulum (Galiano et al., 2004). Cytosolic calcium, together with calmodulin, triggers phosphorylation of CaMKII by binding to the regulatory domain of the kinase. CaMKII is composed of an N-terminal serine-threonine kinase domain, a regulatory region with an autoinhibitory sequence, a calmodulin-binding site and a C-terminal domain (reviewed in Griffith, 2003). Anti-rabbit polyclonal antibody was used in our studies to recognize CaMKIIα phosphorylated at site Thr286. A conformational change of CaMKII can be induced following binding with calcium and calmodulin at the regulatory domain, which transforms the enzyme from an originally inactive form into an active autophosphorylated form at Thr286. As a result, a significantly increased affinity of the kinase for Ca^{2+} and calmodulin is developed. More importantly, the autophosphorylated kinase shows prolonged activity even at low calcium concentration or after dissociation of calmodulin (reviewed in Colbran, 2004; reviewed in Colbran and Brown, 2004). This autonomous activity of CaMKII enables it to play an important role in the regulation of synaptic plasticity.

Translocation of activated CaMKII and interaction with its substrates

CaMKII is present in both the cytosolic fraction and the cell membrane. It is also the major component of the postsynaptic density (PSD), which is an electron-dense

region in the membrane of a postsynaptic neuron, suggesting a role of CaMKII in the regulation of synaptic plasticity. Using green fluorescent protein (GFP)-tagged CaMKII, CaMKII translocation after its activation was monitored. Accumulation of GFP-CaMKII to postsynaptic sites was observed rapidly after glutamate treatment, which suggests that Thr286-autophosphorylation promotes the interaction of CaMKIIα with synapses (Shen and Meyer, 1999; reviewed in Griffith et al., 2003). Furthermore, the requirement of autophosphorylation of CaMKIIα at Thr286 in the modulation of neuronal function was supported by evidence from CaMKIIαT286A mutant mice (Hardingham et al., 2003). After autophosphorylation and translocation, activated CaMKII regulates synaptic function by interacting with many receptors, signaling molecules, and transcription factors. By phosphorylating GluR1 subunits, activated CaMKII facilitates the conductance of AMPA receptors (Derkach et al., 1999). CaMKII may also regulate the redistribution of AMPA receptors and enhance the recruitment of AMPA receptors to postsynaptic silent synapses, which originally lack AMPA receptors (Liao et al., 2001; Shi et al., 2001).

PKC activation is another down-stream event following NK-1 activation in the dorsal horn

Besides the activation of CaMKII, the induced influx of Ca^{2+} and release of diacylglycerol (DAG), another membrane phospholipid hydrolysis product, also catalyze the activation of the PKC pathway. So far, more than 10 isozymes have been found in the PKC family, which can be divided into three major groups: the conventional group (calcium-dependent), the calcium independent group and the alternative group. The conventional group includes α, βI, βII and γ isoforms of PKC, which are predominantly expressed in the central nervous system (Kuo et al., 1980; Minakuchi et al., 1981; Kikkawa et al., 1982). When activated, PKC is involved in signal transduction by phosphorylating serine and threonine residues on substrate proteins. In the nervous system, PKC plays a multifunctional role in regulating cellular functions, such as channel

conductance, neurotransmitter release, LTP, and interaction of receptors (reviewed in Battaini and Pascale, 2005).

Distribution of PKCγ and its role in central sensitization

In our study, a plexus of neuronal processes with dense expression of PKCγ was mainly found in lamina II of the dorsal horn, with some expression in laminae I and III (Figure 26). In addition, STT cells that contain PKCγ were clearly seen in the superficial layers (Figure 27). These results indicate a potential role of PKCγ in nociceptive signaling. The expression pattern of the conventional group of PKC is quite similar in adult rat spinal cord. Akinori (1998) reported that all the four isoforms have abundant expression in the superficial dorsal horn, with less distribution in interneurons of the dorsal horn (reviewed in Akinori, 1998). A further study indicated that most PKCγ-containing neurons in the superficial dorsal horn were non-GABAergic and likely to be excitatory neurons (Polgár et al., 1999).

Spinal application of PKC activators, such as phorbol esters, could regulate A-type K^+ current and induce enhanced NMDA and EAA currents in dorsal horn neurons (Chen et al., 1992; Gerber et al., 1989; Hu et al. 2003, Hu and Gereau, 2003). Moreover, increased responses of STT cells to innocuous stimuli (Palecek et al. 1994; Lin et al. 1996; Peng et al., 1997) and increased release of SP, CGRP (Frayer et al., 1999), and EAA (Gerber et al., 1989) from spinal cord slices were observed following PKC activation. The activation of PKC also attenuated the descending inhibition from the periaqueductal gray (PAG) to STT cells, therefore enhancing central sensitization (Peng et al. 1997). Evidence from behavioral studies demonstrated that activation of PKC results in mechanical hyperactivity in rats, which was dependent on the activation of glutamate receptors (Sluka and Audetta, 2006). Recently, it has been suggested that by phosphorylating Ser185 and Ser191 residues of G protein-coupled inward rectifier K^+ (GIRK) channels, PKC mediates neuronal excitability and neurotransmission (Mao et al., 2004).

The important role of PKC activation in persistent nociception is also illustrated in both inflammatory and neuropathic models. Increased expression of PKCγ was found in rat dorsal horn following peripheral inflammation (Martin et al., 1999) and nerve injury (Mao et al., 1995). Both pre- and post-administration of a PKC inhibitor had an inhibitory effect on capsaicin-evoked central sensitization of primate STT cells (Peng et al., 1997; Sluka et al., 1997a, b). Similar results were observed in formalin- (Coderre et al., 1992; Nakanishi et al., 1999; Yashpal et al., 2001; Souza et al., 2002; Sweitzer et al., 2004) or mustard oil-induced (Munro et al. 1994) pain models. Spinal blockade of PKC activation also inhibited exaggerated pain behaviors resulting from capsaicin injection (Sluka and Willis, 1997), alcoholic-induced neuropathy (Dina et al., 2000), diabetic neuropathy (reviewed in Kamei, 2001), and spinal nerve ligation (Hua et al., 1999). The requirement of PKC activation was further confirmed in PKC mutant animals, which showed attenuated pain-related hyperactivity in the spinal cord following nerve injury (Malmberg et al., 1997). The down-stream events of PKC activation have also been investigated, which shows that activated PKC following peripheral noxious stimulation could induce increased phosphorylation of the NR1 subunit of the NMDA receptor (Zou et al., 2004) and CREB (Miyabe and Miletic, 2005) in the spinal dorsal horn.

Activation of PKCγ following spinal NK-1 activation

The expression change of PKC in the dorsal horn following NK-1 activation was investigated in this study. Colocalization of PKCγ and NK-1 receptors was observed in the superficial layers of the dorsal horn (Figure 26). Spinal SP treatment also induced upregulation of PKCγ in the superficial dorsal horn, compared with the control group (Figure 28). Furthermore, activation of PKCγ was dependent on the activation of spinal NK-1 receptors (Figure 29). These results indicate the involvement of PKCγ activation in NK-1 signaling pathways. Expression of PKCγ in some STT cells in lamina I was observed (Figure 27). However, unlike the results concerning pCaMKII expression in the STT cells, there was no difference in the proportion of PKCγ-containing STT cells

between the SP-treated and saline-treated groups (data not shown). This might be due to the relatively small expression of PKCγ in lamina I.

NPC15437 has been demonstrated to inhibit PKC activity through interference with its regulatory domain (IC_{50} =19 ± 2 μM, Sullivan et al. 1992). As an "allosteric" inhibitor, the binding of NPC15437 with PKC changes the enzyme conformation, which results in inhibition of enzyme activity. In addition, NPC15437 shows high selectivity for PKC over PKA or myosin light chain kinase (IC_{50}>>300 μM, Sullivan et al. 1992). Our results demonstrate that preemptive application of NPC15437 blocked SP-induced effects on WDR neurons (Figs 29-30), which further proves that the PKC pathway is downstream of NK-1 receptor activation.

Possible involvement of PKA activation in NK-1 signaling pathways

Besides the CaMKII and PKC pathways, activation of other secondary messenger systems may be induced following NK-1 activation as well, such as the cyclic AMP (cAMP)-PKA signaling cascade. The critical role of the cAMP-PKA signaling pathway in nociceptive transmission has been illustrated in previous reports. Behaviorally, an analog of cAMP can facilitate hyperalgesia and allodynia in rats in response to mechanical stimuli, whereas an adenylate cyclase inhibitor or a PKA inhibitor can reverse the capsaicin-induced secondary hyperalgesia and allodynia (Sluka and Willis, 1997). In an electrophysiological study, the activation of PKA by 8-bromo-cAMP in the spinal cord significantly enhanced the responses of STT cells to mechanical stimuli, and this was prevented by a PKA inhibitor (Lin et al. 2002). Inhibition of SP-induced responses to mechanical stimuli due to H89 pretreatment was observed in our experiment, which means that besides CaMKII and PKC pathways, the cAMP-PKA pathway acts as another important component of the SP signaling network.

Growing evidence has demonstrated that there exists crosstalk between the PKA and PKC pathways (Cooper et al. 1995; Mau et al. 1997; Badie-Mahdavi et al. 2001; reviewed in Willis, 2001a; Sun et al. 2004). First, for SP signaling pathways, in rat pituitary cell cultures, SP could facilitate cAMP production after forskolin treatment

whereas inhibitors of PKA and cAMP blocked the effect of forskolin and vasoactive intestinal peptide (VIP) on PKC isoenzyme dislocation (Mau et al. 1997). In vitro experiments show that SP-induced dorsal horn neuron excitability and the expression of Fos, a transcription factor in the dorsal horn, was reduced following treatment with either a PKA inhibitor or a PKC inhibitor (Badie-Mahdavi et al. 2001). Our lab has also demonstrated that PKC and PKA are both downstream in the CGRP signaling pathway (Sun et al. 2004). Another instance is the involvement of PKC and PKA, as well as CaMKII, in the induction of spinal cord LTP. Application of inhibitors of those kinases abolished the formation of early phase LTP (Yang et al. 2004). In a study of synaptic plasticity in an *Aplysia* model, PKC activation resulted in more accumulation of cAMP in sensory neurons. The underlying mechanism may be due to the regulation by PKC of a specific adenylyl cyclase (Sugita et al. 1997).

Thus, it is believed that rather than working in isolation, cAMP-PKA and IP_3 - PKC pathways form an intricate intracellular network following the activation of NK-1 receptors. Their effects may be mediated through coupling to the same receptors or converging onto a common target. For instance, although working on different amino acids, both PKC and PKA can phosphorylate NMDA receptor 1 (NR_1) subunits to enhance the responsiveness of NMDA receptors during central sensitization (Leonard and Hell, 1997; Tingley et al. 1997; Zou et al. 2002, 2004; Fang et al. 2003). However, a lot of details are missing in this network and further investigation is required to characterize the interaction of these two pathways.

In summary, the essential role of NK-1 activation in capsaicin induced central sensitization and the molecular events following NK-1 activation in the nociceptive dorsal horn cells are discussed in the present study. The results show that peripheral capsaicin injection induces increased release of SP in the dorsal horn and subsequent activation of NK-1 receptors, which is required for the development of central sensitization and behavioral hyperactivity. Moreover, following spinal NK-1 receptor activation, increased intracellular calcium mediates the phosphorylation of CaMKIIα and

activation of PKC in the rat dorsal horn. These events result in a prolonged increase in the excitability of nociceptive dorsal horn neurons.

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Abstracts

Yan JY, Sun RQ, Zou XJ, and Willis WD. Activation of protein kinase C in rat dorsal horn following spinal substance P treatment. 26th Annual Scientific Meeting of American Pain Society, Washington DC, USA, #613, 2007

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