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Tumor-evoked hyperalgesia and sensitization of nociceptive dorsal horn neurons in a murine model of cancer pain

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Abstract

Pain associated with cancer, particularly when tumors metastasize to bone, is often severe and debilitating. Better understanding of the neurobiological mechanisms underlying cancer pain will likely lead to the development of more effective treatments. The aim of this study was to characterize changes in response properties of nociceptive dorsal horn neurons following implantation of fibrosarcoma cells into and around the calcaneus bone, an established model of cancer pain. Extracellular electrophysiological recordings were made from wide dynamic range (WDR) and high threshold (HT) dorsal horn neurons in mice with tumor-evoked hyperalgesia and control mice. WDR and HT neurons were examined for ongoing activity and responses to mechanical, heat, and cold stimuli applied to the plantar surface of the hind paw. Behavioral experiments showed that mice exhibited hyperalgesia to mechanical and heat stimuli applied to their tumor-bearing hind paw. WDR, but not HT, nociceptive dorsal horn neurons in tumor-bearing mice exhibited sensitization to mechanical, heat, and cold stimuli and may contribute to tumor-evoked hyperalgesia. Specifically, the proportion of WDR neurons that exhibited ongoing activity and their evoked discharge rates were greater in tumor-bearing than in control mice. In addition, WDR neurons exhibited lower response thresholds for mechanical and heat stimuli, and increased responses to suprathreshold mechanical, heat, and cold stimuli. Our findings show that sensitization of WDR neurons contribute to cancer pain and support the notion that the mechanisms underlying cancer pain differ from those that contribute to inflammatory and neuropathic pain.

Keywords

cancer pain; hyperalgesia; spinal cord; dorsal horn; electrophysiology; sensitization

1. Introduction

Pain is one of the most common symptoms reported by patients with cancer (Foley, 2000). Indeed, nearly 90% of patients with end-stage cancer report pain (Foley, 2000; Peng et al., 2006; Portenoy, 1989). Metastasis of tumor cells to bone is particularly common in patients with lung, breast, and prostate cancer (Rubens, 1998) and patients with bone metastasis are more likely to experience severe pain (Ahles et al., 1984; Brescia et al., 1992; Daut and

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Cleeland, 1982; Mercadante, 1997; Portenoy et al., 1999). Once metastatic bone cancer is diagnosed, the frequency of pain is greater than 60% for patients with sarcomas, breast cancer, multiple myeloma, or lung cancer (Pecherstorfer and Vesely, 2000). Thus, pain associated with tumor cells that have metastasized to bone is a frequent and debilitating complication of cancer.

Understanding the neurobiological mechanisms underlying cancer pain is critical for improved management. Animal models of cancer pain using mice (Asai et al., 2005; Baamonde et al., 2004; Lee et al., 2005; Menendez et al., 2003; Sabino et al., 2003; Sasamura et al., 2002; Schwei et al., 1999; Shimoyama et al., 2002; Wacnik et al., 2001; Wacnik et al., 2003) and rats (Medhurst et al., 2002) have been developed and are providing new information on the mechanisms that contribute to cancer-related pain. Implantation of tumor cells into bone produces behavioral signs of ongoing pain (Menendez et al., 2003; Schwei et al., 1999; Wacnik et al., 2001), as well as increased nocifensive responses to mechanical (Luger et al., 2002; Medhurst et al., 2002; Schwei et al., 1999; Wacnik et al., 2001), heat (Menendez et al., 2003), and cold stimuli (Urch et al., 2003; Wacnik et al., 2001) applied to the hind paw ipsilateral to tumor growth.

Using a model in which fibrosarcoma cells are implanted into and around the calcaneus bone (Wacnik et al., 2001), we have shown that ~35% of C nociceptors exhibited ongoing, spontaneous activity and were sensitized to heat stimuli (Cain et al., 2001). Furthermore, tumor growth produced peripheral neuropathy as evidenced by a decrease in the number of epidermal nerve fibers (ENFs) in the plantar skin overlying the tumor site (Cain et al., 2001) with sparing of the ENFs that contained calcitonin gene-related peptide, CGRP (Gilchrist et al., 2005; Wacnik et al., 2005), a neuropeptide associated with nociceptive signaling (Lee et al., 1985; Sun et al., 2003; Sun et al., 2004).

Although C nociceptors exhibited sensitization to heat stimuli, hyperalgesia to heat has not been reported for this model of cancer pain. Furthermore, mice with fibrosarcoma cells implanted into and around the calcaneus bone exhibit hyperalgesia to mechanical and cold stimuli but C nociceptors examined in this model did not exhibit decreased thresholds to mechanical or cold stimuli and may not have been sensitized to these stimuli. One possibility is that the mechanical and cold hyperalgesia in this model are mediated by central sensitization (Coderre et al., 1993; Woolf, 1983). Studies using other models of cancer pain have shown neurochemical changes in the dorsal horn consistent with central sensitization (Medhurst et al., 2002; Schwei et al., 1999; Zhang et al., 2005) and direct evidence for central sensitization has been shown in electrophysiological studies (Donovan-Rodriguez et al., 2004; Urch et al., 2003). The aim of the present study was to characterize changes in response properties of nociceptive dorsal horn neurons following implantation of fibrosarcoma cells into and around the calcaneus bone. We also determined whether this model of cancer pain produces hyperalgesia to heat as a behavioral correlate to the sensitization of C nociceptors to heat stimuli.

2. Results

2.1. Tumor-evoked mechanical and heat hyperalgesia

Consistent with our earlier reports (Cain et al., 2001; Hamamoto et al., 2007), implantation of fibrosarcoma cells into and around the calcaneus bone in mice produced mechanical hyperalgesia (Fig. 1A). The mean frequency of paw withdrawals to the von Frey filament (3.4 mN bending force) for the tumor-bearing hind paw increased from baseline levels of $15 \pm 2.6\%$ to $49.0 \pm 3.8\%$ by day 3 after implantation ($P < 0.001$). Paw withdrawal frequency further increased until day 10 ($70.7 \pm 4.0\%$) and remained elevated until the last testing session on day 24. In contrast, paw withdrawal frequency for the nontumor-bearing hind paw was unchanged

during the time course and was lower than for the tumor-bearing hind paw at all test days after implantation ($P < 0.05$).

Implantation of fibrosarcoma cells also produced hyperalgesia to heat (Fig. 1B). Mean paw withdrawal latency to the radiant heat stimulus was 9.2 ± 0.5 s prior to implantation of fibrosarcoma cells. By day 3 after implantation, paw withdrawal latency began to decrease (7.9 ± 0.5 s) in the tumor-bearing hind paw. Paw withdrawal latency decreased further to 5.3 ± 0.5 s by day 18 ($P < 0.01$). In contrast, paw withdrawal latencies for the nontumor-bearing hind paw remained unchanged and higher than those of the tumor-bearing hind paw from day 13 after implantation through day 24 ($P < 0.05$). Thus, implantation of fibrosarcoma cells into and around the calcaneus bone in mice produced mechanical and heat hyperalgesia.

Heat hyperalgesia was exhibited by a decrease in paw withdrawal latency. To compare the level of heat hyperalgesia to the electrophysiological responses of nociceptive dorsal horn neurons to the heat stimuli delivered by the contact Peltier device, we determined the temperature at which mice withdrew their hind paws from the radiant heat stimulus. Thus, temperatures under the skin of the hind paws of anesthetized mice ($n=8$) were determined during application of the radiant heat stimulus used in the behavioral study. Before application of radiant heat (time = 0), paw temperature was $33.5 \pm 0.3^\circ\text{C}$. After a lag of 0.5 s, paw temperature increased in a linear fashion at a rate of 0.52°C/s . Paw temperatures were higher than at baseline beginning 1.5 s after initiating the heat stimulus ($P < 0.05$). Paw temperature at the time that unanesthetized mice withdrew their hind paw (paw withdrawal latency) at baseline (9.3 ± 0.5 s) was $38.1 \pm 0.2^\circ\text{C}$. On days 3–5 after implantation, mice withdrew their tumor-bearing hind paw at 7.8 ± 0.3 s, which corresponded to a paw temperature of $37.6 \pm 0.2^\circ\text{C}$. When mice further developed heat hyperalgesia (days 18–24), paw withdrawal latency was 5.3 ± 0.5 s, which corresponded to a paw temperature of $36.3 \pm 0.2^\circ\text{C}$. Thus when mice exhibited the greatest heat hyperalgesia, the threshold temperature of the tumor-bearing hind paw was $\sim 2^\circ\text{C}$ lower than the threshold temperature of the hind paw before implantation of fibrosarcoma cells.

2.2. General characteristics of mouse spinal neurons

Forty-five nociceptive dorsal horn neurons were studied in control nontumor-bearing mice ($n=25$) and 42 nociceptive dorsal horn neurons were studied in tumor-bearing mice ($n=29$) on days 10–21 after implantation. Figure 2 shows the locations of histologically determined recording sites for 24 of 45 neurons in control mice and for 31 of 42 neurons in tumor-bearing mice. The depths of the recording sites from the surface of the spinal cord did not differ between those that were recovered histologically ($387.3 \pm 41.3\mu\text{m}$) and those that were not ($450.3 \pm 28.4\mu\text{m}$). Nociceptive dorsal horn neurons were separated into superficial (recording site $< 200\mu\text{m}$) or intermediate/deep (recording site $> 200\mu\text{m}$). There were no differences in the proportion of WDR or HT neurons in the superficial or deep dorsal horn of tumor-bearing compared to control mice (Table 1). Overall, tumor-bearing mice exhibited similar proportions of WDR and HT neurons as control mice with $\sim 60\%$ of the nociceptive dorsal horn neurons studied being WDR neurons in both groups. Moreover, there were no differences in the response properties of WDR neurons between those located in the superficial or deep dorsal horn for either control mice or tumor-bearing mice. Similarly, there were no differences in the response properties of HT neurons between those located in the superficial or deep dorsal horn for either control mice or tumor-bearing mice. Therefore, responses of superficial and deep dorsal horn neurons were analyzed together to determine if there were differences in response properties of nociceptive dorsal horn neurons between control and tumor-bearing mice.

A greater proportion (76%) of WDR neurons exhibited ongoing activity in tumor-bearing mice than in control mice (54%, $P < 0.05$) and the discharge frequency of the ongoing activity for WDR neurons in tumor-bearing mice was ~ 3 times greater than that of WDR neurons in control

mice ($P < 0.01$). In contrast, there was no difference in the proportion of HT neurons that exhibited ongoing activity between control and tumor-bearing mice.

Nociceptive dorsal horn neurons were initially identified using mechanical stimuli, and thus all of the neurons studied were mechano-sensitive. However, implantation of fibrosarcoma cells resulted in an increase in the proportion of WDR neurons that also responded to thermal stimuli (Table 1). In control mice, the ratio of mechano-sensitive to mechanothermal sensitive WDR neurons was 43:57% whereas in tumor-bearing mice the ratio was 24:76%. However, the proportion of mechanothermal sensitive WDR neurons in each of the three subclasses (mechano/heat/cold (~80%), mechano/heat (~15%), and mechano/cold (~5%)) did not differ between control and tumor-bearing mice. For HT neurons, there was no difference in the ratio of mechano-sensitive to mechanothermal sensitive neurons in tumor-bearing compared to control mice. In both groups of mice, nearly all HT neurons studied responded to mechanical and thermal stimuli. Thus, there was no change in the proportion of WDR neurons studied in the tumor-bearing compared to control mice but the proportion WDR neurons that responded to thermal stimuli increased in mice with tumor-evoked hyperalgesia.

2.3. Responses to mechanical stimuli

Implantation of fibrosarcoma cells into and around the calcaneus bone increased the responses of WDR neurons to mechanical stimuli applied to the hind paw. Examples of responses of WDR neurons to mechanical stimuli in a control and a tumor-bearing mouse are shown in Fig. 3A. In the control mouse, the WDR neuron responded to innocuous (i.e., brush and 4.8 mN) and noxious (i.e., 54.7, 97.8 mN and pinch) mechanical stimuli in a graded fashion. In the tumor-bearing mouse, the WDR neuron exhibited ongoing activity of 1.2 imp/s and responses to the innocuous and noxious stimuli (after subtracting the level of ongoing activity) were greater than that of the WDR neuron in the control mouse. Figure 3B shows that the mean number of impulses evoked by the mechanical stimuli were greater for WDR neurons in tumor-bearing mice than control mice ($P < 0.05$). Of note is that WDR neurons in tumor-bearing mice exhibited greater responses (9.5 ± 1.0 imps) to the mechanical stimulus used in the behavioral studies (3.4 mN) than did WDR neurons in control mice (1.4 ± 0.7 imps; $P < 0.0001$).

In contrast to WDR neurons, HT neurons in control and tumor-bearing mice exhibited similar responses to mechanical stimuli. Examples of responses of HT neurons to mechanical stimuli in a control and a tumor-bearing mouse are shown in Fig. 3C. In both the tumor-bearing and control mice, the HT neurons responded minimally or not at all to innocuous (i.e., brush and 4.8 mN) mechanical stimuli but responded in a graded fashion to noxious (i.e., 54.7, 97.8 mN and pinch) mechanical stimuli. Figure 3D shows that there were no differences in the number of impulses evoked by the mechanical stimuli between HT neurons in tumor-bearing and control mice.

Mechanical thresholds of WDR neurons determined using von Frey filaments were lower in tumor-bearing mice than those of WDR neurons in control mice. The median mechanical threshold for WDR neurons ($n=25$) in tumor-bearing mice was 2.4 mN compared to 5.7 mN for WDR neurons ($n=28$) in control mice ($P < 0.001$). Importantly, the median mechanical threshold of WDR neurons in tumor-bearing mice was lower than the force of the von Frey filament (3.4 mN) used to evoke paw withdrawal responses in the behavioral studies. The distributions of mechanical thresholds of WDR neurons for tumor-bearing and control mice are shown in Fig. 4A. Only 28.6% (8/28) of WDR neurons in control mice had mechanical thresholds at or below 3.4 mN. In contrast, a greater proportion of WDR neurons (64%; 16/25) in tumor-bearing mice had mechanical thresholds at or below 3.4 mN ($P < 0.0001$). Thus, sensitization of WDR neurons to mechanical stimuli could contribute to the tumor-evoked mechanical hyperalgesia in this murine model of cancer pain.

Mechanical thresholds of HT neurons in tumor-bearing mice were similar to those in control mice (Fig 4B). The median mechanical threshold of HT neurons ($n=17$) in tumor-bearing mice was 78.2 mN compared to 54.7 mN for HT neurons ($n=17$) in control mice. Thus, HT neurons in cancer mice did not exhibit sensitization to mechanical stimuli and likely did not contribute to tumor-evoked mechanical hyperalgesia.

2.4. Responses to heat stimuli

WDR neurons in tumor-bearing mice exhibited sensitization to heat applied to the hind paw. Examples of responses of WDR neurons to heat in a control and a tumor-bearing mouse are shown in Fig. 5A. In the control mouse, the WDR neuron responded to innocuous (i.e., 37°C) and noxious (i.e., 45 and 51°C) heat in a graded fashion. In the tumor-bearing mouse, the WDR neuron also responded to both innocuous and noxious heat but responses to the noxious stimuli (after subtracting the level of ongoing activity) were greater than that of the WDR neuron in the control mouse. Figure 5B shows that WDR neurons in tumor-bearing mice exhibited greater responses to temperatures at 43°C and above than did the WDR neurons in control mice ($P < 0.05$).

Heat thresholds of WDR neurons ($35.0 \pm 0.0^\circ\text{C}$; $n=5$) recorded on days 18–21 after implantation were $\sim 2^\circ\text{C}$ lower than thresholds of WDR neurons ($37.4 \pm 0.4^\circ\text{C}$; $n=16$) in control mice ($P < 0.005$). This lower heat threshold corresponds to the $\sim 2^\circ\text{C}$ difference in skin temperatures at the time when mice withdrew their tumor-bearing hind paw compared to control mice in the behavioral assay. This decrease in heat threshold for WDR neurons in tumor-bearing mice may contribute to tumor-evoked heat hyperalgesia.

HT neurons in tumor-bearing mice also exhibited sensitization to heat applied to the hind paw. Examples of responses of HT neurons to heat in a control and a tumor-bearing mouse are shown in Fig. 5C. The HT neurons in both the control and tumor-bearing mice responded to noxious (i.e., 45 and 51°C) but not innocuous (i.e., 37°C) heat. Figure 5D shows that HT neurons ($n=14$) in tumor-bearing mice exhibited greater responses to the 49 and 51°C stimuli than did the HT neurons ($n=16$) in control mice ($P < 0.05$). However, there was no difference in heat threshold for HT neurons in tumor-bearing ($39.4 \pm 0.8^\circ\text{C}$) compared to control ($38.9 \pm 0.4^\circ\text{C}$) mice. Thus, HT neurons in cancer mice exhibited increased responses only to temperatures above those that induced paw withdrawals in our behavioral studies (33–39°C).

2.5. Responses to cold stimuli

WDR neurons in tumor-bearing mice also exhibited sensitization to cold applied to the hind paw. Examples of responses of WDR neurons to cold in a control and a tumor-bearing mouse are shown in Fig. 6A. The WDR neurons in both the control and tumor-bearing mouse responded to noxious (i.e., 8 and 0°C) cold in a graded fashion. However, responses of the WDR neuron in the tumor-bearing mouse were greater than those of the WDR neuron in the control mouse. Figure 6B shows that WDR neurons ($n=16$) in tumor-bearing mice exhibited greater responses to temperatures of 12°C and below than did the WDR neurons ($n=14$) in control mice ($P < 0.05$). Response thresholds of WDR neurons to cold stimuli in tumor-bearing mice ($23.1 \pm 1.7^\circ\text{C}$) were not different from those of WDR neurons in control mice ($19.3 \pm 2.3^\circ\text{C}$).

There were no differences in responses of HT neurons to cold stimuli between tumor-bearing and control mice. Examples of responses of a HT neuron in a control and tumor-bearing mouse are shown in Fig. 6C. HT neurons in both tumor-bearing ($n=8$) and control ($n=6$) mice exhibited increased responses to increasingly colder temperatures applied to the hind paw (Fig. 6D). However, there were no differences in the number of impulses evoked at each temperature or in the cold threshold temperature for HT neurons between control ($18.7 \pm 2.9^\circ\text{C}$) and tumor-

bearing ($16.5 \pm 3.2^{\circ}\text{C}$) mice. Thus, HT neurons likely did not contribute to the cold hyperalgesia exhibited by tumor-bearing mice shown previously (Wacnik et al., 2001).

3. Discussion

Implantation of fibrosarcoma cells into and around the calcaneus bone in mice produces mechanical and cold hyperalgesia (Cain et al., 2001; Hamamoto et al., 2007; Wacnik et al., 2001). The results of the present study demonstrate that tumor-bearing mice also exhibit hyperalgesia to heat. Tumor-evoked hyperalgesia to mechanical, heat, and cold stimuli are, at least in part, due to sensitization of WDR neurons. The proportion of WDR neurons that exhibited ongoing activity was greater and the discharge frequency of that ongoing activity was higher in tumor-bearing than control mice. This ongoing activity likely contributed to ongoing nocifensive behaviors exhibited in this model (Wacnik et al., 2001). Also, WDR neurons exhibited lower mechanical thresholds and greater responses to mechanical stimuli in tumor-bearing compared to control mice. The proportion of WDR neurons that responded to thermal stimuli was higher, their responses to heat and cold stimuli greater, and their heat thresholds (for days 18–21 after-implantation) were at lower temperatures in tumor-bearing compared to control mice. In contrast, there were no differences in mechanical, heat or cold thresholds or suprathreshold responses to mechanical, heat (except at 49°C or above) or cold stimuli for HT neurons in tumor-bearing compared to control mice. Thus, sensitization of WDR, but not HT, neurons likely contributed to tumor-evoked hyperalgesia in this murine model of cancer pain. Our results are consistent with earlier reports by Dickenson and colleagues (Urch et al., 2003) who showed that neurons classed as WDR were sensitized following implantation of mammary carcinoma cells into the tibia of rats.

Implantation of mammary carcinoma cells into the tibia of rats resulted in an increase in the proportion of WDR neurons in the superficial dorsal horn (47%) when compared to those in control rats (26%) (Urch et al., 2003). This finding suggests that the thresholds of some HT neurons decreased so as to be indistinguishable from WDR neurons (Urch et al., 2003). In the present study, the proportion of superficial WDR neurons was not different in tumor-bearing (64%) compared to control (56%) mice, although it is possible that some WDR neurons were sensitized HT neurons. Also, a greater percentage of WDR neurons exhibited ongoing activity in tumor-bearing (76%) compared to control (54%) mice. The higher level of ongoing activity in tumor-bearing mice may be due in part to the ongoing activity of sensitized C nociceptors that we have observed (Cain et al., 2001). In contrast, there were no differences in the percentage of nociceptive dorsal horn neurons that exhibited ongoing activity between tumor-bearing (17% of superficial and 18% of deep neurons) and control (21% of superficial and 15% of deep neurons) rats (Urch et al., 2003). These differences between characteristics of WDR neurons in a murine compared to a rat model of cancer pain could be due to differences in the type of tumor cells, implantation site, species differences, or method of sampling. Additionally, tumor-bearing mice were anesthetized with sodium pentobarbital whereas tumor-bearing rats were anesthetized with halothane (Urch et al., 2003). Pentobarbital may affect the electrophysiological properties of nociceptive dorsal horn neurons differently than halothane (Hori et al., 1984).

Differential sensitization of WDR but not HT neurons observed in our study and by Dickenson and colleagues (Urch et al., 2003) may be unique to cancer pain in that both WDR and HT neurons exhibit increased levels of ongoing activity in models of incisional pain (Vandermeulen and Brennan, 2000), arthritis (Grubb et al., 1993; Neugebauer and Schaible, 1990), paw inflammation (Randich et al., 1997), skin freeze injury (Khasabov et al., 2001), activation of C nociceptors by mustard oil (Woolf et al., 1994), capsaicin evoked pain (Johanek and Simone, 2005; Simone et al., 1989) and neuropathic pain (Sotgiu et al., 1994). Furthermore, recent studies have demonstrated that neurochemical changes in the spinal cord of mice with

tumor-evoked hyperalgesia differ from those in mice with hyperalgesia produced by inflammation or nerve injury (Honore et al., 2000). Thus, changes in response properties of nociceptive dorsal horn neurons may differ depending upon the nature of the injury.

3.1. Tumor-evoked mechanical hyperalgesia and sensitization of nociceptive dorsal horn neurons to mechanical stimuli

Injury to peripheral tissues sensitizes nociceptors and nociceptive dorsal horn neurons, and produces hyperalgesia (Mannion and Woolf, 2000; Millan, 1999; Treede et al., 1992). We previously reported that a proportion of C nociceptors were sensitized to heat stimuli in mice with tumor-evoked hyperalgesia (Cain et al., 2001). This sensitization likely contributed to the tumor-evoked hyperalgesia to heat reported in the present study. Thresholds to mechanical stimuli in sensitized C nociceptors were not decreased suggesting that these C nociceptors may not contribute to tumor-evoked mechanical hyperalgesia. However, responses to suprathreshold mechanical stimuli were not examined. In inflamed skin, mechanical thresholds did not change but nociceptors exhibited increased responses to suprathreshold stimuli (Andrew and Greenspan, 1999). Thus, C nociceptors in tumor-bearing mice may have been sensitized although their thresholds to mechanical stimuli may not have decreased.

Another possibility is that sensitization of nociceptive dorsal horn neurons may contribute to tumor-evoked mechanical hyperalgesia. Indeed, central sensitization of nociceptive dorsal horn neurons contributes to mechanical hyperalgesia in models of inflammatory and neuropathic pain (Laird and Bennett, 1993; Ma and Sluka, 2001; Palecek et al., 1992). In the present study, responses of WDR neurons to mechanical stimuli were higher in tumor-bearing compared to control mice. In mice with tumor-evoked hyperalgesia, responses of WDR neurons to the von Frey filament (3.4 mN bending force) used to assess mechanical hyperalgesia were almost 7 times greater than the responses of WDR neurons in control mice and the median threshold force was lower for WDR neurons in tumor-bearing mice (2.4 mN) than control (5.7 mN) mice. These findings are consistent with our previous report that threshold force for evoking a paw withdrawal decreases in tumor-bearing mice (Hamamoto et al., 2007). Moreover, the proportion of WDR neurons with thresholds below 3.4 mN was higher in tumor-bearing (64%) compared to control (28.6%) mice. These changes were not observed in HT neurons. Thus, WDR neurons likely contribute to tumor-evoked mechanical hyperalgesia in this model of cancer pain. This finding is consistent with the findings of Dickenson and colleagues in that central sensitization of superficial WDR, but not HT, neurons contributed to tumor-evoked mechanical hyperalgesia in rats (Urch et al., 2003). Sensitization of WDR but not HT neurons to mechanical stimuli may be unique to cancer pain as both WDR and HT neurons are sensitized to mechanical stimuli in models of diabetic neuropathy (Chen and Pan, 2002), peripheral nerve injury (Sotgiu et al., 1995) inflammatory (Grubb et al., 1993; Ma and Sluka, 2001; Neugebauer and Schaible, 1990; Ren et al., 1994), and capsaicin-evoked (Simone et al., 1989) pain.

3.2 Tumor-evoked hyperalgesia and sensitization of nociceptive dorsal horn neurons to thermal stimuli

In their initial description of the model of cancer pain used in the present study, Wacnik and colleagues reported that implantation of fibrosarcoma cells into and around the calcaneus produced hyperalgesia to mechanical and cold stimuli applied to the plantar surface of the hind paw (Wacnik et al., 2001). Whether tumor-bearing mice exhibited hyperalgesia to heat was not reported. We now demonstrate that this model of cancer pain also produces hyperalgesia to heat. In a previous study, we found that C nociceptors located in the plantar skin of the hind paw were sensitized to heat, as evidenced by decreased threshold temperatures and increased responses to suprathreshold stimuli (Cain et al., 2001). Thus, tumor-evoked sensitization of C nociceptors is associated with tumor-evoked heat hyperalgesia.

Interestingly, when this same line of fibrosarcoma cells was implanted into the tibia of mice, a transient period of reduced responsiveness (hypoalgesia) to heat developed followed by heat hyperalgesia after 4 weeks (Menendez et al., 2003). In the present study, tumor-evoked hyperalgesia to heat developed by day 10 after implantation and was greatest by day 24. In both studies, heat stimuli were delivered to the plantar surface of the hind paw. When implanted into and around the calcaneus bone, fibrosarcoma cells form tumor masses adjacent to the calcaneus and subcutaneously along the plantar surface of the hind paw (Wacnik et al., 2005). The fibrosarcoma cells likely affected the primary afferent fibers near their terminations in the plantar skin. Indeed, innervation in the plantar skin initially increased but then decreased as the tumor continued to grow (Cain et al., 2001). As the number of epidermal nerve fibers (ENFs) decreased, there was sparing of ENFs that contained CGRP and the proportion of remaining ENFs that co-expressed CGRP and the P2X₃ receptor increased (Gilchrist et al., 2005; Wacnik et al., 2005). CGRP is a neuropeptide that is found in nociceptive nerve fibers and terminals (Lee et al., 1985) and adenosine triphosphate (ATP) binding to purinergic receptors such as the P2X₃ receptor contributes to nociceptive signaling (Hamilton and McMahon, 2000). These changes in ENFs may contribute to sensitization of C nociceptors that terminate in the plantar skin.

In contrast, when the fibrosarcoma cells were implanted into the tibia, tumor growth may have affected nerves as they passed by on their way to innervating the plantar surface of the hind paw. The fibrosarcoma cells eroded through the bone and infiltrated the perineurium of nerves by 4 weeks after implantation, the time when hyperalgesia to heat began to develop (Menendez et al., 2003). Indeed, when tumor cells were implanted directly around the sciatic nerve, hyperalgesia to heat developed more quickly (i.e., by 10 days after implantation) (Shimoyama et al., 2002). Fibrosarcoma cells may release biochemical mediators, such as TNF α , that when outside of the tibia may affect nearby nerves as intraneural injection of TNF α produces hyperalgesia to heat and mechanical stimuli (Zelenka et al., 2005). The later onset of hyperalgesia to heat when fibrosarcoma cells are implanted into the tibia may be due to the additional time needed for the fibrosarcoma cells to erode through the bone and interact with nearby nerves. In contrast, implantation of fibrosarcoma cells into and around the calcaneus bone allows the tumor cells to interact with cutaneous nerve terminals more quickly. The importance of the site of implantation for development of heat hyperalgesia is supported by the finding that B16-BL6 melanoma cells implanted subcutaneously into the plantar surface of the hind paw in mice produced heat hyperalgesia beginning 7 days after implantation (Sasamura et al., 2002). Thus, the difference in the time course of the development of hyperalgesia to heat may be due to the difference in the site of implantation.

The findings of the present study confirm the findings of Dickenson and colleagues that nociceptive dorsal horn neurons are sensitized to heat in tumor-bearing rodents. In the present study, responses of WDR neurons in tumor-bearing mice exhibited higher number of impulses evoked by 41–51°C than those in control mice. Similarly, superficial WDR neurons in tumor-bearing rats exhibited higher number of impulses evoked by 40–50°C than those in control rats (Urch et al., 2003). In contrast, Dickenson and colleagues reported that there were no differences in the number of impulses evoked by 32–50°C in superficial HT neurons in tumor-bearing and control rats. We also found that responses of HT neurons in tumor-bearing mice were similar to those in control mice with the exception that HT neurons in tumor-bearing mice exhibited higher number of impulses to 49 and 51°C. In comparison, WDR neurons in models of neuropathic pain do not show sensitization to heat (Laird and Bennett, 1993; Palecek et al., 1992; Pertovaara et al., 1997; Takaishi et al., 1996). Following zymosan-induced inflammation, intradermal injection of capsaicin, and freeze injury of the skin, both WDR and HT neurons exhibited sensitization to heat with decreased response thresholds and increased responses to suprathreshold stimuli (Johanek and Simone, 2005; Khasabov et al., 2001; Randich et al., 1997).

Few studies have compared the responses of WDR and HT neurons to cold stimuli in animals exhibiting hyperalgesia to cold. In a model of spinal cord injury, rats exhibited nociceptive behaviors to innocuous cold stimuli (Hao et al., 2004). In these rats, the percentage of both WDR and HT neurons that responded to cold stimuli increased. Similarly, we reported that a mild freeze injury to the plantar skin of rats lowered response thresholds and increased responses of both WDR and HT neurons to suprathreshold cold (and heat) stimuli (Khasabov et al., 2001). In the present study, WDR (but not HT neurons) in tumor-bearing mice exhibited sensitization to cold stimuli as responses of these neurons to noxious cold stimuli ($\leq 12^{\circ}\text{C}$) were greater than responses of WDR neurons in control mice. Sensitization of WDR neurons in tumor-bearing mice is consistent with the hyperalgesia to cold reported previously (Wacnik et al., 2001). In rats, implantation of mammary carcinoma cells into the tibia also produced cold hyperalgesia (Medhurst et al., 2002). Thus, tumor-evoked sensitization of WDR neurons to thermal stimuli contributes to tumor-evoked hyperalgesia to heat and cold stimuli.

3.3. Roles of WDR and HT neurons in tumor-evoked hyperalgesia

In the spinal cord, WDR and HT neurons respond to nociceptive stimuli and both classes of neurons project to areas involved with nociceptive processing (Price, 1988). However, there is debate regarding the roles of WDR and HT neurons in processing of nociceptive stimuli (Craig, 2003; Price et al., 2003). Both WDR and HT neurons appear to encode the onset of noxious stimuli (Hoffman et al., 1981), although detection latency of near-threshold changes in intensity of noxious heat stimuli were correlated with neuronal discharge of WDR and not HT neurons (Maixner et al., 1986). WDR and HT neurons may play different roles in nociception. HT neurons have smaller receptive fields than WDR neurons (Price et al., 1978), suggesting that HT neurons may contribute to localization of painful stimuli (Hoffman et al., 1981). In contrast, WDR neurons appear to encode nociceptive stimulus intensities more accurately because WDR neurons have steeper stimulus response functions (Hoffman et al., 1981; Price et al., 1976) allowing better discrimination between small changes in stimulus intensities (Price et al., 1976; Price et al., 1978). Moreover, the stimulus response functions of WDR neurons more closely parallel those from psychophysical ratings of pain intensity than do the stimulus response functions of HT neurons (Price, 1988). However, Craig found that electrophysiological responses of lamina I nociceptive neurons corresponded better than those of lamina V WDR neurons to the profile of burning pain in human subjects produced by brief contact heat paradigm (Craig, 2004). Thus, the precise roles of WDR and HT neurons in nociceptive processing under normal conditions may be dependent in part on the nature of the stimuli, and remain to be resolved.

The roles of WDR and HT neurons may also differ in states of prolonged pain. Responses of HT neurons to prolonged nociceptive stimuli decreased more rapidly than those of WDR neurons or psychophysical ratings of pain produced by subcutaneous injection of capsaicin (Simone et al., 1991) and repeated application of noxious heat (Coghill et al., 1993). In the present study, the proportion of WDR neurons exhibiting ongoing activity increased and their discharge rates were greater in mice with tumor-evoked hyperalgesia compared to control mice. In contrast, these changes were not exhibited by HT neurons in mice with tumor-evoked hyperalgesia. Thus, WDR neurons appear to play a greater role in processing prolonged, ongoing nociception such as that associated with tumor growth. However, HT neurons may also contribute to tumor-evoked nociception as response thresholds of some HT neurons might have decreased such that they were classed as WDR neurons (Urch et al., 2003).

3.4. Conclusions

Implantation of fibrosarcoma cells into and around the calcaneus produced hyperalgesia to mechanical and heat stimuli and sensitization of WDR, but not HT, nociceptive dorsal horn neurons to mechanical, heat, and cold stimuli. Tumor-evoked hyperalgesia to heat is consistent

with our previous finding of tumor-evoked sensitization of C nociceptors and sensitization of WDR neurons to heat in the present study. Sensitization of WDR neurons to mechanical and cold stimuli likely contributes to tumor-evoked mechanical and cold hyperalgesia. Our findings support the notion that the mechanisms underlying cancer pain differ from those that contribute to inflammatory and neuropathic pain in that only WDR neurons became sensitized in mice with tumor-evoked hyperalgesia compared to sensitization of both WDR and HT neurons in other models of chronic pain. Understanding the unique changes in the physiology and neurochemistry of the dorsal horn produced by tumor growth may provide new opportunities for improved management of cancer pain.

4. Experimental Procedure

4.1. Subjects

Adult (> 5 weeks old), male, C3H/HeNCR mice (National Cancer Institute, Frederick, MD) weighing 20–30 g were used in all experiments. Mice were housed 4 per box, had free access to food and water, and were maintained on a 12-h light/dark schedule. All studies were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

4.2. Implantation of fibrosarcoma cells

NCTC 2472 fibrosarcoma cells (ATCC, Manassas, VA) were maintained as described previously (Clohisy et al., 1996). Briefly, fibrosarcoma cells were grown to confluency in 75 cm² flasks in NCTC 135 medium (pH 7.4) containing 10% horse serum and prepared for implantation by creating a cell suspension with trypsin. Fibrosarcoma cells were counted using a hemocytometer, pelleted and resuspended in phosphate buffered saline for implantation.

Fibrosarcoma cells were implanted into the hind paw as described previously (Wacnik et al., 2001). Mice were briefly anesthetized with halothane (2–3%) and fibrosarcoma cells (2×10^5 cells/10 μ l) were injected into and around the calcaneus bone of the left hind paw using a 0.3 cc insulin syringe with a 29.5 gauge needle. None of the mice showed signs of motor dysfunction after implantation of fibrosarcoma cells.

4.3. Mechanical hyperalgesia

Mice were placed on a wire mesh platform, covered with a glass container (10 $\times$ 6.5 $\times$ 6.5 cm), and allowed to acclimate for at least 30 min. A von Frey (Semmes-Weinstein) filament (Stoelting Co, Wood Dale, IL) with a bending force of 3.4 mN was applied to the plantar surface of the hind paw at random locations avoiding the heel and toes. For each trial, the filament was applied for 1–2 s with an interstimulus interval of approximately 5 s. Only vigorous paw withdrawals were counted. Tumor-evoked mechanical hyperalgesia was defined as an increase in the frequency of paw withdrawals from baseline values. Only mice that exhibited sufficient mechanical hyperalgesia, defined as paw withdrawals in at least 50% of the 10 trials, were used in the electrophysiological experiments. Approximately 90% of mice implanted with fibrosarcoma cells exhibited sufficient mechanical hyperalgesia by day 10 after implantation.

4.4. Heat hyperalgesia

Heat hyperalgesia was examined by determining paw withdrawal latency to a radiant heat source (Hargreaves et al., 1988). Mice were placed on a temperature controlled (30°C) glass platform, covered with ventilated plastic containers (diameter = 10 cm) and allowed to acclimate for at least 30 min. Radiant heat was applied to the middle of the plantar surface of the hind paw and latency (s) to paw withdrawal was determined. Testing consisted of 4 trials for each hind paw with each trial separated by at least 5 min. Mean paw withdrawal latency was calculated from the last 3 trials. The intensity of the heat source was adjusted so that, at

baseline, mice withdrew their hind paws at ~ 9 s. A cut off time of 16 s was chosen to avoid tissue damage. Tumor-evoked heat hyperalgesia was defined as a decrease in paw withdrawal latency from baseline values. Mechanical and heat hyperalgesia were determined for each mouse for 3 days before implantation of fibrosarcoma cells and on days 3, 4, 5, 6, 10, 11, 13, 18, 21 and 24 following implantation (day 0).

4.5. Paw temperature

In a separate experiment, temperature of the hind paw was determined during application of radiant heat. A needle thermocouple probe (MT26-4, Physitemp Instruments, Clifton, NJ) was placed just under the skin of anesthetized mice ($n = 8$). Mice were placed with the hind paw resting flat on the temperature controlled glass surface. Radiant heat was applied such that the tip of the thermoprobe was in the center of the light source. The thermocouple probe was connected to an electronic thermometer (BAT 10R, Physitemp Instruments) and temperatures were recorded every 0.5 s. Three trials were recorded for each hind paw, which were averaged, with at least 3 minutes between trials to allow the hind paw temperature to return to baseline levels.

4.6. Electrophysiology

4.6.1. Electrophysiological recordings from spinal dorsal horn neurons—Mice with tumor-evoked hyperalgesia were studied on days 10–21 after implantation of fibrosarcoma cells. Mice with tumor-evoked hyperalgesia and control mice were anesthetized with acepromazine maleate (20 mg/kg, i.p.) and sodium pentobarbital (Nembutal, 48 mg/kg, i.p.). Supplemental doses of sodium pentobarbital (15 mg/kg) were added as needed to maintain areflexia. After removal of hair, an incision was made in the skin overlying the thoracic and lumbar parts of the vertebral column and the lumbar enlargement was exposed by a laminectomy. Mice were secured in a spinal frame and the spinal cord was continually bathed in warm (37°C) mineral oil. Extracellular recordings from dorsal neurons at the L3 – L5 spinal levels with receptive fields (RFs) located on the plantar surface of the hind paw were obtained using stainless steel microelectrodes (10 m Ω ; Frederick Haer and Co., Brunswick, ME) lowered into the spinal cord using an electronic micromanipulator (Burleigh, Fisher, NY) in 3 μ m steps. Neurons recorded at depths of 20 to 200 μ m from the surface of the spinal cord were considered to be located in superficial laminae and those recorded at depths greater than 200 μ m were considered to be located in intermediate to deep laminae (Cuellar et al., 2004; Mazario and Basbaum, 2007; Weng et al., 2001). Only single neurons with easily discriminated action potentials were studied. Electrophysiological activity of dorsal horn neurons was amplified (DAM80, World Precision Instruments, Sarasota, FL), audio-monitored (AM8 audiomonitor, Grass Instruments, West Warwick, RI), and displayed on a storage oscilloscope. Neuronal activity, discriminated impulses, and stimulus temperatures were collected using a customized data acquisition program (Lab View, National Instruments Co., Austin, TX) for off-line analyses.

4.6.2. Functional classification of spinal neurons—Dorsal horn neurons were identified using mechanical stimulation (stroking the skin and mild pinching with the experimenter's fingers) of the plantar surface of the hind paw. Each spinal neuron was characterized based upon its responses to graded intensities of mechanical stimuli applied to the RF. Innocuous stimuli consisted of stroking the skin with a cotton swab or the experimenter's fingers. Noxious stimuli consisted of mild pinching with the experimenter's fingers or with serrated forceps, but this latter stimulus was applied sparingly to avoid neuronal sensitization. Neurons were classed functionally according to responses evoked by mechanical stimuli as (1) low threshold - excited maximally by innocuous stimulation; (2) wide dynamic range (WDR) - responded in a graded fashion to increasing intensity of stimulation beginning

with innocuous stimuli; and (3) high threshold (HT) - response evoked by noxious stimulation only. Only nociceptive dorsal horn neurons (WDR and HT) were studied further.

4.6.3. Mechanical and thermal stimulation—Following identification and general characterization of each nociceptive dorsal neuron, the level of ongoing activity was recorded for 2 min. Next, mechanical thresholds were determined using calibrated von Frey filaments (bending force from 0.1 to 189.1 mN) and were expressed as the minimum force needed to evoke a response in at least 50% of the trials. Responses to mechanical stimuli (brush, von Frey filaments (3.4, 4.8, 54.7, and 97.8 mN), and pinching with serrated forceps) applied for 2 s were determined. Interstimulus intervals were 10 s. Thermal stimuli were applied using a Peltier-type thermode (contact area of 1 cm²) starting from a base temperature of 32°C. Heat stimuli (35 to 51°C, 5 s duration) were delivered in ascending order of 2°C increments at a ramp rate of 18°C/s with an interstimulus interval of 60 s. Cold stimuli (28 to 0°C, 10 s duration) were applied in descending order of 4°C decrements with a ramp rate of 8°C/s and interstimulus interval of 180 s.

4.7. Histology

At the end of the experiment, mice received an overdose of pentobarbital sodium and were perfused with normal saline followed by 10% formalin containing 1% potassium ferrocyanide. Serial transverse sections (50 µm) were stained with neutral red. Recording sites were identified by Prussian Blue marks or by small lesions.

4.8. Statistical analyses

Frequency of paw withdrawals (mechanical hyperalgesia) and paw withdrawal latency (heat hyperalgesia) are presented as mean ± sem. Each dependent measure was compared between hind paws (tumor-bearing or control) and across days after implantation (days 0, 3, 4, 5, 6, 10, 11, 13, 18, 21, and 24) using a one-way repeated measures ANOVA. Individual pairwise comparisons were made using paired and unpaired t-tests with the Bonferroni correction for multiple comparisons. Paw temperatures during application of the radiant heat source used in the behavioral studies were determined every 0.5 s and measured 3 times in each paw. Paw temperatures were averaged at 0.5 s intervals and were compared to baseline paw temperatures using a repeated-measures ANOVA followed by paired t-tests with the Bonferroni correction.

Proportions of HT and WDR neurons, proportions of neurons exhibiting ongoing activity, and proportions of mechano- and mechanothermal sensitive neurons in control and tumor-bearing mice were compared using the Chi Square test. Discharge frequencies of ongoing activity of neurons were compared using a one way ANOVA. Mechanical threshold forces of WDR and HT neurons were compared between control and tumor-bearing mice using the Mann-Whitney Rank Sum Test. The number of impulses evoked by mechanical and thermal stimuli were determined by subtracting any ongoing activity from the response that occurred during the stimulus. The number of impulses evoked by mechanical stimuli were compared between groups by using a one way ANOVA. Responses of WDR and HT neurons to heat and cold stimuli were compared using two-way repeated measures ANOVAs followed by paired and unpaired t-tests with the Bonferroni correction. For all statistical analyses, a probability value < 0.05 was considered significant.

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Abbreviations

CGRP	calcitonin gene-related peptide
ENFs	epidermal nerve fibers
HT	high threshold
TNFα	tumor necrosis factor alpha
WDR	wide dynamic range

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Figure 1.

Implantation of fibrosarcoma cells into and around the calcaneus produces hyperalgesia to mechanical and heat stimuli. A) Mean ($\pm$ sem) frequency of paw withdrawal increased in the tumor-bearing hind paw by 3 days after implantation compared to before implantation (day 0) and was higher than the frequency for the control paw at all times after implantation. B) Mean ($\pm$ sem) paw withdrawal latency decreased in the tumor-bearing hind paw by day 10 after implantation and was lower than the latencies for the control paw by day 13. * $P < 0.05$ and ** $P < 0.01$ for each day after implantation compared to baseline (day 0). # $P < 0.05$ and ## $P < 0.01$ for tumor-bearing compared to contralateral hind paw at each day after implantation.

Figure 2.
Schematic representation of recording sites of WDR and HT neurons in control and tumor-bearing (Cancer) mice.

Figure 3.

Responses of nociceptive dorsal horn neurons to mechanical stimuli. A) Examples of the responses of WDR neurons from a control and a tumor-bearing (Cancer) mouse to mechanical stimuli. B) Mechanical stimuli evoked greater mean ($\pm$ sem) number of impulses from WDR neurons in tumor-bearing compared to control mice. C) Examples of the responses of HT neurons from a control and a tumor-bearing mouse to mechanical stimuli. D) There were no differences in the mean ($\pm$ sem) number of impulses from HT neurons evoked by the mechanical stimuli between tumor-bearing and control mice. * $P < 0.05$, **** $P < 0.0001$ for number of impulses between tumor-bearing and control mice for each mechanical stimulus.

Figure 4.

Mechanical thresholds of nociceptive dorsal horn neurons in control and tumor-bearing (Cancer) mice. A) The proportion of WDR neurons with mechanical thresholds lower than the force used in the behavioral studies (3.4 mN) was greater for tumor-bearing than control mice. B) There were no HT neurons with mechanical thresholds below the force used in the behavioral studies for control or tumor-bearing mice.

Figure 5.

Responses of nociceptive dorsal horn neurons to heat stimuli. A) Examples of the responses of WDR neurons from a control and a tumor-bearing (Cancer) mouse to heat stimuli. B) Heat stimuli evoked greater mean ($\pm$ sem) number of impulses from WDR neurons in tumor-bearing compared to control mice. C) Examples of the responses of HT neurons from a control and a tumor-bearing mouse to heat stimuli. D) There were no differences in the mean ($\pm$ sem) number of impulses from HT neurons evoked by the heat stimuli below 49°C between tumor-bearing and control mice. * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$ for number of impulses between tumor-bearing and control mice at each temperature.

Figure 6.

Responses of nociceptive dorsal horn neurons to cold stimuli. A) Examples of the responses of WDR neurons from a control and a tumor-bearing (Cancer) mouse to cold stimuli. B) Cold stimuli evoked greater mean ($\pm$ sem) number of impulses from WDR neurons in tumor-bearing compared to control mice. C) Examples of the responses of HT neurons from a control and a tumor-bearing mouse to cold stimuli. D) There were no differences in the mean ($\pm$ sem) number of impulses from HT neurons evoked by cold stimuli between tumor-bearing and control mice. * $P < 0.05$, **** $P < 0.001$ for number of impulses between tumor-bearing and control mice at each temperature.

Table 1
Characteristics of Nociceptive Dorsal Horn Neurons

	Control		Cancer	
	WDR	HT	WDR	HT
Proportion of superficial neurons	56% (9/16)	44% (7/16)	64% (9/14)	36% (5/14)
Proportion of deep neurons	66% (19/29)	34% (10/29)	57% (16/28)	43% (12/28)
Proportion of all neurons	62% (28/45)	38% (17/45)	60% (25/42)	40% (17/42)
Presence of ongoing activity	54% (15)	65% (11)	76% [*] (19)	65% (11)
Discharge frequency of ongoing activity (mean $\pm$ sem, imp/s)	0.9 $\pm$ 0.4	2.2 $\pm$ 0.8	2.9 $\pm$ 0.7 ^{**}	1.3 $\pm$ 0.4
Mechano-sensitive	43% (12/28)	7% (1/17)	24% [*] (6/25)	12% (2/17)
Mechanothermal sensitive	57% (16/28)	93% (16/17)	76% [*] (19/25)	88% (15/17)

Unless indicated, parentheses indicate the number of neurons and the total number of neurons.

^{*} P < 0.05 and

^{**} P < 0.01 for WDR neurons in tumor-bearing (Cancer) compared to control mice.