Multiple Sodium Channel Isoforms and Mitogen-Activated Protein Kinases Are Present in Painful Human Neuromas

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Objective: Although axons within neuromas have been shown to produce inappropriate spontaneous ectopic discharges, the molecular basis for pain in patients with neuromas is still not fully understood. Because sodium channels are known to play critical roles in neuronal electrogenesis and hyperexcitability, we examined the expression of all the neuronal voltage-gated sodium channels (Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9) within human painful neuromas. We also examined the expression of two mitogen-activated protein (MAP) kinases, activated p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2), which are known to contribute to chronic pain, within these human neuromas.

Methods: We used immunocytochemical methods with specific antibodies to sodium channels Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9, and to activated MAP kinases p38 and ERK1/2 to study by confocal microscopy control and painful neuroma tissue from five patients with well-documented pain.

Results: We demonstrate upregulation of sodium channel Nav1.3, as well as Nav1.7 and Nav1.8, in blind-ending axons within human painful neuromas. We also demonstrate upregulation of activated p38 and ERK1/2 MAP kinases in axons within these neuromas.

Interpretation: These results demonstrate that multiple sodium channel isoforms (Nav1.3, Nav1.7, and Nav1.8), as well as activated p38 and ERK1/2 MAP kinases, are expressed in painful human neuromas, indicating that these molecules merit study as possible therapeutic targets for the treatment of pain associated with traumatic neuromas.

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Injury to peripheral nerves associated with trauma, amputation, compression, or surgery can lead to the formation of painful neuromas, tangled masses of blind-ending axons, and proliferating connective tissue.[1] In humans, these neuromas can be debilitating, causing chronic and severe pain, which is frequently refractory to medical treatment. Axons in both experimental and human neuromas have been shown to produce spontaneous ectopic discharges,[2–4] which have been implicated in neuropathic pain,[5] but the molecular mechanisms responsible for this pain-producing hyperexcitability in neuromas are not fully understood.

Considerable attention has been focused recently on understanding the contribution of voltage-gated sodium channels in the pathogenesis of neuropathic pain.[6,7] It is now clear that there are nine distinct isoforms of sodium channels, with different amino acid sequences and distinct physiological profiles.[8] Sodium channel isoforms Nav1.3, Nav1.7, Nav1.8, and Nav1.9 have been shown to exhibit physiological properties and patterns of expression within the nervous system that poise them to play important roles in chronic pain. Significantly, Nav1.3 is present at very low levels, if at all, in adult rat dorsal root ganglia (DRG) neurons, but the expression of Nav1.3 is upregulated at the transcriptional level after peripheral axotomy of DRG neurons[9,10] or inflammation in their peripheral projection fields.[11] Nav1.3 produces persistent currents and depolarizing responses that amplify small stimuli close to resting potential, and recovers rapidly from inactivation, thereby increasing neuronal excitability when expressed at higher than normal levels.[12–14] Nav1.7 produces a depolarizing response to small, slow stimuli such as generator potentials,[15] thus setting the

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gain on nociceptors.[16] Consistent with a prominent role for this channel in nociception, Nav1.7 is expressed in 85% of functionally identified nociceptive neurons within DRG[17] and has been localized to sensory nerve endings.[18] Gain-of-function mutations in Nav1.7 have been shown to produce severe chronic pain,[15,19] whereas loss-of-function mutations in this channel produce profound insensitivity to pain in humans.[20–22] Nav1.8, which is present within approximately 90% of C- and Aδ-nociceptive DRG neurons,[23] produces a majority of the current underlying the upstroke of the action potential in these neurons and supports repetitive firing when these cells are depolarized.[24] To date, two studies have examined expression of Nav1.7 and Nav1.8 in human neuromas.[25,26] However, there have been no studies on the expression of Nav1.3 and other sodium channel isoforms within human neuromas.

Mitogen-activated protein kinases (MAPKs) transduce extracellular stimuli into intracellular posttranslational and transcriptional responses in a variety of cell types.[27] Current evidence has implicated activation of MAPK signaling pathways as a major contributor to the development and persistence of pain.[28,29] It has recently been demonstrated that sodium channels can be direct targets of activated MAPK. The activities of Nav1.6,[30] Nav1.7,[31] and Nav1.8[32,33] have been shown to be modulated by MAPK. However, at this time, it is not known whether activated MAPKs are present in human painful neuromas.

Although the effect of surgical excision of neuromas is controversial, several studies in amputees have reported good results.[34–36] One goal of such neuroma surgeries is moving the nerve stump to a deeper location so that if a new neuroma re-forms, as it often does, it will not be so superficially located and vulnerable to mechanical stimulation. In this study, we have examined the expression of all the neuronal sodium channels, Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9, and activated MAPKs p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2) within painful neuromas that were surgically extirpated from five patients. We demonstrate that Nav1.3, which is not detectable in control nerve, is accumulated together with Nav1.7 and Nav1.8 in human painful neuromas. We also show that activated p38 and ERK1/2 are accumulated in the majority of human painful neuromas. These results identify Nav1.3, as well as Nav1.7 and Nav1.8, and MAPKs p38 and ERK1/2 as potential therapeutic targets in painful human neuromas.

Patients and Methods

Patients

Patients with verified peripheral nerve injury and palpable neuromas, referred by other physicians because of intractable pain to the neuropathic pain clinic at Aarhus University Hospital, were eligible to enter the study. The patients had tried a range of medications, including tricyclic antidepressants and anticonvulsants, but most of them wanted to stop these medications either because of side effects or because of lack of effect of treatment. The decision to operate and remove neuromas was made after discussion with a specialist in hand surgery, before patients consented to participate in the research study, and was based on the clinical status of patients, including elicitation of severe pain after palpation/percussion of the neuroma. Patients were told that removal of such neuromas and the subsequent burying of the excised nerve stump in surrounding muscle might result in reduced evoked pain, but that there was also a risk for worsened pain. Six patients were enrolled in the study (Table 1). Patients were informed about the study, and written informed con-

Table 1. **Patient Baseline Characteristics**

Patient No.	Sex/Age (yr)	Cause of Pain/ Location	Injured Nerve	Previous Neuroma Removal	Pain Duration (mo)	Spontaneous Pain (NRS, 0-10): Stump/Phantom[a]	Analgesic Treatment
1	M/37	Amputation (transmetacarpal)/5th finger, right hand	Ulnar	2003, 2004	71	9.4/6.7	None
2	M/58	Amputation/4th finger, left hand	Ulnar and median	—	24	6.1/5.2	None
3	M/61	Amputation/2nd finger, right hand	Median (common and proper palmar digital branches)	—	19	4.3/3.9	Pregabalin
4	F/26	Fracture and subsequent surgery/ right volar wrist	Radial	2004	28	1	None
5	M/38	Fracture and subsequent surgery/left volar wrist	Median	—	82	5.3	Oxycodone, ibuprofen (Ibumetin)
6	F/57	Amputation/2nd finger, right hand	Median	1994, 1996	192	7.7/6.1	Tramadol, paracetamol

[a]NRS = numeric rating scale (0 = no pain; 10 = worst possible pain). Intensity of pain was calculated as a mean of the previous seven daily pain scores.

sent was obtained. The protocol was approved by the regional ethics committee (No. 2006-0044; Aarhus, Denmark).

Assessment of Pain

During the study period, the intensity of pain was recorded during the evening for a 1-week period commencing at 7 days before the operation and at 1, 3, and 6 months after the operation. A numerical rating scale with 0 as "no pain" and 10 as "worst possible pain" was used. Amputees recorded both stump and phantom pain; stump pain was defined as pain localized to the region of the stump, and phantom pain was defined as pain experienced in the missing part of the limb. Mean intensity of pain was calculated from the previous seven daily pain scores (Table 2).

Surgery and Handling of Neuromas

Patients underwent general anesthesia or axillary brachial plexus blockade, and the same hand surgeon performed all operations. The surgical approach was as follows: (1) any previous skin scar was reopened and excised; (2) the nerve lesion was shown, and soft tissue scars were carefully excised; (3) the nerve was mobilized from the scar tissue and the nerve-end neuroma excised; and (4) the mobilized nerves were wrapped in a sheet of Divide®, (Johnson, Johnson, Birkerod, Denmark) an adhesion barrier used to prevent scar adherences in areas close to joints, if considered appropriate by the surgeon. The neuromas were not locally anesthetized with lidocaine or similar agents. A small area of normal-appearing nerve trunk located approximately 2cm proximal to the neuroma was excised in most cases, providing nerve tissue outside of the neuroma from the same patient, to serve as control tissue.

Control and neuroma tissue were immediately snap-frozen in dry ice. Within 20 minutes after removal, the tissue was transferred to a freezer and stored at −80°C until shipping from Denmark to Connecticut. During shipping, the tissue was kept frozen in dry ice.

Immunocytochemistry

Ten-micrometer cryosections were processed for immunocytochemistry from six control nerves, and nine painful neuromas were removed from six patients as described previously.[37] Control and neuroma tissue were processed in parallel. Sections of control and neuroma tissue were mounted on the same slides, and were immersed for 5 minutes in 4% paraformaldehyde in 0.14M Sorensen's phosphate buffer, pH 7.4, rinsed several times in phosphate-buffered saline (PBS), and incubated in blocking solution (PBS with 5% normal goat serum, 1% bovine serum albumin, 0.1% Triton X-100 [Sigma, St. Louis, MO], 0.02% sodium azide) for 30 minutes at room temperature. Sections were then incubated individually or in combination with primary antibodies [rabbit anti-Nav1.1 (1:100; Alomone, Jerusalem, Israel), rabbit anti-Nav1.2 (1:100, Alomone), rabbit anti-Nav1.3 (#16153; 1:500),[38] rabbit anti-Nav1.6 (PN4; 1:100; Sigma), rabbit anti-Nav1.7 (Y083, 1:250; generated from rat a.a. sequence 514–532), rabbit anti-Nav1.8 (1:200; Alomone), rabbit anti-Nav1.9 (#6464; 1:500),[39] mouse anti-phosphorylated and anti-nonphosphorylated neurofilament (each 1:10,000; SMI 31 and SMI 32; Covance, Princeton, NJ), and guinea pig anti-Caspr (1:2,000)[40]] for 24 to 48 hours at 4°C. Sections were subsequently washed with PBS, incubated in appropriate secondary antibodies [goat anti–mouse IgG Alexa Fluor 488 or 633; 1:1,000; Molecular Probes, Eugene, OR), goat anti–guinea pig IgG Alexa Fluor 488 (Molecular Probes), and goat anti–rabbit IgG Cy3 (1:2,000, Amersham, Piscataway, NJ)] for 12 to 24 hours at 4°C, washed with PBS, and coverslipped with Aqua Poly mount (Polysciences, Warrington, PA).

Tissue Analysis

To validate sodium channel antigenicity within control and neuroma samples, we initially reacted sections from all samples with antibodies to Nav1.6 and Caspr. Nav1.6 is the predominant sodium channel at nodes of Ranvier where it is expressed at a high density[41] and Caspr demarcates paranodal regions.[42] Samples that did not exhibit Nav1.6 labeling at nodes, as identified by paranodal Caspr staining, were not further examined for this study. Using this criterion, we included in our analyses five of six control samples from four patients and seven of nine neuroma samples from five patients (patients 1, 2, 3, 5, 6). For analyses of control and neuroma sections, multiple images were accrued with a Nikon C1 confocal microscope (Nikon USA, Melville, NY). Imaging settings were selected with control tissue, and images of neuroma immunolabeling were acquired with the same settings. Control and neuroma images were composed and processed in parallel with enhanced contrast using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Table 2. **Intensity of Pain (NRS: 0-10) before Surgery and after 1, 3, and 6 Months**

Patient No.	Before	After 1 Month	After 3 Months	After 6 Months	Effect of Surgery on Pain
1	9.4/6.7	9.6/7.3	9.7/6.6	9/6.7	↔
2	6.1/5.2	4.9/3.3	7.9/5.3	7/5.6	↓ →
3	4.3/3.9	1.7/2.7	2.1/2	2.1/3.1	↓
4	1	1.6	2.6	5.4	↑
5	5.3	4.4	4.1	ND	→
6	7.7/6.1	3.6/3	1.6/1.4	2.4/3	↓

Intensity of pain (stump/phantom in patients with amputations) was calculated as a mean of the previous 7 daily pain scores.
NRS = numeric rating scale (0 = no pain; 10 = worst possible pain); ND = not determined.

Results

Six patients with neuropathic pain after peripheral nerve injury, not adequately controlled by medications, and palpable neuromas participated in the study (see Table 1). Nine nerve-end neuromas and six control samples were obtained from these six patients. All neuromas were painful. Movement exacerbated pain in all patients, but Tinel's sign could not be elicited at locations proximal to the neuroma. Three patients had previously undergone neuroma removal, but the pain-attenuating effect of that surgery was transient. Medical treatment had limited or no effect on the painful neuromas analyzed in this study.

Voltage-Gated Sodium Channels

In this study, we analyzed seven painful human nerve-end neuromas from five patients who met our criterion for preserved sodium channel antigenicity. As exemplified in Figure 1, neurofilament-positive axons, which run in parallel within the nerve trunk, course in a disorganized pattern throughout neuroma, which often becomes club shaped. Some of the axons within the neuroma are grouped into mini-fascicles, which consist of both myelinated and unmyelinated fibers.

NAV1.3 IS UPREGULATED IN HUMAN PAINFUL NEUROMAS.

Previous work with rat experimental neuromas demonstrated an accumulation of Nav1.3 within the distal axon stumps.[10] Because Nav1.3 produces persistent and ramp currents, and recovers rapidly from inactivation,[13,14] the expression of Nav1.3 in injured neurons and their extensions has been suggested to contribute to their hyperresponsiveness.[7,12] In this study, we asked whether Nav1.3 was accumulated within painful human neuromas. Nav1.3 was not detectable in control nerves but was clearly present within axons in four of seven (approximately 60%) of the neuromas. In double-label immunofluorescence studies, Nav1.3 immunolabeling was colocalized with neurofilament labeling, consistent with the accumulation of Nav1.3 within axons in neuromas (Fig 2). Nav1.3 was present within the blindly ending tips of axons within these neuromas but did not appear to be confined to the tips of axons and was generally also present more proximally within the neuroma (see Fig 2).

NAV1.7 AND NAV1.8 ARE UPREGULATED IN HUMAN PAINFUL NEUROMAS.

In agreement with previous observations,[25] we detected substantially increased Nav1.7 and Nav1.8 immunofluorescence within axons in painful neuromas compared with that exhibited in control samples (Fig 3). Increased Nav1.7 immunoreactivity was always detected in the neuromas examined (7/7), whereas increased Nav1.8 immunolabeling was observed in 3 of 7 (43%) of the neuromas.

Similar to the observation of Nav1.8 localization at nodes of Ranvier in normal human tooth pulp,[43] we observed Nav1.8 immunoreactivity at nodes in our control human tissue (see Fig 3, inset); approximately 50% (16/31) of the nodes exhibited Nav1.8 immunolabeling. Nav1.8 was also observed at nodes within painful neuromas, where 60% (21/35) of the nodes displayed Nav1.8 immunoreactivity.

NAV1.1, NAV1.2, NAV1.6, AND NAV1.9 ARE NOT UPREGULATED IN HUMAN PAINFUL NEUROMAS.

Nav1.1 was present at low levels in both control nerves and neuromas, with no qualitative differences in the level of expression (Fig 4). Only background levels of Nav1.2 immunolabeling were observed in control nerves and neuromas (see Fig 4). Nav1.9 immunoreactivity was detected in neuromas at low levels, where its level of expression was similar to that in control nerves (see Fig 4). Nodes of Ranvier in both control nerve and neuromas displayed strong Nav1.6 immunoreactivity (eg, Fig 5), with no apparent difference in the degree of immunoreactivity. Faint immunolabeling for Nav1.6 along nonmyelinated axons was present in

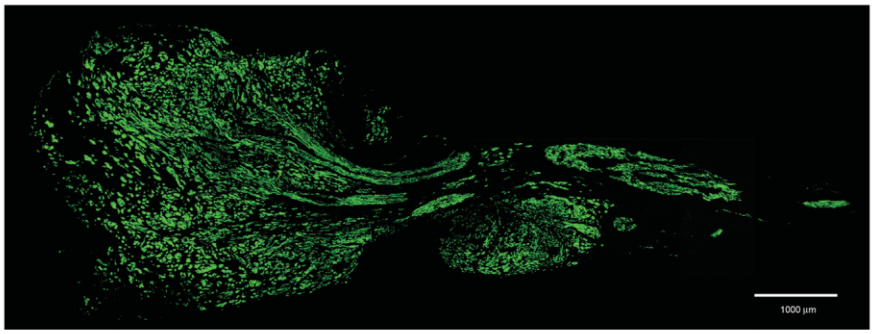

Fig 1. Human painful neuroma. A montage of low-magnification images of a neurofilament-labeled section from a human painful neuroma. Neurofilament-positive axons within the nerve trunk (right side of montage) are parallel in orientation, whereas within the club-shaped nerve-end neuroma, the axons are tangled and disorganized.

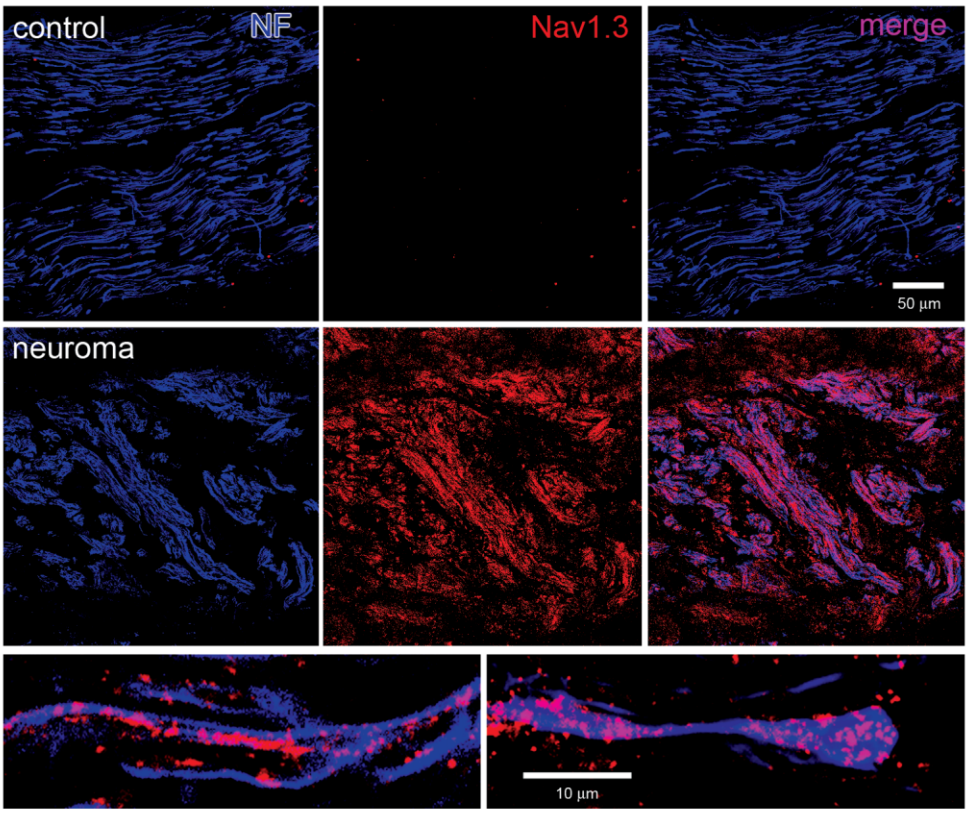

Fig 2. Sodium channel Nav1.3 accumulates in human painful neuromas. Control human tissue exhibits low levels of Nav1.3 immunolabeling. Painful neuromas display substantially increased Nav1.3 immunoreactivity (red) compared with control tissue. Colocalization (magenta) of neurofilament (blue) and Nav1.3 (red) demonstrates that Nav1.3 is present within axons. At increased magnification (bottom two panels), axons (blue) within neuromas display Nav1.3 immunolabeling. Nav1.3 immunolabeling is exhibited by an apparently blind-ending axon (bottom right panel).

both control nerve and neuromas, but there were no apparent differences in the level of labeling.

ACTIVATED P38 AND EXTRACELLULAR SIGNAL-REGULATED KINASES 1 AND 2 ARE UPREGULATED IN PAINFUL HUMAN NEUROMAS.

MAPK pathways have been implicated as contributing to the development of pain syndromes,[28,44] and modulation of sodium channels by p38 MAPK[30,32,33] and ERK1/2[31] has been reported. We therefore asked whether the expression of activated p38 and ERK1/2 was increased in painful human neuromas. We could not detect activated p38 or ERK1/2 in control nerve. In contrast, immunofluorescence for both activated p38 and ERK1/2 were clearly present in neuromas (Fig 6). Double-label immunofluorescence studies with antibodies to activated p38 or ERK1/2 and neurofilament demonstrated that these activated MAPKs were expressed within axons. In favorable sections, activated p38 and ERK1/2 were detected in apparently blind-ending axons (see Fig 6, inset). Accumulations of activated p38 and ERK1/2 were observed in 4 of 7 neuromas for each MAPK, and there was a tendency (3/7) for both p38 and ERK1/2 to be expressed in the same neuroma.

Discussion

The mechanisms underlying pain associated with nerve injury, including that seen after limb amputation, are not fully understood; the available evidence suggests, however, that both peripheral and central mechanisms may contribute.[45] Although it is clear that ectopic impulse activity in neuromas can contribute to chronic pain, the molecular basis for this hyperexcitability is not fully understood. In this study, we examined the expression of neuronal voltage-gated sodium channels Nav1.1, Nav1.2, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9, and the activated MAPK p38 and ERK1/2 in painful human neuromas. Consistent with previous reports,[25,26] we detected enhanced expression of Nav1.7 and Nav1.8 in human neuromas compared with control tissue obtained more proximally from the same nerves. In addition, we report novel observations of accumulation of Nav1.3 and activated MAPK in painful neuromas.

Current evidence strongly supports a major role for

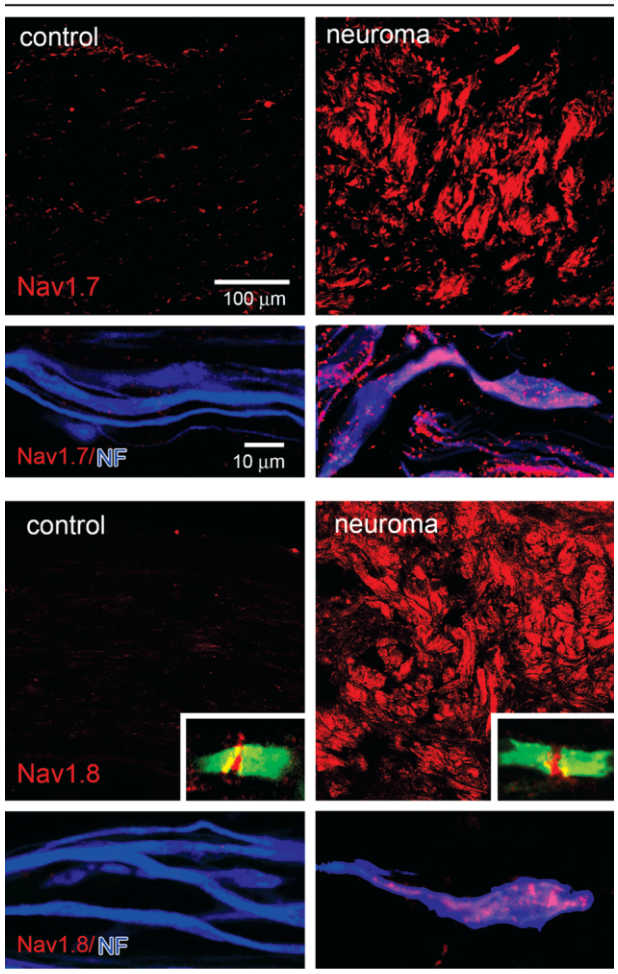

Fig 3. Nav1.7 and Nav1.8 accumulate in human painful neuromas. Control human tissue exhibits low levels of both Nav1.7 and Nav1.8 immunoreactivity. Human painful neuromas display increased Nav1.7 and Nav1.8 immunolabeling (red) compared with control tissue. At increased magnification, both Nav1.7 and Nav1.8 immunolabeling (red) is displayed in apparently blind-ending axons (blue) within neuromas (bottom right panels for Nav1.7 and Nav1.8); colocalization is indicated by magenta color. (insets) Nav1.8 (red) immunolabeling is displayed at nodes of Ranvier (bounded by Caspr-positive (green) paranodes) in both control nerve and neuromas.

sodium channels[6,7,46,47] and MAPK pathways[28,29] in the cause of neuropathic pain. The presence of sodium channels Nav1.3, Nav1.7, and Nav1.8, and activated p38 and ERK1/2 within these neuromas, at greater levels than within control tissue more proximally from these nerves, suggests that these proteins participate in the pathogenesis of pain associated with human neuromas.

Each of the neuromas examined in this study was associated with pain. Consistent with the neuroma per se being the site of ectopic impulse activity, the level of pain was reduced by one point or more on the numerical rating scale at the first postoperative assessment (1 month) in three of the six patients studied (Patients 2, 3, and 6); in one of these three patients (Patient 2), pain subsequently returned, consistent with development of a new neuroma or development of hyperexcitability at a more proximal site. In three of the six patients (Patients 1, 4, and 5), pain was not ameliorated after excision of the neuroma. We did not find an association between the presence or absence of any particular sodium channel isoform or MAPK and the degree of pain or response to neuroma excision.

Although it might be argued that our use of nerve tissue obtained more proximally from the nerve causing the neuroma introduces the possibility of retrograde changes in the control tissue, we would stress that our use of this tissue as a control permitted comparison of axons within the neuroma, and axons outside of the neuroma, that were obtained nearly simultaneously from the same patient and processed in an identical manner. Importantly, we found that expression of Nav1.3, Nav1.7, Nav1.8, and activated p38 and ERK1/2 were *increased* within the neuroma compared with the tissue obtained more proximally, where these channels and kinases were undetectable using our methods. Thus, although we cannot exclude the possibility that other channels such as Nav1.1 or Nav1.2 may have been upregulated in both the neuroma and more proximal parts of the nerve, our results demonstrate an accumulation of Nav1.3, Nav1.7, Nav1.8, and activated p38 and ERK1/2 within the neuroma.

Ectopic spontaneous action potential discharges have been reported in experimental[3,4,48] and human[2] neuromas, and in humans with peripheral neuropathies and paresthesias.[49,50] Evidence that sodium channels contribute to the spontaneous ectopic discharges is provided by studies in which sodium channel blockers, including tetrodotoxin, lidocaine, and carbamazepine, inhibit the spontaneous activity in experimental neuromas.[51–53]

Our study examined, for the first time, expression of Nav1.3 within human neuromas and demonstrates a distinct upregulation of Nav1.3 in more than half of the painful neuromas that we studied. These results extend observations in experimental rat neuromas, in which increased Nav1.3 immunolabeling was detected in the distal stumps of transected rat sciatic nerves,[10] and a report of increased Nav1.3 immunoreactivity in injured axons within peripheral nerve trunks.[54] Significantly, contactin, which has been shown to associate with Nav1.3 and to increase the density of this channel at the cell surface, has also been observed in experimental neuromas.[55] Nav1.3 exhibits several properties that can contribute to neuronal hyperexcitability, including rapid recovery from inactivation, which can support high-frequency firing, and production of persistent current and ramp responses to small, slow depolarizations.[12–14] In conjunction with their unique physiological properties, the localization of Nav1.3

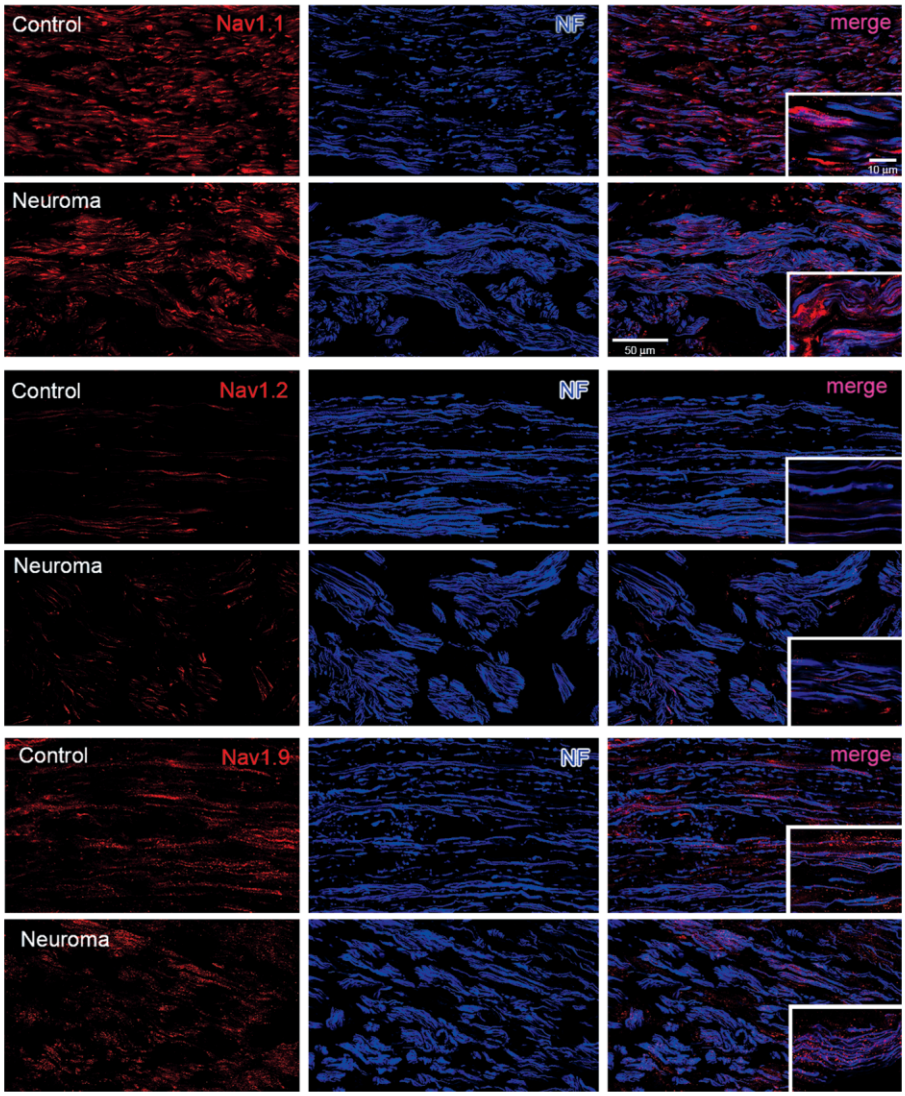

Fig 4. Nav1.1, Nav1.2 and Nav1.9 are not accumulated in neuromas. Control and neuroma tissue sections were reacted with isoform-specific antibodies to Nav1.1, Nav1.2 and Nav1.9. Low levels of Nav1.1 immunolabeling are exhibited in both control nerves and neuromas, whereas only background levels of Nav1.2 labeling are present in control tissue and neuromas. Low levels of Nav1.9 immunolabeling are present in control nerves and neuromas. NF = neurofilament.

channels within blind-ending axons of painful human neuromas suggests that these channels can participate in the generation of ectopic discharges associated with chronic pain. We detected Nav1.3 in four of seven painful neuromas; whether Nav1.3 was present within the other three neuromas, at levels too low for immunocytochemical detection but high enough to support electrogenesis,[56] is not known.

We also detected increased immunoreactivity for sodium channels Nav1.7 and Nav1.8 in painful human neuromas compared with control tissue, but not for Nav1.1, Nav1.2, Nav1.6, or Nav1.9. Our observations extend previous reports of accumulation of Nav1.7 and Nav1.8 in human neuromas.[25,26] Interestingly, Kretschmer and colleagues[25] reported that painful neuromas of peripheral nerves exhibited greater Nav1.7 labeling than nonpainful neuromas, whereas no difference was detected between painful and nonpainful human lingual nerve neuromas.[26] In our study, all seven neuromas, which were always from the upper extremity and were all painful, displayed enhanced Nav1.7 immunoreactivity.

The prevalence of Nav1.7 within painful neuromas, coupled with its voltage dependence and kinetic properties, support a major contributory role for this channel in neuroma neuropathic pain. Nav1.7 is highly expressed in DRG neurons, where 100% of C-nociceptive and 93% of Aδ-nociceptive neurons exhibit Nav1.7 immunolabeling.[17] The slow closed-state inactivation and voltage dependence of Nav1.7 chan-

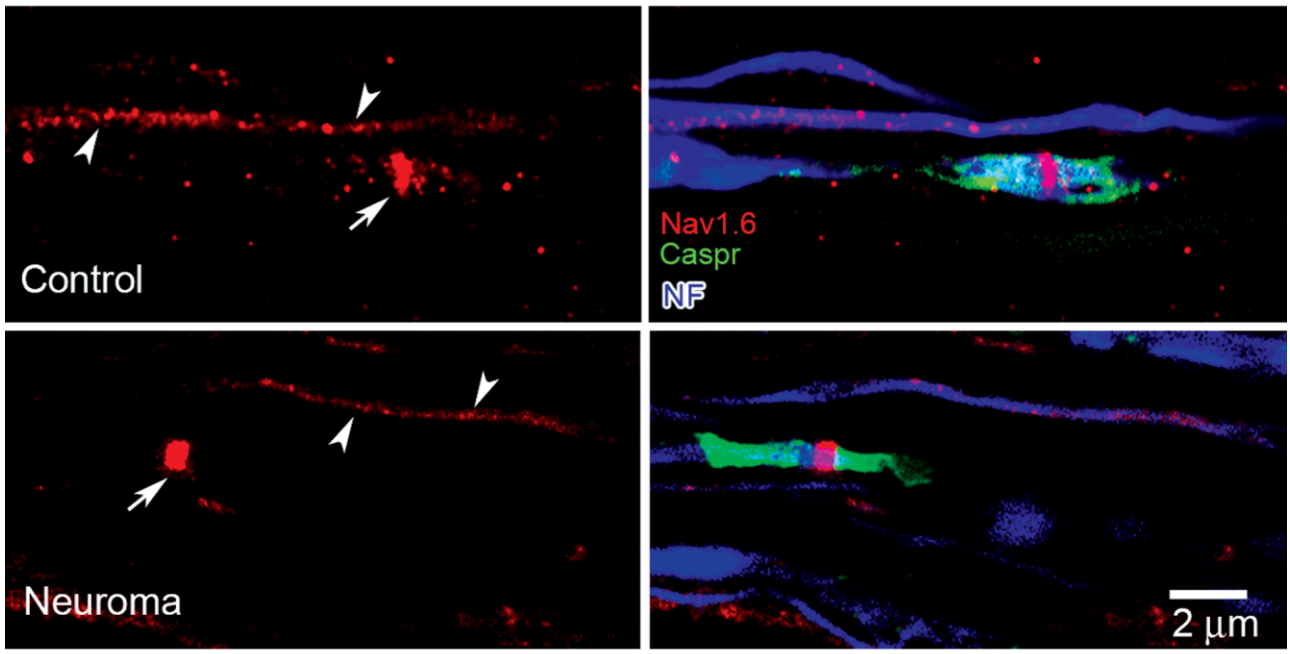

Fig 5. Nav1.6 is expressed at nodes of Ranvier in control nerves and neuromas. Sections of control nerves and neuromas were triple immunolabeled for Nav1.6, neurofilament (NF), and Caspr. Left column shows Nav1.6 (red) signal only, whereas the right column shows merged image of Nav1.6 (red), NF (blue), and Caspr (green) images. Nodes (arrows) *in both control nerves and neuromas exhibit Nav1.6 immunolabeling. Nonmyelinated axons* (arrowheads) *in control nerves and neuromas display a low level of Nav1.6 immunoreactivity. There is no apparent accumulation of Nav1.6 in neuromas compared with control nerves.*

nels permit them to produce relatively large responses to small, subthreshold depolarizations,[57,58] and thus poise these channels to set the gain on nociceptors.[16] Point mutations that hyperpolarize the activation voltage dependence and slow deactivation of Nav1.7 have been linked to the human pain disorder erythromelalgia,[15,19] whereas impaired inactivation has been linked to paroxysmal extreme pain disorder.[59] Recently, loss-of-function mutations in Nav1.7 have been shown to produce insensitivity to pain.[20–22] These observations provide strong evidence that Nav1.7 is essential to nociception in humans and suggest that the aberrant accumulation of Nav1.7 within neuromas plays a role in the onset and/or maintenance of pain associated with the neuromas.

Accumulation of Nav1.8 is also expected to make axons within neuromas hyperexcitable. Nav1.8 has been shown to produce the majority of the inward current responsible for the action potential upstroke in the DRG neurons in which it is expressed and produces high-frequency firing of the cells when they are depolarized.[24] Moreover, experimental expression of Nav1.8, in cells that do not normally express it, markedly enhances the excitability of these cells.[60,61]

MAPKs are a family of serine/threonine protein kinases that transduce extracellular stimuli into cellular responses via transcriptional and posttranslational modifications.[27,62] MAPKs have received considerable attention for their involvement in nociception and sensitization.[28,63,64] For instance, ERK1/2 plays an important role in inflammatory responses,[65,66] and p38 activation is induced by noxious stimuli.[67] Recently, it has been demonstrated that sodium channels are substrates for MAPKs, which can modulate their activities. Phosphorylation of Nav1.8 by activated p38 significantly increases the current density of this channel in DRG neurons[32,33] and would be expected to enhance the excitability of these cells. It has also been demonstrated that phosphorylation by ERK1/2 hyperpolarizes the activation curve of Nav1.7,[31] lowering the threshold for activation of this channel.

In summary, our observations of upregulation of Nav1.3, as well as Nav1.7 and Nav1.8, in conjunction with the localization of activated p38 and ERK1/2 within painful neuromas, implicate MAPKs and at least three isoforms of sodium channels as contributors to the pain associated with neuromas. Our results add to the evidence supporting the development of subtype-specific sodium channel blockers as potential treatments for neuropathic pain and suggest that, together with sodium channels, MAPKs may be opportune therapeutic targets for chronic pain after traumatic nerve injury in humans.

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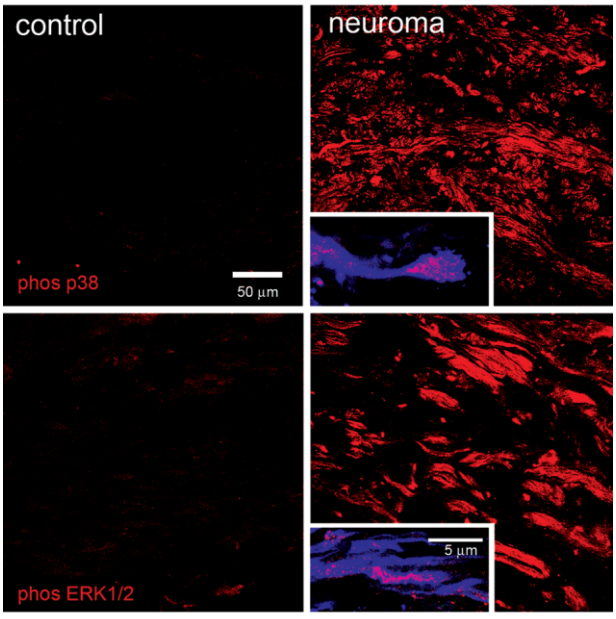

Fig 6. Mitogen-activated protein (MAP) kinases accumulate in human painful neuromas. Control human tissue displays low levels of activated (phosphorylated) p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2). In contrast, painful neuromas exhibit substantially increased immunolabeling for p38 and ERK1/2 compared with control tissue. (insets) At increased magnification, activated p38 and ERK1/2 are localized within neurofilament-positive (blue) axons. In favorable section, activated p38 is accumulated at an apparent axon end-bulb.

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References

1. Cravioto H, Battista A. Clinical and ultrastructural study of painful neuromas. Neurosurgery 1981;8:181–190.
2. Nystrom B, Hagbarth K. Microelectrode recording from transected nerves in amputees with phantom limb pain. Neurosci Lett 1981;27:211–216.
3. Devor M, Govrin-Lippmann R. Axoplasmic transport block reduces ectopic impulse generation in injured peripheral nerves. Pain 1983;16:73–85.
4. Burchiel KJ. Effects of electrical and mechanical stimulation on two foci of spontaneous activity which develop in primary afferent neurons after peripheral axotomy. Pain 1984;18:249–265.
5. Devor M. Neuropathic pain: what do we do with all these theories? Acta Anaesthesiol Scand 2001;45:1121–1127.
6. Amir R, Argoff CE, Bennett GJ, et al. The role of sodium channels in chronic inflammatory and neuropathic pain. J Pain 2006;7:S1–S29.
7. Cummins TR, Sheets PL, Waxman SG. The roles of sodium channels in nociception: implications for mechanisms of pain. Pain 2007;131:243–257.
8. Catterall WA, Goldin AL, Waxman SG. International union of pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev 2005;57:397–409.
9. Waxman SG, Kocsis JD, Black JA. Type III sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is reexpressed following axotomy. J Neurophysiol 1984;72:466–472.
10. Black JA, Cummins TR, Plumpton C, et al. Upregulation of a silent sodium channel after peripheral, but not central, nerve injury in DRG neurons. J Neurophysiol 1999;82:2776–2785.
11. Black JA, Liu S, Tanaka M, et al. Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. Pain 2007;108:237–247.
12. Cummins TR, Waxman SG. Downregulation of tetrodotoxin-resistant sodium currents and upregulation of a rapidly reprimming tetrodotoxin-sensitive sodium current in small spinal sensory neurons after nerve injury. J Neurosci 1997;17:3503–3514.
13. Cummins TR, Aglieco F, Renganathan M, et al. Nav1.3 sodium channels: rapid repriming and slow closed-state inactivation display quantitative differences after expression in a mammalian cell line and in spinal sensory neurons. J Neurosci 2001;21:5952–5961.
14. Lampert A, Hains BC, Waxman SG. Upregulation of persistent and ramp sodium current in dorsal horn neurons after spinal cord injury. Exp Brain Res 2006;174:660–666.
15. Dib-Hajj SD, Cummins TR, Black JA, Waxman SG. From genes to pain: Nav 1.7 and human pain disorders. Trends Neurosci 2007;30:555–563.
16. Waxman SG. Neurobiology: a channel sets the gain on pain. Nature 2006;444:831–832.
17. Djouhri L, Newton R, Levinson SR, et al. Sensory and electrophysiological properties of guinea-pig sensory neurones expressing Nav1.7 (PN1) Na^+ channel alpha subunit protein. J Physiol 2003;546:565–576.
18. Toledo-Aral JJ, Moss BL, Koszowski AG, et al. Identification of PN1, a predominant voltage-dependent sodium channel expressed principally in peripheral neurons. Proc Natl Acad Sci USA 1997;94:1527–1532.
19. Waxman SG, Dib-Hajj S. Erythermalgia: molecular basis for an inherited pain syndrome. Trends Mol Med 2005;11:555–562.
20. Cox JJ, Reimann F, Nicholas AK, et al. An SCN9A channelopathy causes congenital inability to experience pain. Nature 2006;444:894–898.
21. Ahmad S, Dahllund L, Eriksson AB, et al. A stop codon mutation in SCN9A causes lack of pain sensation. Hum Mol Genet 2007;16:2114–2121.
22. Goldberg YP, MacFarlane J, MacDonald ML, et al. Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations. Clin Genet 2007;71:311–319.
23. Djouhri L, Fang X, Okuse K, et al. The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J Physiol 2003;550:739–752.
24. Renganathan M, Cummins TR, Waxman SG. Contribution of Nav1.8 sodium channels to action potential electrogenesis in DRG neurons. J Neurophysiol 2001;86:629–640.
25. Kretschmer T, Happel LT, England JD, et al. Accumulation of PN1 and PN3 sodium channels in painful human neuroma: evidence from immunocytochemistry. Acta Neurochir 2002;144:803–810.

26. Bird EV, Robinson PP, Boissonade FM. Nav1.7 sodium channel expression in human lingual nerve neuromas. Arch Oral Biol 2007;52:494–502.
27. Seger R, Krebs EG. The MAPK signaling cascade. FASEB J 1995;9:726–735.
28. Obata K, Noguchi K. MAPK activation in nociceptive neurons and pain hypersensitivity. Life Sci 2004;74:2643–2653.
29. Cheng J-K, Ji R-R. Intracellular signaling in primary sensory neurons and persistent pain. Neurochem Res 2008;33: 1970–1978.
30. Wittmack EK, Rush AM, Hudmon A, et al. Voltage-gated sodium channel Nav1.6 is modulated by p38 mitogen-activated protein kinase. J Neurosci 2005;25:6621–6630.
31. Stamboulian S, Choi J-S, Tyrrell LC, et al. The sodium channel $Na_v1.7$ is a substrate and is modulated by the MAP kinase ERK. Soc Neurosci Abstr 2008;466.20.
32. Jin X, Gereau RW. Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-α. J Neurosci 2006;26:246–255.
33. Hudmon A, Choi J-S, Tyrrell L, et al. Phosphorylation of sodium channel Nav1.8 by p38 mitogen-activated protein kinase increases current density in dorsal root ganglion neurons. J Neurosci 2008;28:3190–3201.
34. Koch H, Haas F, Hubner M, et al. Treatment of painful neuroma by resection and nerve stump transplantation into a vein. Ann Plast Surg 2003;51:45–50.
35. Krishnan KG, Pinzer T, Schackert G. Coverage of painful peripheral nerve neuromas with vascularized soft tissue: method and results. Neurosurgery 2005;56:369–378.
36. Ducic I, Mesbahi AN, Attinger CE, Graw K. The role of peripheral nerve surgery in the treatment of chronic pain associated with amputation stumps. Plast Reconstr Surg 2008;121: 908–914.
37. Black JA, Newcombe J, Trapp BD, Waxman SG. Sodium channel expression within chronic multiple sclerosis plaques. J Neuropathol Exp Neurol 2007;66:828–837.
38. Hains BC, Black JA, Waxman SG. Primary motor neurons fail to up-regulate voltage-gated sodium channel Na(v)1.3/brain type III following axotomy resulting from spinal cord injury. J Neurosci Res 2002;70:546–552.
39. Fjell J, Hjelmström P, Hormuzdiar W, et al. Localization of the tetrodotoxin-resistant sodium channel NaN in nociceptors. Neuroreport 2000;11:199–202.
40. Rush AM, Wittmack EK, Tyrrell L, et al. Differential modulation of sodium channel Na(v)1.6 by two members of the fibroblast growth factor homologous factor 2 subfamily. Eur J Neurosci 2006;23:2551–2562.
41. Caldwell JH, Schaller KL, Lasher RS, et al. Sodium channel Nav1.6 is localized at nodes of Ranvier, dendrites and synapses. Proc Natl Acad Sci 2000;97:5616–5620.
42. Peles E, Nativ M, Lustig M, et al. Identification of a novel contactin-associated transmembrane receptor with multiple domains implicated in protein-protein interactions. EMBO J 1997;16:978–988.
43. Henry MA, Sorensen HJ, Johnson LR, Levinson SR. Localization of the Nav1.8 sodium channel isoform at nodes of Ranvier in normal human radicular tooth pulp. Neurosci Lett 2005; 380:32–36.
44. Ji R-R, Suter MR. p38 MAPK, microglial signaling, and neuropathic pain. Mol Pain 2007;3:33–41.
45. Nikolajsen L, Jensen TS. Phantom limb. In: McMahon SB, Kolzenburg M, eds. Wall and Melzack's textbook of pain. 5th ed. London: Churchill-Livingstone, 2006:561–571.
46. Rogers M, Tang L, Madge DJ, Stevens EB. The role of sodium channels in neuropathic pain. Semin Cell Dev Biol 2006;17: 571–581.
47. Hains BC, Waxman SG. Sodium channel expression and the molecular pathophysiology of pain after SCI. Prog Brain Res 2007;161:195–203.
48. Wall PD, Gutnick M. Ongoing activity in peripheral nerves: the physiology and pharmacology of impulses originated from a neuroma. Exp Neurol 1974;43:580–593.
49. Ochoa JL, Torebjörk HE. Paraesthesiae from ectopic impulse generation in human sensory nerves. Brain 1980;103:835–853.
50. Norden M, Nystrom B, Wallin U, Hagbarth K-E. Ectopic sensory discharge and paresthesiae in patients with disorders of peripheral nerves, dorsal roots and dorsal columns. Pain 1984;20: 231–245.
51. Burchiel KJ. Carbamazepine inhibits spontaneous activity in experimental neuromas. Exp Neurol 1988;102:249–253.
52. Devor M, Wall PD, Catalan N. Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. Pain 1992;48:261–268.
53. Matzner O, Devor M. Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na^+ channels. J Neurophysiol 1994;72:349–359.
54. Coward K, Aitken A, Powell A, et al. Plasticity of TTX-sensitive sodium channels PN1 and Brain III in injured human nerves. Neuroreport 2001;12:495–500.
55. Shah BS, Rush AM, Liu S, et al. Contactin associates with sodium channel Nav1.3 in native tissues and increases channel density at the cell surface. J Neurosci 2004;24:7387–7399.
56. Waxman SG, Black JA, Kocsis JD, Ritchie JM. Low density of sodium channels supports action potential conduction in axons of neonatal rat optic nerve. Proc Natl Acad Sci 1989;86: 1406–1410.
57. Cummins TR, Howe JR, Waxman SG. Slow closed-state inactivation: a novel mechanism underlying ramp currents in cells expressing the hNE/PN1 sodium channel. J Neurosci 1998;18:9607–9619.
58. Herzog RI, Cummins TR, Ghassemi F, et al. Distinct reprimming and closed-state inactivation kinetics of Nav1.6 and Nav1.7 sodium channels in mouse spinal sensory neurons. J Physiol 2003;551:741–750.
59. Fertleman CR, Baker MD, Parker KA, et al. SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron 2006;52: 767–774.
60. Renganathan M, Gelderblom M, Black JA, Waxman SG. Expression of Nav1.8 sodium channels perturbs the firing patterns of cerebellar Purkinje cells. Brain Res 2003;959:235–242.
61. Rush AM, Dib-Hajj SD, Liu S, et al. A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons. Proc Natl Acad Sci USA 2006a;103:8245–8250.
62. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37–40.
63. Ji R-R. Mitogen-activated protein kinases as potential targets for pain killers. Curr Opin Investig Drugs 2004;5:71–75.
64. Ji RR, Kawasaki Y, Zhuang ZY, et al. Protein kinases as potential targets for the treatment of pathological pain. Handb Exp Pharmacol 2007;177:359–389.
65. Dai Y, Iwata K, Fukuoka T, et al. Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. J Neurosci 2002;22:7737–7745.
66. Zhuang ZY, Xu H, Clapham DE, Ji RR. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. J Neurosci 2004;24:8300–8309.
67. Mizushima T, Obata K, Yamanaka H, et al. Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. Pain 2005;113:51–60.