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Pain and anesthesia

# Rodent models clarify the role of cells expressing the substance P receptor in pain

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The recent failure of substance P receptor (NK1) antagonists in clinical trials for treatment of pain spawned additional studies asking why preclinical models implicating SP in chronic pain did not translate into a viable target for analgesic development. This review focuses on recent pain model system studies, suggesting that it is not SP or NK1 *per se*, but rather the cells that express NK1 that are crucial for maintaining chronic pain.

## Introduction

According to the American Chronic Pain Association, more than 50 million people in the USA alone are disabled to some extent by chronic pain. After tissue injury, spinal cord dorsal horn neurons, which are the first CNS processing station for pain signal from the periphery, show a decreased threshold for action potential firing, increased responsiveness to a given stimulus, and receptive field enlargement. These electrophysiological changes are collectively referred to as central sensitization and are thought to underlie the development of chronic pain [1–4].

For years, researchers have been trying to elucidate the molecular mechanisms that underlie central sensitization to develop novel approaches for treating chronic pain conditions. A variety of studies led to the hypothesis that substance

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For pain research, it is traditional to search for a pain transmitter. Through designing selective chemical antagonists for such pain transmitter receptors, we hope to control chronic pain in patients. However, recent studies of substance P (SP) receptor antagonists failed to do this. There are many possible reasons to explain why the drugs did not give analgesic effects in patients. In this manuscript, Yarimar Carrasquillo and Robert Gereau IV review recent failure and progress in SP-related studies and suggest that the cells expressing SP receptors (neurokinin 1) are crucial for chronic pain.

P (SP) and its neurokinin 1 (NK1) receptor were involved in central sensitization. However, highly specific antagonists of NK1 failed to show significant analgesic efficacy in clinical trials [5]. Recent studies have shown that NK1 antagonists are not the magic bullet we were hoping for because it is not the NK1 receptor itself, but rather the spinal cord neurons that express NK1 that are pivotal for central sensitization and chronic pain [6–10].

## Evidence from *in vivo* models

At the heart of the belief that SP and its NK1 receptor were crucial for central sensitization and chronic pain were the beautiful studies from Mantyh and colleagues, which were based largely on the observations that SP is released upon peripheral noxious stimulation and activates spinal cord nociceptive-specific neurons [11–14], and that when SP binds to NK1, both SP and NK1 are internalized [14,15]. Mantyh

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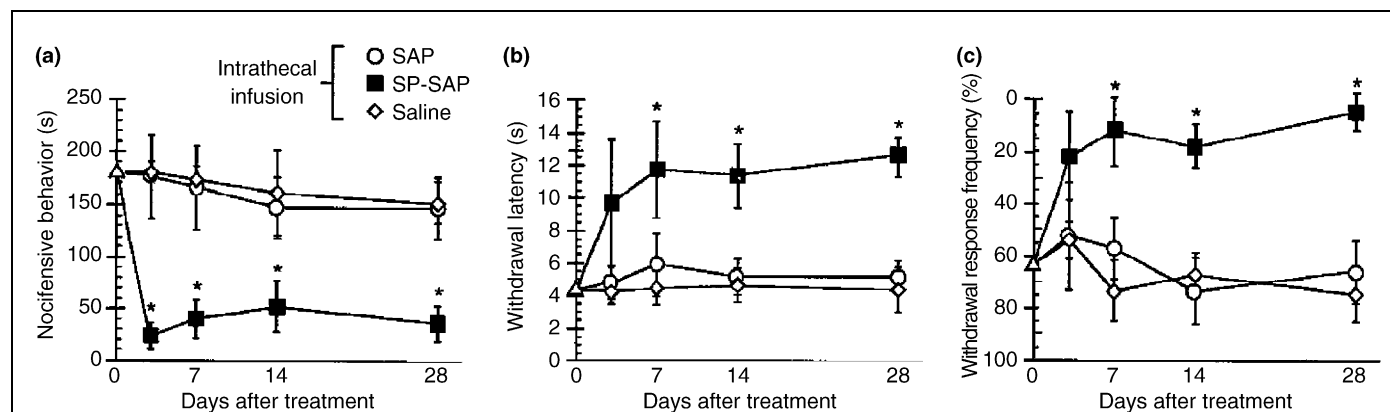
*et al.* took advantage of the NK1 internalization to create a targeted neurotoxin by conjugating SP to the ribosome-inactivating protein, saporin (SAP), which is cytotoxic when internalized [16]. SP-SAP was used to selectively ablate NK1-expressing neurons in the spinal cord dorsal horn of rats. This ablation treatment has been used in combination with different behavioral models of inflammatory and neuropathic pain as well as with various cellular models of pain sensitization to characterize the pivotal role of spinal cord NK1-expressing neurons on pain transmission and central sensitization (Table 1) [6–9].

#### SP-SAP treatment attenuates nociceptive behavior in rats

In their first two studies, Mantyh *et al.* investigated the behavioral effects of SP-SAP on models of inflammatory pain and neuropathic pain in rats, all of which are thought to involve central sensitization [6,7]. Intraplantar injection of capsaicin, formalin, carrageenan, or complete Freund's adjuvant (CFA) produced local inflammation, thermal hyperalgesia and mechanical allodynia. Ablation of dorsal horn NK1-expressing neurons with SP-SAP attenuated spontaneous nociceptive behavior in all four inflammatory pain models and dramatically reduced inflammation-induced thermal hyperalgesia and mechanical allodynia (Fig. 1) [6,7]. The effects of SP-SAP on neuropathic pain were also evaluated using the nerve ligation model. Nerve ligation produced long-lasting mechanical allodynia that was significantly inhibited in SP-SAP-treated rats, independent of whether the SP-SAP treatment was administered before or after the induction of nerve injury. Importantly, SP-SAP treatment left responses to mild acute noxious stimuli unchanged and did not affect morphine analgesia. These results suggest that spinal dorsal horn NK1-expressing neurons modulate

transmission of chronic noxious stimuli without interfering with acute nociception, a vital physiological function. In addition, these neurons do not seem to be the major site of morphine action, suggesting that opiates could still serve as a therapeutic tool for acute pain relief after SP-SAP treatment.

The dramatic effects of SP-SAP on nociceptive behavior demonstrate that NK1-expressing neurons in the spinal cord are pivotal for the transmission of highly noxious stimuli and the generation and maintenance of pain hypersensitivity associated with inflammation and chronic neuropathy. However, the failure of NK1 antagonists in clinical trials for treatment of pain suggest that the anti-hyperalgesic effects observed in SP-SAP-treated rats is not solely mediated by SP or NK1. The SP-SAP treatment does not block or inactivate NK1 receptors only; this treatment kills a population of neurons in the spinal cord that express a variety of other receptors and molecules that could also be involved in the processing of pain. It is possible that, upon peripheral noxious stimulation, the NK1 receptor is activated by SP initiating a series of cellular events that result in the development and maintenance of persistent pain and central sensitization. This sensitization process might be initiated in NK1-expressing neurons but could be maintained by processes not involving the NK1 receptor itself. The effective attenuation of allodynia in SP-SAP-treated rats, independent of whether the SP-SAP treatment was administered before or after the induction of nerve injury, supports the pivotal role of these neurons in both the development and maintenance of persistent pain. Identifying the cellular and molecular mechanisms that these neurons undergo during persistent pain states could help in the understanding and management of chronic pain.



**Figure 1.** Reduced capsaicin-induced pain behavior in substance P-saporin (SP-SAP)-treated rats [6]. In untreated rats, intraplantar injection of capsaicin produced nociceptive behavior for approximately 3 min and an approximate 50% decrease in thermal withdrawal latency and withdrawal frequency from mechanical stimuli. (a) SP-SAP treatment markedly reduced capsaicin-induced nociceptive behavior and (b, c) attenuated both thermal hyperalgesia and mechanical allodynia. Infusion of saline or saporin (SAP) produced no significant change compared with untreated rats in any of the capsaicin-induced behavioral responses measured. All data points are expressed as the mean  $\pm$  S.E.M., and significant differences were calculated by a one-way analysis of variance (ANOVA) and Bonferroni comparisons ( $P < 0.01$ ). Adapted from [6], with permission.

### *SP-SAP treatment decreases capsaicin-induced neuronal responses in the spinal dorsal horn and eliminates central sensitization in vivo*

To start characterizing the cellular mechanisms underlying the decreased pain hypersensitivity observed in SP-SAP-treated rats, Khasabov *et al.* [8] evaluated *in vivo* the effects of SP-SAP treatment on general neuronal response properties, central sensitization, and the development of windup on the remaining dorsal horn neurons after the ablation treatment. Both, central sensitization and windup have long been used as cellular models of pain sensitization [4]. As discussed earlier in this review, central sensitization has been defined as a decreased threshold for action potential firing, increased responsiveness to a given stimulus, and the receptive field enlargement of dorsal horn neurons [1–4]. Windup, by contrast, has been defined as facilitated or enhanced responses of dorsal horn neurons by repeated stimulation of C-fiber afferents [4]. Khasabov *et al.* evaluated the effects of SP-SAP treatment on these cellular models of pain sensitization by performing *in vivo* single-unit electrophysiological recordings of spinal cord dorsal horn neurons from vehicle, SAP, or SP-SAP-treated rats. The *in vivo* electrophysiological approach allows one to record changes in firing properties of spinal dorsal horn neurons in response to peripheral stimulation such as thermal and mechanical stimuli or capsaicin injection.

SP-SAP treatment resulted in approximately 62% decrease of NK1-expressing neurons in the dorsal horn, when compared to vehicle-treated rats. Capsaicin-evoked responses of the remaining wide dynamic range (WDR) and high-threshold (HT) neurons and the duration of their responses were significantly decreased in SP-SAP-treated rats (Fig. 2a). In addition, none of the WDR or HT neurons exhibited capsaicin-evoked sensitization to thermal and mechanical stimuli nor did they exhibit windup (Fig. 2b). Analogous to the behavioral results, the responses of the remaining WDR and HT neurons to basal mechanical or thermal stimuli (before capsaicin injection) were unaltered in SP-SAP-treated rats; however, the number of HT neurons found in SP-SAP-treated rats was significantly lower than in vehicle-treated rats. [8]. The results from these *in vivo* recording experiments nicely correlate the behavioral effects of SP-SAP treatment and further confirm the crucial role of NK1-expressing neurons in central sensitization and persistent pain. Equally important, these results suggest that SP-SAP is selectively targeting HT neurons that mediate hyperalgesia and allodynia but not acute pain transmission.

### *SP-SAP treatment disrupts supraspinal descending facilitating pathways to the spinal cord*

NK1-expressing neurons constitute less than 5% of the total neurons in the dorsal horn [7,17]. How can elimination of this relatively small population of dorsal horn neurons have such a profound effect on pain behavior and central sensi-

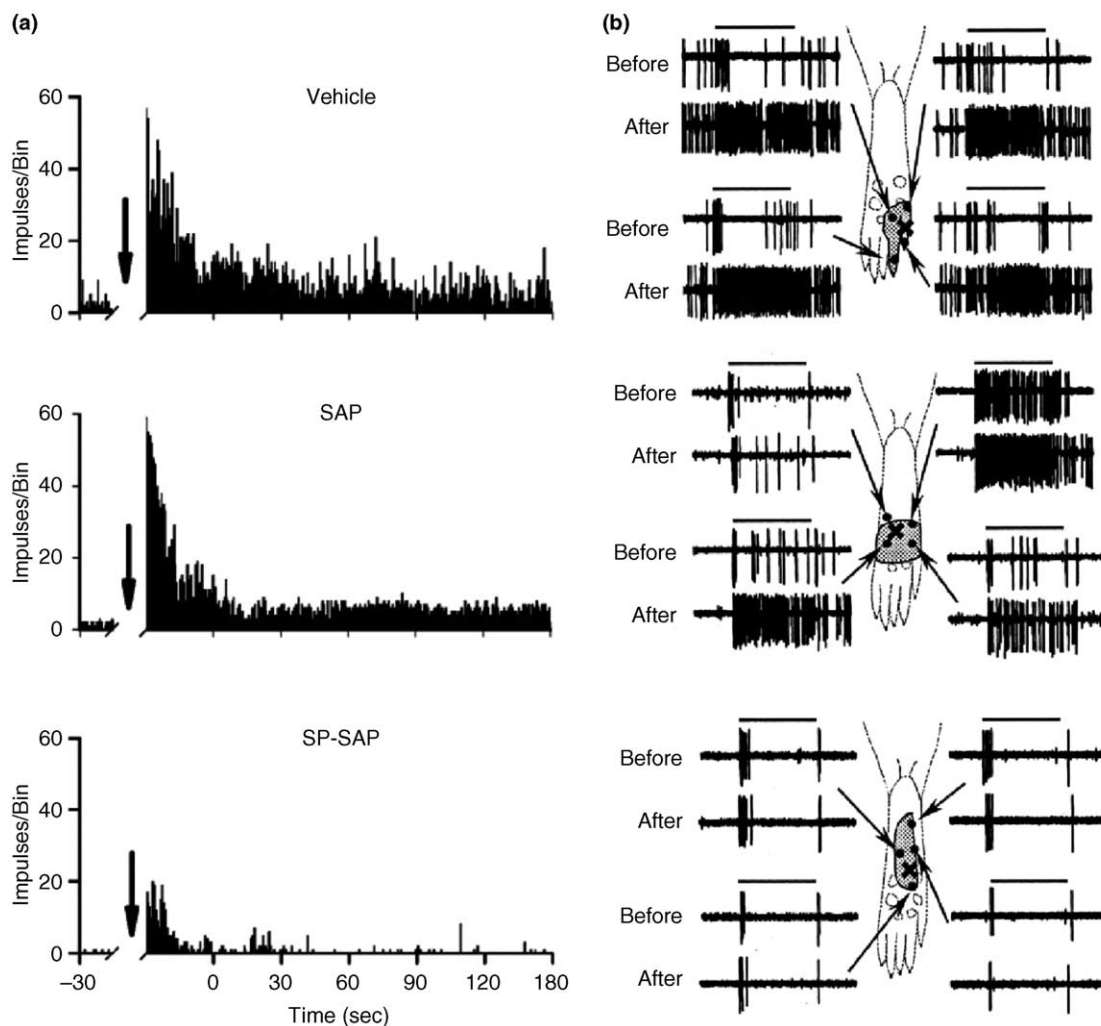
zation? A possible explanation is that the NK1-expressing neurons are part of a supraspinal circuit that activates descending facilitation mechanisms thereby increasing the excitability of dorsal horn neurons. Ablation of NK1-expressing neurons might disrupt this supraspinal circuit, preventing the development of central sensitization and hyperalgesia. This hypothesis is supported by the fact that the majority of NK1-expressing neurons in the dorsal horn of the spinal cord are projection neurons that belong to the spinoparabrachial tract, one of the main ascending pathways important for pain transmission and modulation and for the development of central sensitization [18]. Suzuki *et al.* [9] addressed this hypothesis more directly using a combination of electrophysiological, pharmacological and immunohistochemical approaches in SP-SAP-treated rats. As previously shown by other groups, SP-SAP treatment produced a selective and significant ablation of NK1-expressing neurons in the spinal cord, reduced inflammation-induced mechanical allodynia without affecting baseline thresholds, and markedly attenuated both central sensitization and windup in the remaining dorsal horn neurons after the ablation. In addition, this study demonstrated that pharmacological blockade of descending serotonergic (5HT) pathways in vehicle or SAP-treated rats reproduced the behavioral and electrophysiological changes observed in SP-SAP-treated rats, but had little or no effect on SP-SAP-treated rats. Furthermore, using Fos immunoreactivity as a neuronal activity marker, a significant decrease in nociceptive-induced activation of brainstem serotonergic neurons was found in SP-SAP-treated rats when compared to vehicle or SAP-treated rats. These elegant studies suggest that the behavioral and cellular changes observed in SP-SAP-treated rats are mediated, at least in part, through alterations in supraspinal descending facilitating pathways to the spinal cord.

The results from the SP-SAP *in vivo* experiments discussed in this review demonstrate that spinal cord neurons that express NK1 are crucial for the development and maintenance of central sensitization and chronic pain. It is important to specify that these results do not demonstrate a crucial role for NK1, but rather that the NK1-expressing neurons, through an as yet undefined molecular mechanism, are crucial for pain processing. The combination of behavioral, pharmacological and cellular approaches used in these studies provide strong evidence in support of this hypothesis. Furthermore, the results obtained from these experiments offered a potential anatomical and cellular explanation for the pronounced effects observed with the ablation treatment.

### **Evidence from *in vitro* models**

#### *Synaptic mechanisms underlying sensitization and plasticity of the spinal cord NK1-expressing neurons*

The realization that NK1-expressing neurons in the spinal cord play a pivotal role in persistent pain and central



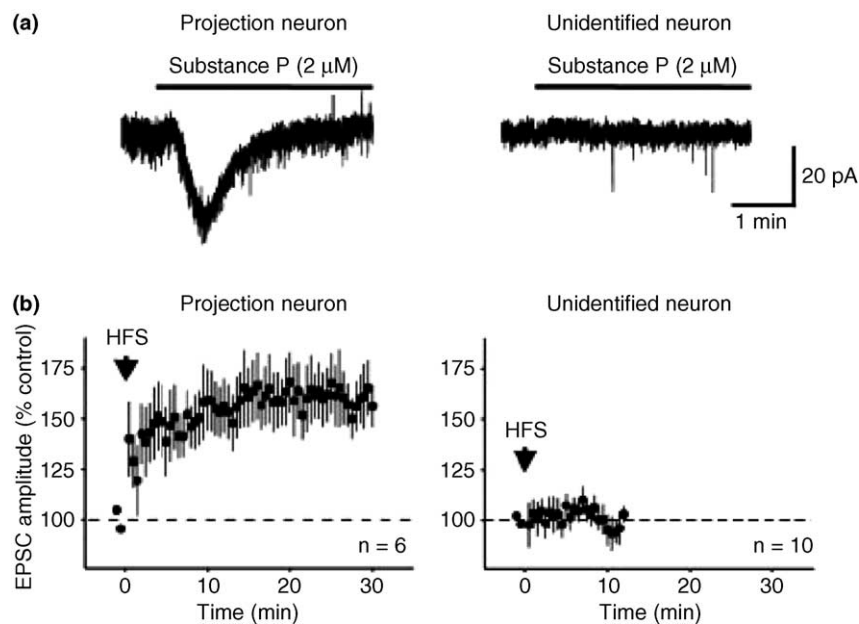
**Figure 2.** Effects of substance P-saporin (SP-SAP) on capsaicin-induced neuronal responses and central sensitization *in vivo* [8]. (a) SP-SAP treatment reduced the responses of dorsal horn wide dynamic range (WDR) neurons evoked by intraplantar injection of capsaicin. The arrow points to the time of capsaicin injection. Bin size is 500 ms. (b) Representative examples illustrating responses of WDR neurons to mechanical stimuli before and after capsaicin in vehicle-, saporin (SAP)- and SP-SAP-treated groups. Sensitization of nociceptive neurons to mechanical stimuli after capsaicin injection does not occur in animals pretreated with SP-SAP. Receptive fields (RF) are indicated by the gray shaded areas, and test sites for mechanical stimulation are indicated by the dots within the RF. Arrows point to specific test sites at which pairs of responses (before and after capsaicin) were obtained. The capsaicin injection is indicated by the  $\times$ . Horizontal bars denote time of stimulation (2 s). Adapted from [8], with permission.

sensitization suggested that understanding the molecular events underlying the sensitization of these neurons during persistent pain conditions might lead to the identification and development of novel treatments for chronic pain. Ikeda *et al.* initiated this molecular and cellular characterization with a pioneering study that identified for the first time synaptic mechanisms underlying the sensitization of spinal cord NK1-expressing neurons. In this study, whole-cell patch-clamp recordings were performed from dorsal horn NK1-expressing neurons in an acute spinal cord slice preparation.

Most of the NK1-expressing neurons in the dorsal horn project to the parabrachial area [18]; Ikeda *et al.* took advantage of this fact and retrogradely labeled with Dil the spinal cord dorsal horn neurons that project to the parabrachial area to identify neurons that could potentially express NK1. Neu-

ronal responses to bath application of SP were used to identify the expression of functional NK1 receptors in retrogradely labeled projection neurons. The unlabeled neurons or the labeled neurons that did not respond to SP were used as control neurons. Most of the projection neurons (77%) responded with an inward current to SP (Fig. 3a). These neurons had larger membrane capacitance, more negative resting membrane potentials, and more negative thresholds for firing action potentials than unlabeled neurons.

Ikeda *et al.* used the induction of activity-dependent synaptic long-term potentiation (LTP) as a cellular model of afferent-induced hyperalgesia. LTP is characterized by long-lasting increases in the efficacy of synaptic transmission after brief high-frequency stimulation (HFS). This cellular phenomenon can be induced in both humans and animals



**Figure 3.** Long-term potentiation (LTP) is preferentially induced in lamina I projection neurons that express NK1 [10]. (a) Projection neurons (PNs), but not unidentified neurons (UNs), respond with an inward current to bath application of substance P (SP). (b) High-frequency stimulation (HFS) induced synaptic LTP in all PNs that responded to bath application of SP but failed to change synaptic strength in the UNs that did not respond to SP. Adapted from [11], with permission.

and has been strongly correlated with pain behavior [4,19–21]. Moreover, SP has been shown to be released in the spinal cord dorsal horn *in vivo* during LTP, suggesting that SP might be involved in the induction of LTP, possibly through activation of the NK1 receptor [22]. Ikeda *et al.* tested this hypothesis using a spinal cord acute slice preparation and recording only from dorsal horn neurons that project to the parabrachial area. HFS was applied to the spinal dorsal root and the evoked excitatory postsynaptic currents (EPSCs) were recorded from retrogradely labeled projection neurons. LTP was preferentially induced in dorsal horn NK1-expressing projection neurons (identified by their responses to SP) but not in the projection neurons that did not express functional NK1 receptors (Fig. 3b). NK1 receptors in dorsal horn projection neurons are required for the induction of LTP because HFS failed to induce LTP in NK1-expressing neurons in the presence of an NK1 antagonist. Furthermore, this study demonstrated that in dorsal horn projection neurons that express NK1, but not in other projection neurons, synergistic activation of both NK1 and low-threshold voltage-gated calcium channels facilitate activity and calcium-dependent sensitization [10]. This study was the first to identify synaptic mechanisms underlying sensitization and plasticity of the spinal cord NK1-expressing neurons that mediate pain hypersensitivity.

The functional role of NK1 receptor activation in the induction of LTP supports the hypothesis that NK1 activation is important for the initiation of the cellular changes that could underlie persistent pain. The answer to whether NK1

activation has any role in the maintenance of persistent pain and the cellular changes underlying this behavior remains elusive. In most of the clinical trials that tested the analgesic action of NK1 antagonists in humans, the treatment was administered to reduce an already established pain rather than to prevent the initiation of pain. All the evidence so far points to a role of NK1 receptor activation in the induction of pain and central sensitization; however, the role of NK1 activation on pain and central sensitization maintenance has not been established. Additional studies aimed at identifying the downstream effects of NK1 activation could help elucidate the cellular and molecular mechanisms underlying central sensitization maintenance and persistent pain.

#### *Cultured dorsal horn neurons serve as a model to study the cellular mechanisms and biochemical cascades underlying central sensitization*

Studies to identify the cellular mechanisms and biochemical cascades underlying central sensitization have been performed using cultured dissociated dorsal horn neurons. By performing whole cell recordings from cultured dorsal horn neurons, Hu *et al.* [23] showed that protein kinase A (PKA), protein kinase C (PKC), and the extracellular signal-regulated kinase (ERK) exert inhibitory effects on transient A-type potassium currents of spinal cord dorsal horn neurons. Transient A-type potassium currents are known to be important determinants of neuronal excitability. Consistent with this and in support of the modulatory actions that PKA and PKC

**Table 1. Comparison summary table**

	<i>In vivo</i> models	<i>In vitro</i> models
<b>Pros</b>	<ol style="list-style-type: none"> <li>1. Closely resemble human pain conditions</li> <li>2. The effects of drugs on pain behavior can be measured</li> </ol>	<ol style="list-style-type: none"> <li>1. The cellular and molecular mechanisms underlying pain can be investigated at the synaptic level</li> <li>2. Allows accurate control of drug concentrations, providing greater specificity of action</li> </ol>
<b>Cons</b>	<ol style="list-style-type: none"> <li>1. The cellular and molecular mechanisms of pain cannot be assessed at the synaptic level</li> <li>2. Concentrations of pharmacologic agents are difficult to control</li> </ol>	<ol style="list-style-type: none"> <li>1. The relevance and specific contribution to pain behavior of the results obtained from <i>in vitro</i> models is difficult to determine</li> </ol>
<b>Best use of model</b>	<ol style="list-style-type: none"> <li>1. To study the effects of drugs on pain behavior</li> <li>2. To correlate changes in neuronal firing properties with specific behavior</li> </ol>	<ol style="list-style-type: none"> <li>1. To evaluate pain transmission at a cellular and molecular level</li> <li>2. Identify biochemical cascades and synaptic mechanisms underlying central sensitization</li> </ol>
<b>References</b>	[6–9,22]	[10,23,24]

exert on A-type potassium currents, activation of PKA and PKC was shown to increase action potential firing, which reflects an increase in the excitability of superficial dorsal horn neurons [24]. Although not tested in this study, it is possible that the activation of this signaling cascade and the accompanying changes in neuronal excitability do occur in the dorsal horn neurons that express NK1 and mediate pain hypersensitivity. ERK activation in superficial spinal cord neurons has been shown to induce NK1 upregulation and to contribute to pain sensitization [25]; however, the specific cellular mechanisms and biochemical cascades underlying this pain sensitization remain elusive. Studies using cultured neurons like those described above could help to further identify the cellular mechanisms underlying pain and central sensitization in NK1-expressing neurons.

### Evidence from *in silico* models

*In silico* models to study central sensitization are not currently available. However, our rapidly increasing knowledge on the neural mechanisms underlying central sensitization could aid in the development of an *in silico* model in the near future. The development of this type of model would provide us with new tools to study the mechanisms of central sensitization at a theoretical level.

### Conclusions

Treatments currently available for chronic pain conditions are few and their use is limited by the severe side effects of these medications. The identification and development of

novel effective treatments for chronic pain with minimal side effects is still a challenge to the field of pain. The combination of *in vivo* and *in vitro* pain model system studies discussed in this review demonstrate that dorsal horn NK1-expressing neurons are pivotal for the development and maintenance of chronic pain (Table 1). However, the cellular and molecular mechanisms underlying the sensitization of these neurons during persistent pain remain elusive. Future studies aimed at identifying the neural bases of sensitization in this subpopulation of dorsal horn neurons could help in the understanding and management of persistent pain. Development of pharmacological agents targeting the cellular machinery of spinal cord SPR-expressing neurons might offer an alternative approach to reduce the severe side effects normally associated with pain treatments.

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### Links

- The American Chronic Pain Association <http://www.theacpa.org/>
- NINDS Chronic Pain Information Page [http://www.ninds.nih.gov/health\\_and\\_medical/disorders/chronic\\_pain.htm](http://www.ninds.nih.gov/health_and_medical/disorders/chronic_pain.htm)

## Outstanding issues

- Identify the molecular mechanisms of LTP induction and maintenance in spinal cord NK1-expressing neurons and its relevance to the initiation and maintenance of chronic pain.
- Identify the cellular and molecular mechanisms underlying sensitization of spinal cord NK1-expressing neurons during persistent pain.
- Development of new therapeutic strategies targeting these sensitization mechanisms in spinal cord NK1-expressing neurons.

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