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Pro-neurotrophins, sortilin, and nociception

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Abstract

Nerve growth factor (NGF) signaling is important in the development and functional maintenance of nociceptors, but it also plays a central role in initiating and sustaining heat and mechanical hyperalgesia following inflammation. NGF signaling in pain has traditionally been thought of as primarily engaging the classic high-affinity receptor tyrosine kinase receptor TrkA to initiate sensitization events. However, the discovery that secreted proforms of nerve NGF have biological functions distinct from the processed mature factors raised the possibility that these proneurotrophins (proNTs) may have distinct function in painful conditions. ProNTs engage a novel receptor system that is distinct from that of mature neurotrophins, consisting of sortilin, a type I membrane protein belonging to the VPS10p family, and its co-receptor, the classic low-affinity neurotrophin receptor p75NTR. Here, we review how this new receptor system may itself function with or independently of the classic TrkA system in regulating inflammatory or neuropathic pain.

Introduction

The neurotrophin family consists of the founding member nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 (NT-4). The mature forms of the neurotrophins all interact with their respective high-affinity tropomyosin kinase receptors (TrkA, NGF; TrkB, BDNF and NT-4; and TrkC, neurotrophin-3) but also bind to the low-affinity p75 neurotrophin receptor (p75NTR; Chao, 2003; Skeldal *et al.*, 2011). Neurotrophin signaling is extremely important for the development of the peripheral nervous system (Lewin & Barde, 1996), including the specification of neuronal diversity (Lewin, 1996), and is also critical in regulating synaptic plasticity (Greenberg *et al.*, 2009; Park & Poo, 2013). Of the four neurotrophic factors, NGF plays a central role in the genesis of post-inflammatory pain hypersensitivity. Indeed, in the last 20 years the link between the biology of NGF and pain has become very well established (Heppenstall & Lewin, 2000; Pezet & McMahon, 2006; Mantyh *et al.*, 2011). Several major pharmaceutical companies are running clinical trials of humanized antibodies designed to sequester NGF for the treatment of pain in conditions as varied as osteoarthritis, lower back pain, and interstitial cystitis (Cattaneo, 2010; Lane *et al.*, 2010; Evans *et al.*, 2011a; Brown *et al.*, 2012, 2013). The mechanism of action of anti-NGF drugs has been predicated on the basis that NGF sequestration primarily reduces signaling through TrkA receptors on nociceptive sensory neurons. Over 10 years ago, a new level of complexity in neurotrophin signaling arrived with the discovery that secreted proforms of the neurotrophins NGF (proNGF) and BDNF (proBDNF) have biological functions distinct from those of the processed mature factors (Lee *et al.*, 2001; Teng *et al.*, 2005). Proneurotrophins (proNTs) were shown to actually initiate apoptosis in sympathetic neurons, and induce synaptic long-term depression and growth cone collapse of hippocampal neurons by binding to p75NTR (Lee *et al.*, 2001; Woo *et al.*, 2005; Martinowich *et al.*, 2007; Deinhardt *et al.*, 2011). Later, it was shown that sortilin, a type I membrane protein belonging to the VPS10p family, is a an essential co-receptor for p75NTR that is required for proNT to engage p75NTR with high affinity and transmit the apoptotic signal (Nykjaer *et al.*, 2004; Jansen *et al.*, 2007; Skeldal *et al.*, 2012). As an NGF signaling axis is essential in the etiology of pain and hyperalgesia, it is also of interest to understand how proNT signaling might fit into the well-characterized nociceptive pathways that are controlled by NGF. Here, we review the mechanistic basis of how NGF functions in nociception and chronic pain, and discuss how proNT signaling may fit into the biology of NGF-dependent pain.

proNTs

It has long been known that neurotrophins are first produced as proforms that are processed by proteolytic cleavage to the mature secreted forms (Teng *et al.*, 2010). There is now abundant evidence that, under physiological conditions, proNTs exit the cell and engage a distinct receptor complex consisting of p75NTR and sortilin that can initiate cell death, especially in cases of neuronal trauma or stress (Jansen *et al.*, 2007; Nykjaer & Willnow, 2012). The p75NTR–sortilin receptor complex engaged by proNTs essentially initiates the opposite signal (death vs. survival) to that of the classic high-affinity

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Trk receptors, which are receptor tyrosine kinases (Lewin & Barde, 1996; Reichardt, 2006). Importantly, the data in the literature have mostly shown that proNTs do not activate Trk receptors directly (Boutilier *et al.*, 2008; Howard *et al.*, 2013). However, as in all signaling events initiated by secreted ligands, the cellular response may be very dependent on the repertoire of receptors available and the cellular context. For example, it is known that, in cells lacking p75NTR, the affinity and specificity of neurotrophin signaling via their cognate Trk receptors is much reduced (Chao, 2003). Thus, the potential signaling events in cells responding to proNGF will depend on the complement of cell surface receptors available. In this context, it is important to note that p75NTR is known to be expressed by approximately half of all sensory neurons (Lee *et al.*, 1992; Arnett *et al.*, 2007; Vaegter *et al.*, 2011), but also has important biological functions in other cell types, notably Schwann cells and oligodendrocytes (Carter *et al.*, 1996; Casaccia-Bonnefil *et al.*, 1996; Gentry *et al.*, 2004). Sortilin is also widely expressed in neuronal and non-neuronal cells (Petersen *et al.*, 1997; Sarret *et al.*, 2003); sometimes, sortilin is also referred to as neurotensin receptor 3, as it is also a high-affinity receptor for the neurotensin peptide (Mazella *et al.*, 1998). Sortilin is co-expressed with p75NTR in many sensory neurons that are not destined for apoptosis, suggesting that the molecular makeup of a neuron may dictate whether the cell responds to proNTs by apoptosis signaling or with as yet unknown activities (Vaegter *et al.*, 2011; Nykjaer & Willnow, 2012). An important fact to appreciate about sortilin is that, as its name implies, it was first identified as a receptor involved in sorting intracellular cargoes between different membrane compartments. In the context of sensory neurons and other central nervous system cells, this fact means that only a small proportion of the total sortilin will be available on the surface to bind ligands, and that the presence of the receptor may be subject to strong regulation. Moreover, recent evidence suggests an important role for sortilin in facilitating anterograde Trk transport along neurites. Sortilin physically interacts with Trk, and mice lacking sortilin expression show reduced peripheral Trk targeting and a blunted response to mature neurotrophins (Vaegter *et al.*, 2011). Another layer of complexity is added by the fact that the extracellular domain of sortilin is also subject to ADAM10-mediated shedding. In fact, significant amounts of soluble sortilin can be detected in human serum samples, where it correlates with receptor expression in the brain (Evans *et al.*, 2011b). Whether the ectodomain acts as a decoy receptor to scavenge proNTs in the fluid phase or functions to increase its activity is currently not known. However, it is noteworthy that soluble sortilin has been shown to prevent proBDNF cleavage *in vitro*, suggesting that it may function as a rheostat to balance synaptic input and regulate long-term depression (proBDNF) and long-term potentiation (BDNF).

If proNTs play a functional role in peripheral or central sensitization processes that underpin hyperalgesia, then it is important to determine whether these factors are present in somatic sensory neurons and/or their peripheral and central targets. Western blots from skin, peripheral nerve and dorsal root ganglia indicate that proNGF is actually quite abundant in all of these tissues (Reinshagen *et al.*, 2000; Anand, 2004; Bierl *et al.*, 2005; Arnett *et al.*, 2007). The question arises of whether the proNTs are released under physiological conditions associated with pain or hyperalgesia in inflammatory or neuropathic conditions.

NGF and hyperalgesia: the linchpin theory

Hyperalgesia is defined as an increase in the felt intensity of a noxious stimulus, usually following an injury or an inflammatory process. Secondary hyperalgesia is the area of hypersensitivity surrounding an injured area that cannot be signaled directly by peripheral sensitization, as the afferents that innervate the secondary area are not sensitized by the injury. This type of hyperalgesia is thought to result from sensitization of central circuits to afferent input coming from near the initial injury site (Treede *et al.*, 1992; Lewin & Moshourab, 2004). It is thought that strong activation of nociceptors leads to rapid and long-lasting plasticity at synapses between primary sensory neurons and dorsal horn neurons, and this long-lasting change in synaptic strength can sustain hyperalgesia. It was first noticed that rats that had been exposed to daily injections of NGF were behaviorally more sensitive to mechanical and heat stimuli than untreated animals (Lewin *et al.*, 1993). Even a single systemic injection of NGF (1 mg/kg body weight) produced profound heat and mechanical hyperalgesia that lasted for several days. Interestingly, heat and mechanical hyperalgesia appeared to be mechanistically distinct, as heat hyperalgesia appeared within minutes, but mechanical hyperalgesia first became apparent at ~7 h after the injection, and was maximal and sustained at 24 h (Lewin *et al.*, 1993). The fact that a single molecule, NGF, could set into train a series of rapid functional changes with all of the hallmarks of hyperalgesia, normally seen after a sterile inflammation, raised the obvious question of whether NGF was necessary for inflammatory hyperalgesia. The use of NGF-blocking antibodies gave rise to a new model of inflammatory hyperalgesia, in which NGF represents a linchpin molecule that provides the key humoral link between inflammation and the nociceptive sensory neurons that initiate and sustain heat and mechanical hyperalgesia (Lewin & Mendell, 1993; Lewin *et al.*, 1994; Woolf *et al.*, 1994). Later, the use of improved molecular tools to sequester NGF, namely TrkA–IgG fusion proteins that specifically bind endogenous NGF, was shown to ameliorate heat and mechanical hyperalgesia associated with carrageenan-evoked inflammation in rats (McMahon *et al.*, 1995). The key finding that blockade of NGF signaling reduces the impact of increased NGF production in inflammatory conditions that is associated with pain has now been repeated in many models (Pezet & McMahon, 2006; Mantyh *et al.*, 2011).

In 1993, Lewin and Mendell proposed a mechanistic model illustrating the various ways in which increased NGF signaling could produce heat and mechanical hyperalgesia in inflammatory conditions. One key feature of this model was the idea that the mechanisms that underlie the NGF-dependent heat hyperalgesia are distinct from those that underlie the mechanical hyperalgesia (Lewin & Mendell, 1993; Lewin *et al.*, 1994). One important difference is that NGF is capable of inducing extremely rapid changes in the peripheral terminals of C-fibers that sensitize them to noxious heat stimuli (Fig. 1A). Mechanical hyperalgesia, on the other hand, seems to require the induction of changes in gene expression that eventually lead to central sensitization, which maintains mechanical hyperalgesia (Lewin & Mendell, 1993; Lewin *et al.*, 1994). Below, we will briefly review what is known of the molecular mechanisms that underpin heat and mechanical hyperalgesia, and review how proNT signaling may be involved.

NGF-dependent heat hyperalgesia: molecular mechanisms

The availability of NGF in the skin was shown early on to regulate the number of C-fibers that respond to noxious heat. Thus, decreasing NGF levels with blocking antibodies reduced the number of C-fibers that respond to heat, and raised NGF levels increased the number of heat-sensitive C-fibers (Lewin & Mendell, 1994). These early experiments demonstrated that the molecular basis of noxious

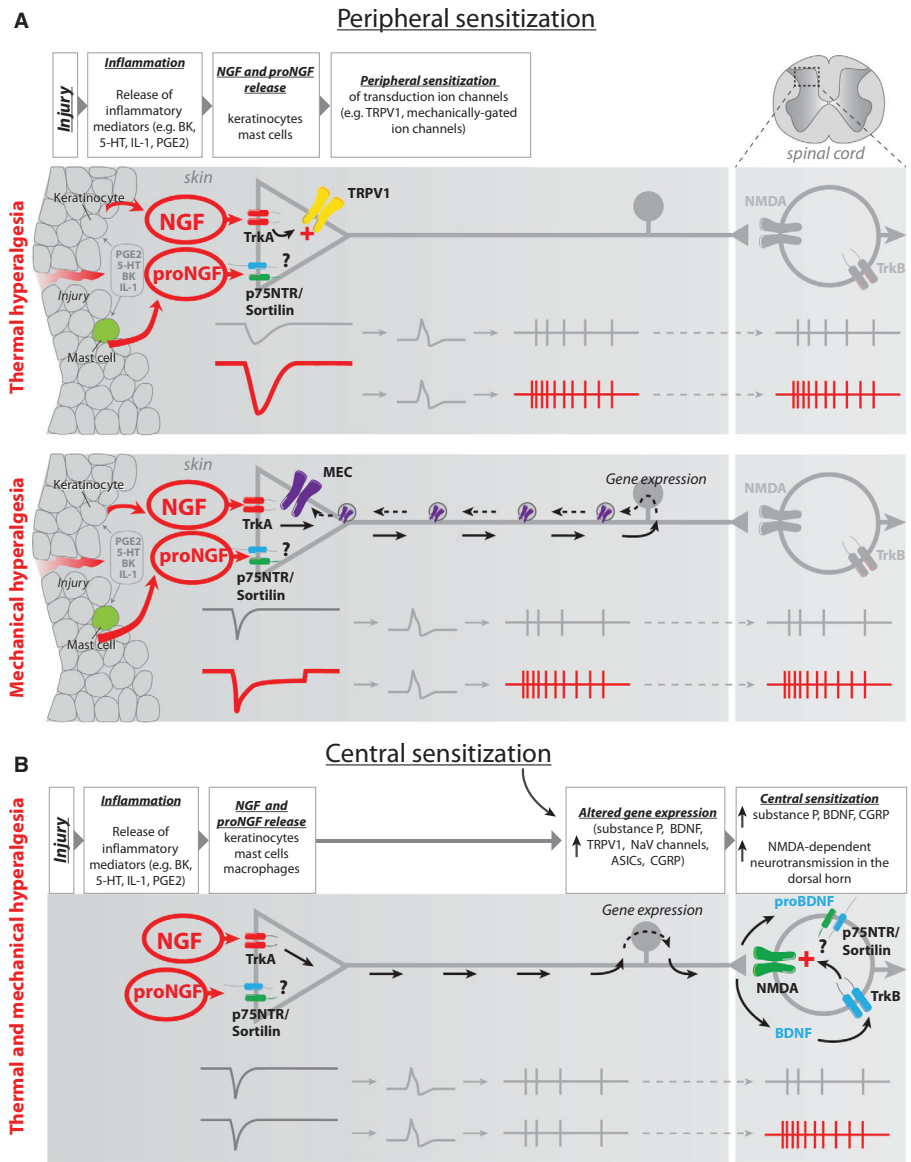

FIG. 1. Schematic representation of mechanisms that contribute to NGF hyperalgesia. (A) Possible mechanisms of peripheral sensitization that are dependent on increased NGF production and release after injury and inflammation. Heat hyperalgesia (top) may be initiated locally by increased levels of NGF released from keratinocytes and mast cells. NGF binds to high-affinity TrkA receptors to initiate rapid and local sensitization of nociceptors to heat that requires TRPV1 ion channels. Transduction currents may be increased (left current traces), leading to greater firing rates of nociceptors (middle action potentials in nociceptors) in response to a heat stimulus (red) than that of resting nociceptors (gray). proNGF may also be released to interact with its receptors p75NTR and sortilin. The signaling pathways initiated by proNGF are poorly understood. Increased NGF release after inflammation may also sensitize nociceptors to mechanical stimuli, including increased transduction currents (left), which would increase firing rates in response to a standard mechanical stimulus (middle; gray, resting; red, sensitized). Mechanical sensitization is long-lasting, and may develop over a longer time frame than heat hyperalgeia. NGF signaling may induce the increased biogenesis and transport of mechanosensitive ion channels (MECs) from the cell boy to the periphery in 'transducosomes'. The role of proNGF signaling in this sensitization event is poorly understood. (B) Possible mechanisms of central sensitization that are dependent on increased NGF production and release after injury and inflammation. Signals initiated by NGF, or proNGF signaling, initiate increases in the expression of sensory neuron proteins in the cell body. Some of the regulated proteins, such as BDNF, can affect the physiology of central spinal cord neurons to produce central sensitization; red traces represent the responses of a sensitized dorsal horn neuron, and gray traces represent a resting neuron. proBDNF may also be released after sensitization, to influence synapses in the dorsal horn. ASIC, acid-sensing ion channel; BK, Bradykinin; 5-HT, 5-hydroxytryptamine; IL-1, interleukin-1; PGE2, prostaglandin E_2.

heat transduction was itself a target of regulation by NGF. The regulation of noxious heat transduction in single C-fibers in an inflammatory pain model was also shown to be dependent on NGF (Koltzenburg *et al.*, 1999). The very rapid NGF-induced heat hyperalgesia was shown to be partly mediated by NGF-induced mast cell degranulation, which can, in turn, release more NGF (Mazurek *et al.*, 1986; Lewin *et al.*, 1994; Andreev *et al.*, 1995). However, subsequent studies have emphasized that most of the rapid heat sensitization initiated by NGF takes place directly at the nociceptor membrane (Fig. 1A). Thus, it was found that distinct isolated sensory neurons possess an ionic inward current that is directly activated by noxious heat, sometimes referred to as I_{heat} (Cesare & McNaughton, 1996). The I_{heat} current in isolated sensory neurons can also be sensitized by algogens such as bradykinin (Cesare & McNaughton, 1996; Cesare *et al.*, 1999). There was great excitement when the capsaicin-gated ion channel transient receptor poten-

tial cation channel subfamily V member 1 (TRPV1) was cloned by Julius and colleagues, and this channel was also gated by heat in a manner similar to I_{heat} (Caterina *et al.*, 1997). Thus, the capsaicin receptor and the noxious heat transduction channel appeared to be one and the same thing. Mendell and Shu then showed that a single short exposure of isolated sensory neurons to NGF (as well as NT-4) greatly potentiated the capsaicin current amplitude measured just minutes later (Shu & Mendell, 1999). NGF-induced heat hyperalgesia was later found to be dependent on the presence of TRPV1, as NGF-induced hyperalgesia was not found in TRPV1$^{-/-}$ mice (Chuang *et al.*, 2001). The persistence of NGF-induced heat hyperalgesia in p75NTR$^{-/-}$ mice suggested that this low-affinity receptor is not a prerequisite for downstream sensitization (Bergmann *et al.*, 1998). As proNT signaling bypasses Trk receptors, this finding suggests that proNGF is not involved in the generation of heat hyperalgesia (Fig. 1A). However, one group has claimed that a recombinant proNGF can, in fact, induce fast and long-lasting heat hyperalgesia in mice (Watanabe *et al.*, 2008). The proNGF protein that they used was mutated to protect it from proteolysis, and was effective at the doses normally used for local injection of NGF (Andreev *et al.*, 1995; Mills *et al.*, 2013). The same group showed that blocking antibodies directed against p75NTR ameliorated the heat hyperalgesia found after complete Freund's adjuvant-induced inflammation, and also ameliorated NGF-induced heat hyperalgesia (Watanabe *et al.*, 2008). If these authors are correct, then proNGF could directly signal via sortilin–p75NTR to potentiate TRPV1-dependent heat responses in the periphery. However, there is as yet no direct genetic evidence that either p75NTR or sortilin is required for the effects described for proNGF. The present consensus is that TRPV1 is a noxious heat-gated ion channel present in most noxious heat-sensitive C-fibers, but its presence does not appear to be necessary for these neurons to respond to noxious heat *in vivo* (Woodbury *et al.*, 2004). Recent studies have implicated new heat-activated ion channels, such as anoctamin-1, a calcium-activated chloride channel, and transient receptor potential cation channel subfamily M member 3 (TRPM3) as being required for heat transduction in nociceptors (Vriens *et al.*, 2011; Cho *et al.*, 2012). However, it is not yet known whether NGF-dependent heat hyperalgesia and nociceptor sensitization are dependent on either anoctamin-1 or TRPM3. The absolute requirement for TRPV1 for NGF-dependent heat hyperalgesia and nociceptor sensitization has led many workers to use increased TRPV1 activity as a molecular surrogate for sensitization. Thus, capsaicin has often being used, rather than heat, to activate TRPV1. Work on dorsal root ganglion (DRG) neurons identified protein kinase activity as being responsible for the sensitization brought about by NGF, with protein kinase C and phosphoinositide 3-kinase (PI3K) being the most likely mediators (Shu & Mendell, 1999; Bonnington & McNaughton, 2003). Whereas protein kinase C acts predominantly via direct phosphorylation of TRPV1 (Numazaki *et al.*, 2002), the PI3K pathway has multiple steps: following TrkA autophosphorylation at Tyr760, PI3K is activated, and in turn activates Src kinase, a non-receptor tyrosine kinase, that subsequently phosphorylates Tyr200 on TRPV1, resulting in translocation to the plasma membrane and increased membrane expression (Zhang *et al.*, 2005; Stein *et al.*, 2006). The fact that a translocation of TRPV1 channels from the cytoplasmic compartment to the plasma membrane is involved in sensitization is intriguing in the context of the role of sortilins in sorting and signaling. Although sortilin deficiency by itself does not impact on heat-induced pain or mechanical sensation, on the genetic background of p75NTR knockout both nociception and tactile sensation are severely impaired (Vaegter *et al.*, 2011). As mice lacking sortilin or p75NTR are unable to respond to proNT stimulation, the sensory deficits in the double-knockout mouse are most likely to be accounted for by reduced Trk signaling from mature neurotrophins. Hence, p75NTR deficiency reduces the affinity and specificity of neurotrophins for their cognate Trk receptors (Chao, 2003), and sortilin reduces anterograde Trk transport and exposure on the plasma membrane. Whether sortilin may also target TRPV1 to the cell surface is unknown, but it is noteworthy that sortilin is required for translocation of vesicles containing glucose transporter type 4 from the intracellular compartment to the plasma membrane (Kandror & Pilch, 2011). It would, of course, be interesting to determine in cellular systems whether sortilin or p75NTR participates in the fast sensitization events. NGF-induced heat hyperalgesia is rapid in onset *in vivo*, but is also very long-lasting, and it has been suggested that NGF can also enhance TRPV1 expression levels via the Ras–mitogen-activated protein kinase pathway (Ji *et al.*, 2002), which could contribute to persistent heat hyperalgesia. It should, however, be noted that there is good evidence that persistent heat hyperalgesia following inflammation or NGF elevation may also be dependent on central sensitization (Fig. 1B).

The fact that TRPV1 is necessary for sensitization, but not for the transduction of noxious heat by nociceptors, is an important fact that requires further investigation (Woodbury *et al.*, 2004; Koerber *et al.*, 2010). One could speculate that freshly phosphorylated TRPV1 or newly inserted TRPV1 molecules in turn directly interact with candidate heat-gated channels, such as anoctamine-1 or TRPM3, to produce sensitization. Alternatively, TRPV1 may itself have a signaling function that is required for the sensitization of heat transduction. In order to answer these questions, definitive identification of the molecule(s) necessary for heat transduction will be required. The signaling pathways that converge onto TRPV1 from TrkA activation are also engaged by other growth factor receptors, such as c-Ret together with its co-receptors GFRα2 and GFRα3 (Stucky *et al.*, 2002; Malin *et al.*, 2006), which are preferentially activated by neurturin and artemin, respectively (Baloh *et al.*, 2000; Bespalov & Saarma, 2007). Neurturin signaling, in particular, may be like NGF signaling, in the sense that it regulates the number of heat-sensitive neurons among the subpopulation of isolectin-B4-positive sensory neurons that, in the adult, lack TrkA receptors (Molliver *et al.*, 1997; Stucky & Lewin, 1999; Stucky *et al.*, 2002). Curiously, the sortilin-related receptor SorLA was recently demonstrated to be critically involved in GFRα and c-Ret sorting and signaling (Glerup *et al.*, 2013). The receptor tyrosine kinase c-Kit is the receptor for stem cell factor (SCF), and was recently found to be expressed by a subpopulation of noxious heat-sensitive nociceptors (Milenkovic *et al.*, 2007). It has been shown that SCF–c-Kit signaling is necessary to maintain nociceptor heat sensitivity, and that SCF can, like NGF, sensitize I_{heat} and produce rapid but short-lasting heat hyperalgesia in a TRPV1-dependent manner (Milenkovic *et al.*, 2007). Interestingly, in the case of glial cell line-derived neurotrophic factor-like ligands and SCF, only heat sensitization has been reported, but mechanical hyperalgesia was absent [but see (Albers *et al.*, 2006)].

Mechanisms of NGF-dependent mechanical hyperalgesia

Mechanical hyperalgesia is the symptom that most concerns patients with painful conditions caused by inflammation or injury. It is very striking to observe that a short burst of elevated NGF can be sufficient to induce mechanical hyperalgesia that can last for days or even weeks in rodents and humans (Lewin *et al.*, 1993; Petty *et al.*,

1994). Systemic or local injection of the NGF peptide is unlikely to lead to sustained TrkA activation, as this small polypeptide would be rapidly degraded by extracellular proteases after injection. Thus, a pulse of NGF is sufficient to set in train a series of events that sustain mechanical hyperalgesia, often for days (Fig. 1A and B). NGF can produce long-lasting changes in gene expression in adult sensory neurons, and the first genes shown to be controlled by NGF were those encoding the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP; Lindsay & Harmar, 1989). Release of neuropeptides from sensory neurons may modulate the strength of spinal cord synapses (Seybold, 2009); however, mice with a targeted mutation of the tachykinin-1 gene coding for SP do not show deficits in inflammation-induced mechanical hyperalgesia (Cao *et al.*, 1998). Thus, considering that NGF is required for inflammation-induced mechanical hyperalgesia, it appears to be unlikely that SP is a major central mediator. Studies on mice lacking a second major neuropeptide, CGRP, expressed in TrkA-positive sensory neurons (Molliver *et al.*, 1997), have indicated broad deficits in inflammatory hyperalgesia, including mechanical hyperalgesia (Salmon *et al.*, 2001). In their study on proNGF in inflammatory pain, Watanabe *et al.* (2008) described p75NTR-dependent increases in CGRP levels in the DRG. It is, however, unclear whether these effects are also dependent on sortilin receptors. Until now, there has been no concrete indication that changes in gene expression can be specifically regulated by p75NTR–sortilin signaling (Fig. 1A and B). The question naturally arises of whether exogenous proNGF can produce mechanical hyperalgesia and, if so, what mechanisms are involved. Recently, it was reported that high doses of recombinant proNGF could provoke significant mechanical hyperalgesia in mice, starting 5 h after local injection into the skin; later time points were not examined (Capsoni *et al.*, 2011). The authors used a recombinant unmodified proNGF molecule that might conceivably be processed to mature NGF in the extracellular space. It is thus possible, but not proven, that the mechanical hyperalgesia observed resulted from activation of the classic TrkA pathway or occurred via sortilin–p75NTR signaling.

Neurotrophins were traditionally thought of as being produced by the targets of sensory neurons, but it became apparent from developmental studies that many sensory neurons can actually express and produce neurotrophins (Ernfors *et al.*, 1990). It was therefore striking when it was discovered that BDNF is normally produced by a subset of TrkA-positive nociceptors, and that the number of TrkA-positive neurons making this factor is dramatically increased by increased NGF levels (Apfel *et al.*, 1996; Michael *et al.*, 1997). Indeed, BDNF was shown to be released by activity in sensory neurons, and its release was enhanced by the elevated NGF levels that follow inflammation (Balkowiec & Katz, 2000; Lever *et al.*, 2001). Thus, increased peripheral NGF levels lead to increased production and release of BDNF from the central synapses of nociceptors in the spinal cord, and this may be critical for certain central sensitization events, especially those involving *N*-methyl-D-aspartate (NMDA) receptors (Kerr *et al.*, 1999). Direct electrophysiological evidence demonstrating that BDNF can rapidly potentiate transmission at synapses formed by nociceptors was provided by Mendell and colleagues (Garraway *et al.*, 2003). The effects of mature BDNF on spinal synapses is rapid, and probably occurs via both presynaptic and postsynaptic TrkB receptors; such effects have been shown to occur via phosphorylation of NMDA receptor subunits (Kerr *et al.*, 1999; Heppenstall & Lewin, 2001; Garraway *et al.*, 2003). It is clear that the classic pathways activated by BDNF are eventually responsible for the phosphorylation of NMDA receptor subunits such as NR1, which eventually mediate some forms of central sensitization (Pezet & McMahon, 2006; Fig. 1B). One complication in examining the central sensitization effects of BDNF is that this factor is also produced within the brain and spinal cord. Furthermore, the production and release of BDNF or proBDNF may be controlled by many factors. For example, it has been proposed that, when activated, spinal microglia cells may release BDNF, which, in turn, can modulate the excitability of dorsal horn neurons. The modulation of the anion gradient in lamina I projection neurons, possibly via the modulation of KCC2 (a potassium chloride co-transporter), can lead to a shift in the reversal potential for anions such as chloride, which makes normally hyperpolarizing inputs from inhibitory interneurons either ineffective or even depolarizing (Coull *et al.*, 2005). This type of BDNF effect is thought to be particularly relevant for sustaining neuropathic pain. Whether sortilin is involved in the BDNF-dependent regulation of KCC2 is currently unknown, but it is thought-provoking that sortilin physically interacts with and is responsible for lysosomal targeting of the related cation-coupled chloride co-transporter NCC2 in the kidney (Zhou *et al.*, 2010).

Global deletion of the gene encoding BDNF leads to early postnatal lethality, which has made the study of BDNF's role in the adult nervous system more difficult (Carroll *et al.*, 1998). Nevertheless, studies using isolated spinal cords from young neurotrophin gene mutant mice have shown that the plasticity of ventral root potentials, which reflects C-fiber drive flexion reflexes, is selectively attenuated in the absence of BDNF, but not in the absence of NT-4 (Heppenstall & Lewin, 2001). A systematic examination of pain-related behaviors in BDNF heterozygote mutant mice also indicated that even reduced gene dosage of this important factor can lead to deficits in acute noxious heat sensitivity and reduced pain behaviors, e.g. in the formalin test (MacQueen *et al.*, 2001). An elegant genetic study using mice in which the BDNF gene was selectively deleted in nociceptive sensory neurons showed that BDNF is required for normal heat hyperalgesia following inflammation (Zhao *et al.*, 2006). However, the authors did not definitively address the question of whether NGF-induced mechanical hyperalgesia depends on sensory neuron-derived BDNF. However, direct injection of NGF into skeletal muscle did not provoke mechanical hyperalgesia in this model, which, in common with other studies, suggests that elevated muscle NGF provokes central sensitization (Lewin *et al.*, 1992; Zhao *et al.*, 2006). In summary, it seems that at least a proportion of the sustained heat hyperalgesia initiated by NGF may be sustained by central sensitization driven by BDNF and subsequent phosphorylation of postsynaptic NMDA receptors (Lewin *et al.*, 1994; Zhao *et al.*, 2006).

It is now clear that many neurons can, in fact, synthesize and release proBDNF, as well as mature BDNF (Pang *et al.*, 2004; Teng *et al.*, 2005; Woo *et al.*, 2005). Sensory neurons contain proBDNF and transport proBDNF; it therefore appears likely that both proBDNF and mature BDNF production could be increased when NGF levels are elevated, e.g. following inflammatory insults (Wang *et al.*, 2006; Fan *et al.*, 2008). Thus, the phenotypic consequences of BDNF deletion in sensory neurons are potentially also attributable to loss of proBDNF; there are, as yet, no data indicating that nociceptors actually release proBDNF at central synapses (Fig. 1B). Recent reports have shown that activated microglia can release proNTs (Srinivasan *et al.*, 2004), and that stimulation of sortilin on microglia can promote their migration and mediate their ability to produce and secrete cytokines (Martin *et al.*, 2003; Dicou *et al.*, 2004). Indeed, it has also been reported that sortilin can facilitate proNT secretion, as exemplified by proBDNF (Chen *et al.*, 2005), and exogenous proNGF itself may have an activating effect on hippocampal glia (Guo *et al.*, 2013). The evidence so far suggests that

neuropathic pain does not depend on increased or decreased NGF signaling (Pezet & McMahon, 2006), but the activation of spinal microglia is central to the initiation of neuropathic symptoms (Tsuda *et al.*, 2013). Thus, activated microglia injected into the spinal cord are by themselves sufficient to induce symptoms of neuropathic pain, such as profound mechanical allodynia (Tsuda *et al.*, 2003). The factor or factors that signal nerve injury to activate spinal cord microglia are, at present, unknown. Inhibition of microglial activation has also been promoted as a potential therapy for neuropathic pain, with drugs such as minocycline, a second-generation tetracycline, showing efficacy in neuropathic pain models and suppressing microglial activation (Raghavendra *et al.*, 2003). Interestingly, the same drug was recently shown to inhibit the production of proNGF by activated microglia after spinal cord injury (Yune *et al.*, 2007). Thus, there is indeed indirect evidence that proNTs may be involved in the pathophysiology of neuropathic pain. There are some reports of changes in the levels of proNGF in peripheral tissues associated with nerve injury or in neuropathy models, but the findings are actually contradictory (Yiangou *et al.*, 2002; Peleshok & Ribeiro-da-Silva, 2012). It is thus conceivable that proNTs could play a role at the critical transition point that converts nerve injury into changes in spinal circuitry that sustain neuropathic pain symptoms (Tsuda *et al.*, 2013).

A key difference between NGF-induced heat and mechanical hyperalgesia lies in the often radically different time courses that these phenomena display. Pure NGF-dependent hyperalgesia has, in the last few years, been increasingly studied in human subjects, as the injection of small amounts of NGF into the muscle or skin offers an excellent model for both short-term and long-term sensitization, while bypassing inflammatory processes. During the first phase I safety trials of recombinant human NGF (rhNGF), it was quickly realized that human subjects experienced local soreness, as well as very long-lasting deep tissue hyperalgesia, or myalgia following rhNGF injection (Petty *et al.*, 1994). In this first human study, dose-dependent myalgia and hyperalgesia was observed to last for up to 7 weeks following a single injection. As in animal models, the mechanisms by which NGF produces mechanical hyperalgesia in humans will probably differ between very early and late phases. Recent studies in humans showed that hyperalgesia, as measured with the use of pressure pain threshold or pinprick sensitivity, first appears after 7 days, and peaks 21 days after an intradermal rhNGF injection (Rukwied *et al.*, 2010, 2013; Obreja *et al.*, 2011a; Weinkauf *et al.*, 2012, 2013). Much more rapid and pronounced hyperalgesia has been noted following injection of rhNGF into human muscles or muscle fascia (Svensson *et al.*, 2003, 2008; Andersen *et al.*, 2008; Deising *et al.*, 2012), and this hypersensitivity differs in several important respects from the NGF-induced mechanical hyperalgesia observed in the skin. First, mechanical hyperalgesia is observed within a few hours of the injection, and the pressure pain hypersensitivity extends well beyond the area of the initial injection (Svensson *et al.*, 2003, 2008; Andersen *et al.*, 2008; Deising *et al.*, 2012). As a rule, the muscle hypersensitivity following rhNGF injection is also observed to subside within a few days of injection, in marked contrast to the very long-lasting hyperalgesia that follows a skin injection. In the skin model, the available studies have noted that the mechanical hyperalgesia remains strictly restricted to the area of the initial rhNGF injection (Rukwied *et al.*, 2010; Obreja *et al.*, 2011a; Weinkauf *et al.*, 2012), which is a strong indication that a peripheral sensitization process may be involved (Treede *et al.*, 1992; Lewin & Moshourab, 2004).

In animal models, systemic injection of NGF provoked mechanical hyperalgesia that first appeared between 1 h and several hours after the injection, and persisted for days (Lewin *et al.*, 1993, 1994; Thompson *et al.*, 1995; Mills *et al.*, 2013). Local skin injection of NGF in rats also provoked a localized mechanical hyperalgesia that persisted for days (Mills *et al.*, 2013). It appears that elevated NGF in skeletal muscle can sensitize muscle afferents to mechanical stimuli, but the evidence from human and animal studies suggests that secondary hyperalgesia is a prominent feature of this model, which involves central sensitization (Lewin *et al.*, 1992; Hoheisel *et al.*, 2007, 2013). The observation that elevated NGF in the skin does not appear to provoke secondary mechanical hyperalgesia suggests that nociceptor sensitization plays a prominent role in this model (Fig. 1A).

Clear direct evidence that cutaneous nociceptors are sensitized to mechanical stimuli after exposure to elevated NGF *in vivo* has been missing, until recently (Hirth *et al.*, 2013). Many nociceptors are polymodal, meaning that they are activated by more than one modality of noxious stimulus; for example, C-fibers activated by noxious mechanical and heat stimuli are termed C-mechanoheat units. With this classification scheme, it is possible to record the following additional types of nociceptor in human skin by the use of microneurography techniques: C-mechanosensitive, C-mechanosensitive and cold, C-mechanosensitive heat and cold, C-mechano-insensitive and heat-insensitive (C-MiHi) cold, and finally C-low threshold mechanoreceptors (Lewin & Moshourab, 2004). Broadly, the same types of nociceptors have been recorded in the skin of rats and mice (Lewin & Mendell, 1994; Koltzenburg *et al.*, 1997), but there appear to be consistent species differences, particularly in the incidence of each fiber type. In particular, C-MiHi fibers, identified in sub-human primates as mechanically insensitive afferents, appear to be rare in rodents (Handwerker *et al.*, 1991; Meyer *et al.*, 1991; Kress *et al.*, 1992; Lewin & Mendell, 1994), but are relatively common in human hairy skin (Schmidt *et al.*, 1995; Weidner *et al.*, 1999). Several studies have strongly implicated C-MiHi fibers in peripheral sensitization processes; thus, these fibers can rapidly acquire mechanosensitivity when stimulated with strong algogens. Recent studies by Schmelz and colleagues have shown that C-MiHi units are also present in the skin of the pig, which they have claimed may be a more suitable animal model for human nociceptors (Obreja & Schmelz, 2010). One feature of C-MiHi fibers recorded in humans and in pigs is that they show a very strong and prominent activity-dependent slowing of their conduction velocity (Weidner *et al.*, 1999; Obreja *et al.*, 2011b; Hirth *et al.*, 2013). Thus, the higher the firing rate, the longer it takes for the action potentials to reach the first spinal synapses. Strikingly, cutaneous NGF elevation in pigs selectively reduced the magnitude of activity-dependent slowing as well as reducing the number of conduction failures at a moderate stimulation frequency of 2 Hz (Obreja *et al.*, 2011a,b). Patients experienced more pain when cutaneous electrical stimuli were employed at the height of the hyperalgesia induced by local intradermal injection of rhNGF. The more reliable initiation and propagation of action potentials in nociceptors under these circumstances may be physiologically relevant, as electrical stimulation could be seen as analogous to the driving depolarization produced by the opening of transduction channels. In a very recent study, Hirth *et al.* (2013) actually provide good evidence for local nociceptor sensitization that is robust only 21 days after the initial NGF injection in a pig model. Essentially, the authors show that, at this point, a significant and large proportion of formerly C-MiHi fibers are now very sensitive to mechanical stimuli; however, the suprathreshold coding properties of these fibers was not examined. There are a couple of interesting features of these findings: one is the extremely long period of time that it apparently takes before sensitization of

nociceptors is overt following local NGF exposure; and second, why does it takes so long for NGF-mediated signaling to induce unmasking of mechanosensitivity whereby acute exposure to strong algogens can unsilence C-MiHi fibers with a very rapid time course (Schmidt *et al.*, 1995)? The human psychophysical data are clear with regard to the fact that acute elevation of NGF in muscle, as opposed to skin, can produce rapid sensitization, but even here there are few data to indicate why this may be the case. In one study in rats, NGF was injected directly into the muscle, and led to apparent activation of C-fiber afferents in the muscle, but did not lead to an acute sensitization of muscle C-fibers to mechanical stimuli (Hoheisel *et al.*, 2005). In common with the innervation of the viscera (McMahon & Koltzenburg, 1990), normal skeletal muscle is innervated by a large number of C-fibers that are insensitive to mechanical stimuli (Jankowski *et al.*, 2013). It is not clear at present whether NGF or proNGF can also lead to unmasking of mechanosensitivity in deep tissue nociceptors such as those innervating skeletal muscle.

Cell biology of long-lasting sensitization induced by neurotrophins

The cell biology of DRG sensory neurons is unusual; these neurons accomplish two fundamentally different tasks at their central and peripheral endings, which are separated by an enormous distance. Synaptic transmission and precise connectivity are established at the spinal cord end, and transduction is accomplished at specialized endings in the periphery. In between, located at approximately two-thirds of the distance between these points, is the cell body, which must provide specialized proteins, membranes and organelles that are sometimes differentially distributed between the peripheral and central branches (García-Añoveros *et al.*, 2001). The retrograde and local signal transduction events initiated by NGF have been studied for decades, and it is clear that NGF can exert some effects locally in the periphery, and that many effects are transported and propagated to the cell body via the so-called signaling endosome (Campenot & MacInnis, 2004).

The proNT co-receptor sortilin has recently been found to play an important role in the trafficking of TrkA-bearing vesicles in sensory neurons axons *in vivo*. Thus, in the absence of sortilin receptors, TrkA-positive vesicles are transported much less in the anterograde direction than in the wild type (Vaegter *et al.*, 2011). The reduced transport, especially of the 140-kDa glycosylated TrkA molecule, reduced the signaling efficiency *in vivo*, with weaker activation of phosphorylated extracellular signal-related kinase 1/2, presumably because fewer TrkA receptors reached the plasma membrane. Sortilin was shown to bind directly to all three of the high-affinity neurotrophin receptors – TrkA, TrkB, and TrkC – and probably has a role in regulating receptor distribution in all neurons that respond to neurotrophins (Vaegter *et al.*, 2011). It was especially striking in this respect that complete deletion of the sortilin gene dramatically exacerbated the phenotype of TrkA deficiency, as $TrkA^{+/-}$ mice were not born in the absence of the sortilin receptor (Vaegter *et al.*, 2011). TrkA-positive vesicles need to reach the peripheral endings of sensory axons to respond to physiological NGF levels, and it is also in peripheral tissues that NGF is elevated during inflammatory conditions. However, sortilin/TrkA-containing vesicles are not the only vesicles whose cargo is also destined to become concentrated at the peripheral endings. Sensory endings possess a robust and stable transduction apparatus equipped to transduce mechanical signals in different ways in different sensory subtypes. Indeed, there are now examples of ion channel proteins that are specifically targeted to the peripheral endings of specific mechanoreceptor types, e.g. the potassium channel KCNQ4 in rapidly adapting mechanoreceptors (Heidenreich *et al.*, 2012). How is this exquisite spatial and functional segregation achieved? The transport of proteins involved in the transduction and transformation of sensory signals at the peripheral endings of sensory neurons is very poorly understood, but represents a clear potential target for NGF modulation of afferent mechanosensitivity. STOML3 is the only protein known to participate directly in fast mechanotransduction, a process that could be directly modulated by inflammatory mediators that sensitize nociceptors to mechanical stimuli (Wetzel *et al.*, 2007). We found that STOML3 is localized to a highly mobile and molecularly distinct transport vesicle within cultured sensory neuron axons (Lapatsina *et al.*, 2012). These vesicles are capable of co-transporting the related stomatin-domain protein, stomatin, together with each of the acid-sensing ion channel family members found in the DRG (Lapatsina *et al.*, 2012). Members of the Rab GTPase family of protein are involved in controlling the organization and identity of different membranous compartments within cells and neurons. For example, Rab5 and Rab7 are localized to signaling endosomes that are thought to retrogradely transport neurotrophin signals from the periphery to the cell body (Deinhardt *et al.*, 2006). Interestingly, the STOML3-containing vesicles do not form part of the signaling endosome pool, as they are Rab5-negative but Rab11-positive. Rab11-positive vesicles have been characterized as composing a slowly recycling endocytic compartment, and may be transported predominantly anterogradely in sensory neurons (Ascaño *et al.*, 2009; Eva *et al.*, 2010). Indeed, gain or loss of function Rab11 mutants radically change vesicle behavior, but these compartments still contain STOML3 (Lapatsina *et al.*, 2012). The STOML3 vesicle is obviously enriched in proteins that are destined to function in transduction at the peripheral endings of sensory neurons, and so we have proposed naming these vesicles 'transducosomes'. The localization of sortilin with TrkA-positive vesicles suggests that the 'transducosome' is distinct from anterogradely transported TrkA-containing vesicles; however, this idea has not been tested directly. *Ex vivo* recordings from sensory afferents innervating the skin have demonstrated that transduction of mechanical stimuli at the peripheral endings of sensory neurons is very stable for many hours in the absence of a connection to the cell body. Indeed, early nerve injury experiments provided evidence that anterogradely transported proteins are first incorporated into cut endings to confer mechanosensitivity at a speed that is consistent with their transport distally via fast axonal transport (Koschorke *et al.*, 1994). The stability of the transduction complexes at sensory endings is likely to be a function of three main factors: the number of 'transducosomes' that arrive per unit of time; the propensity of such vesicles to fuse with the membrane and deliver functional transduction proteins; and, finally, the stability of existing transduction complexes. If this model is correct, it is obvious that the ability of a sensory neuron to become sensitized to mechanical stimuli, or indeed to become newly mechanically sensitive, can be regulated at the levels of vesicle transport, fusion, or endocytosis of, or recovery of, spent transduction complexes (Fig. 1A). It is clear from the time course of fast mechanical hyperalgesia (hours) that local action of NGF might regulate the steps outlined above, but the molecular details are still completely unclear. Long-lasting mechanical hyperalgesia could be sustained by signals that are carried by signaling endosomes to initiate a cell body response, which may or may not include new gene expression, but would change the transduction process via the transport of novel, perhaps modulatory, subunits to the mechanotransducer. Thus, in the context of nociceptor sensitization, it is tempting to consider the TrkA/sortilin vesicle as the signaling part, and the 'transducosome' as a potential long-term effector. We recently identified a large extracel-

lular tether protein that appears to be required for efficient and fast transduction in mechanoreceptors and many nociceptors (Hu *et al.*, 2010). It is obvious that the transport of this protein could provide a way to 'unsilence' nociceptors, but this hypothesis can only be tested once the identity of this protein is known.

NGF, proNTs, and sensory neuropathies

It is clear that the NGF elevation that accompanies inflammation initiates a complex series of events, some of which are local and fast, and others of which are global and long-lasting. Anti-NGF therapy is remarkably effective in a broad variety of pain conditions, ranging from muscle pain to bone cancer pain (Mantyh *et al.*, 2010; Jimenez-Andrade *et al.*, 2011). This remarkable efficacy of anti-NGF therapy is probably attributable to the broad range of molecular events that are set into motion by elevated NGF levels in a variety of different tissues. New molecular tools, such as blocking antibodies specific for proNTs (Paoletti *et al.*, 2012), or genetic models such as sortilin-null mutant mice (Jansen *et al.*, 2007; Vaegter *et al.*, 2011), may reveal distinct or overlapping roles for this distinct signaling system in pain. There are, in fact, intriguing hints in the literature that proNGF and proBDNF signaling may play an important role in the pathophysiology of neuropathic pain that requires microglial activation. It is clear that mature NGF is strongly implicated in hyperalgesia following inflammation, but the evidence is much more equivocal for a role in the pathophysiology of neuropathic pain. This is despite the long-standing hypothesis that reduced NGF levels in the skin may be involved in the pathophysiology of neuropathic pain that is associated with diabetic neuropathy (Anand, 2004). In this context, it is particularly interesting that the loss of p75NTR and sortilin function leads to very severe late-onset sensory neuropathy in mice (Vaegter *et al.*, 2011). Loss of function mutations in the genes encoding the TrkA receptor or its ligand NGF are classic causes of the rare and severe condition of congenital insensitivity to pain (Indo *et al.*, 1996; Einarsdottir *et al.*, 2004; Carvalho *et al.*, 2011). A recent report described a novel loss of function mutation in the human NGF gene that leads to congenital pain insensitivity and an apparently non-functional NGF, because it is defectively processed and accumulates as proNGF (Larsson *et al.*, 2009). Biochemical studies on NGF and proNGF carrying this mutation indicate that they are either without biological activity *in vivo* or are less effective at binding their receptors (Covaceuszach *et al.*, 2010; Capsoni *et al.*, 2011). Recent genetic and functional studies have strongly implicated sortilin and related receptors as key molecules that govern the severity of Alzheimer's disease (Carlo *et al.*, 2013; Gustafsen *et al.*, 2013). Given the importance of this receptor in proNT and neurotrophin signaling, it is tempting to speculate that new variants in the sortilin–p75NTR signaling pathway could be associated with human susceptibility to neuropathic and inflammatory pain.

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Abbreviations

BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; C-MiHi, C-mechano-insensitive and heat-insensitive; DRG, dorsal root ganglion; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; NT-4, neurotrophin-4; p75NTR, p75 neurotrophin receptor; PI3K, phosphoinositide 3-kinase; proBDNF, proform of brain-derived neurotrophic factor; proNGF, proform of nerve growth factor; proNT, proneurotrophin; rhNGF, recombinant human nerve growth factor; SCF, stem cell factor; SP, substance P; TRPM3, transient receptor potential cation channel subfamily M member 3; TRPV1, transient receptor potential cation channel subfamily V member 1.

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