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Neurotrophins, endocannabinoids and thermo-transient receptor potential: a threesome in pain signalling

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Abstract

Because of the social and economic costs of chronic pain, there is a growing interest in unveiling the cellular and molecular mechanisms underlying it with the aim of developing more effective medications. Pain signalling is a multicomponent process that involves the peripheral and central nervous systems. At the periphery, nociceptor sensitisation by pro-inflammatory mediators is a primary step in pain transduction. Although pain is multifactorial at cellular and molecular levels, it is widely accepted that neurotrophin (TrkA, p75NTR, Ret and GFRs), cannabinoid (CB1 and CB2), and thermo-transient receptor potential (TRPs; TRPV1, TRPA1 and TRPM8) receptors play a pivotal role. They form a threesome for which endocannabinoids appear to be a first line of defence against pain, while neurotrophins and thermoTRPs are the major generators of painful signals. However, endocannabinoids may exhibit nociceptive activity while some neurotrophins may display anti-nociception. Accordingly, a clear-cut knowledge of the modulation and context-dependent function of these signalling cascades, along with the molecular and dynamic details of their crosstalk, is critical for understanding and controlling pain transduction. Here, the recent progress in this fascinating topic, as well as the tantalizing questions that remain unanswered, will be discussed. Furthermore, we will underline the need for using a systems biology approach (referred to as systems pain) to uncover the dynamics and interplay of these intricate signalling cascades, taking into consideration the molecular complexity and cellular heterogeneity of nociceptor populations. Nonetheless, the available information confirms that pharmacological modulation of this signalling triad is a highly valuable therapeutic strategy for effectively treating pain syndromes.

Introduction

Pain is the most common complaint for which patients seek treatment from a physician. According to WHO estimates, chronic pain is suffered by up to 20% of individuals worldwide, with a median prevalence of 15% (2–40%), and nearly half a billion new cases diagnosed each year (Gureje *et al.*, 1998; Gaskin & Richard, 2012). These numbers are expected to further grow in the future as the population ages. Chronic pain has a major impact on the patient and family quality-of-life and this is aggravated because the current treatments remain unsatisfactory for > 50% of patients (Turk *et al.*, 2011). Moreover, ~5% experience debilitating pain that results in loss of work, family crises, depression and/or suicide (Turk *et al.*, 2011). The economic and medical costs of inadequate pain therapy in the community are enormous (Turk & Theodore, 2011). A recent study estimated that the annual economic cost of pain in the United States amounted up to $635 billion, which is greater than the combined annual cost of heart disease ($309 billion), cancer ($243 billion) and diabetes ($188 billion) (Gaskin & Richard, 2012). It could be estimated that the worldwide cost may be up to $2.5 trillion. Despite the prevalence and cost of undertreated chronic pain, the pharmacological armamentarium for preventing or reducing it is surprisingly limited, mainly due to our poor understanding of the genetic, molecular and cellular mechanisms underlying various pain syndromes but also because of inter-individual variation. Accordingly, this tremendous burden on society requires a concerted research effort to understand the pathophysiological mechanisms of pain transduction and to develop successful treatments.

Pain sensation is initiated when peripheral terminals from a group of sensory neurons, known as nociceptors, are activated by noxious thermal, mechanical or chemical stimuli (Basbaum *et al.*, 2009; von Hehn *et al.*, 2012). Nociceptors transmit afferent information regarding tissue injury and inflammation through the spinal cord to pain-processing regions in the brain to elicit an avoidance response that prevents or minimises the damage. In addition, injury-induced activation of dorsal root ganglion (DRG) neurons produces vasodilation, plasma extravasation and hypersensitivity (second-order neuronal excitation). The neuronal agents that signal inflammation are bioactive peptides released in the periphery upon stimulation of small unmyelinated afferent C-fibres, a subpopulation of peptidergic A-delta fibres and the endocrine cells present in all organs (Chiu *et al.*, 2012). These neuropeptides act in a paracrine fashion on peripheral immune cells and vascular smooth muscle, producing tissue inflammation. Furthermore, antidromic stimulation of nociceptors and activation of neuronal receptors such as transient receptor potential (TRP) vanilloid (TRPV1) or TRP ankyrin (TRPA1) at the peripheral terminals promote an efferent exocytosis of neuropeptides that helps

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to propagate the inflammatory response (Basbaum *et al.*, 2009; Chiu *et al.*, 2012; von Hehn *et al.*, 2012). The symptoms resulting from inflammatory activation and sensitisation of primary sensory neurons are known as neurogenic inflammation. This phenomenon leads to profound changes in the perception of stimuli in the damaged region, such as hyperalgesia and allodynia. In chronic conditions, this process is exacerbated by synaptic changes in the spinal cord, a process known as central sensitisation (Basbaum *et al.*, 2009).

In recent years, a tremendous effort has been made to unveil the cellular coding and molecular components implicated in the generation and propagation of pain signals, as well as in the chronification of pain. This objective is a daunting enterprise considering that pain transduction is a multifactorial, multicellular and multicomponent process involving a plethora of cell types and a myriad of molecules, from algogens to cellular signalling proteins (Basbaum *et al.*, 2009; Julius & Nathans, 2012). In addition, to complicate matters further, sensory neurons have to simultaneously integrate pain-inducing (pro-algesic agents) and pain-suppressing signals (endocannabinoids) that are co-produced by painful insults (De Petrocellis & Di Marzo, 2009; Julius & Nathans, 2012). Nonetheless, pain signalling may be described in simple terms as a process in which the complex pain signals act on cellular sensors in nociceptive neurons. These sensors convey the input noxious cues through intracellular pathways to a limited number of cellular effectors which, in turn, transmit the information to the brain by modulating the nociceptor neuronal excitability via the activation of voltage-gated sodium channels in the peripheral terminals. A pivotal case that illustrates both the complexity as well as the modality-specific nature of pain transduction is provided by the interplay and cross-communication of neurotrophins, endocannabinoids and thermoTRP channels (Fig. 1). These represent a threesome in pain signalling as, apparently, endocannabinoids are playing against the other two components to mitigate the encoding of painful signals. However, this vision is not as simple as previously thought because endocannabinoids may produce nociception, and some neurotrophins display anti-nociceptive activity. We briefly review our current knowledge of the dynamics of this fascinating system, and provide an overview of the remaining challenges to be addressed in gaining a full understanding of its functionality, paving the way to better medication for the treatment of chronic pain. We do not wish to imply that this three-fold system is the primary mechanism governing pain transduction, as many other pathways are also involved (prostanoids, oxidative stress, voltage-gated ion channels (VGIC), noradrenergic pathways, etc.) and probably influence the proposed signalling threesome; we wish to propose a hypothesis that focuses research on this system rather than on its parts. This appears important because neurotrophins, cannabinoids and thermoTRPs have been involved in the aetiology of inflammatory and neuropathic pain and, therefore, the proposed threesome may play an important role in these types of pain.

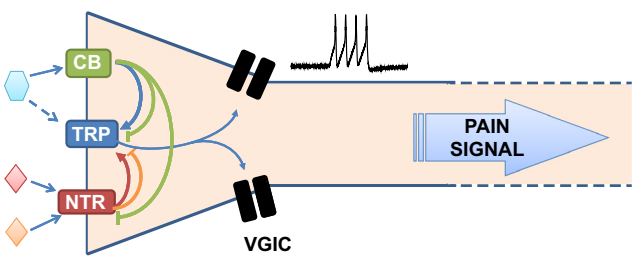

FIG. 1. The thermoTRP, cannabinoid and neurotrophin threesome in peripheral terminals. The diagram depicts the signalling fluxes in this triad. Endocannabinoids may act on metabotropic CB receptors or directly on thermoTRP channels. Nociceptive and anti-nociceptive neurotrophic factors act on their neurotrophin receptors, increasing or decreasing the activity of thermoTRPs. In turn, thermoTRP channels are major triggers of action potentials in sensory neurons by activating voltage-gated Na channels (voltage-gated ion channel; VGIC) through membrane depolarisation. Action potentials (inset) convey the information towards the central nervous system (large arrow). Dashed line denotes lower potency.

ThermoTRP signalling

All organisms have to be able to sense and react to fluctuations in temperature and other environmental changes in order to survive. Humans are endothermic creatures whose body temperature remains fairly constant within a range of environmental temperatures. This property is due primarily to homoeothermic mechanisms triggered by thermosensitive neurons (including nociceptors) that are capable of detecting and transducing temperature changes into neuronal excitability, thereby conveying information to the brain to produce an appropriate metabolic compensatory response (Woolf & Ma, 2007; Julius & Nathans, 2012). Nociceptors are able to detect changes as small as 1 °C and they are tuned to respond to a wide but individually distinct range of temperatures, from noxious cold ($\leq$ 17 °C) to injurious heat ($\geq$ 52 °C). This remarkable dexterity is due to the presence at peripheral terminals of a set of receptor channels, known as thermoTRPs, which are gated by changes in the environmental temperature (Clapham *et al.*, 2005; Basbaum *et al.*, 2009; Julius & Nathans, 2012). ThermoTRPs display distinct activation temperatures and are impressively sensitive to temperature ($Q_{10} \geq 20$). Virtually all thermoTRPs are polymodal receptor channels as they can also be gated by chemical stimuli, in particular natural compounds found mostly in food spices or environmental contaminants (Venkatachalam & Montell, 2007; Julius & Nathans, 2012). Moreover, dysfunction of these channels has profound physiological effects that include alterations in body temperature, and thermal hypo- and hypersensitivity (Wang & Woolf, 2005; Julius & Nathans, 2012).

Molecularly, thermoTRPs are a family of ion channels that belong to the superfamily of TRP channels. These channels are involved in various types of sensory reception, including thermoreception, chemoreception, mechanoreception and photoreception (Julius & Nathans, 2012). The mammalian TRP superfamily comprises six subfamilies known as the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipins), TRPP (polycystin) and the TRPA (ANKTM1, ankyrin) ion channels (Venkatachalam & Montell, 2007; Zheng, 2013). TRP channels are mostly expressed at the plasma membrane of distinct cell types in many tissues, and they all assemble as tetramers. The channel subunits share a common structural homology with six putative transmembrane (TM) segments, a pore region between the fifth and sixth TM segments, and intracellular N- and C-terminal domains. Across the superfamily there is only about 20% homology in the amino acid sequences of the entire TRP channel family, primarily corresponding to the TM domain, although a higher degree of homology exists within each family (Venkatachalam & Montell, 2007). Functionally, the TRP channels display different modes of gating, as well as distinct ion selectivity, consistent with their involvement in sensory physiology, and in transepithelial Ca^{2+} and Mg^{+} transport (Venkatachalam & Montell, 2007). Given their contribution to the pathophysiology of several human maladies, these proteins have been validated as targets for drug intervention (Messeguer *et al.*, 2006; Cortright & Szallasi, 2009; Ferrer-Montiel *et al.*, 2012; Brederson *et al.*, 2013).

ThermoTRP channels are widely expressed in the peripheral neuroimmune system as well as in a variety of other tissues, including the central nervous and urinary systems (Ferrer-Montiel *et al.*, 2012; Brederson *et al.*, 2013). In nociceptors, thermoTRP channels display a broad cellular expression, being found mostly, but not exclusively, in the non-myelinated nociceptor population (Belmonte & Viana, 2008; Dubin & Patapoutian, 2010). Notably, nociceptors expressing thermoTRPs are quite heterogeneous in terms of their molecular composition. For instance, TRPV1 is expressed by peptidergic and nonpeptidergic nociceptors and among each subpopulation there is additional heterogeneity in the receptor expression and function (Price & Flores, 2007; Cavanaugh *et al.*, 2011; McCoy *et al.*, 2012). A similar account can be seen for other thermoTRP channels such as TRPA1 and TRPM8 (Barabas *et al.*, 2012; McCoy *et al.*, 2012). This assorted expression appears essential for a context-dependent mode of action that expands the means of integrating and transducing incoming noxious signals, and helps to understand the pleiotropic consequences of their contribution to pain symptoms.

Cumulative evidence demonstrates that some thermoTRPs are gateways for thermosensory perception, and that their malfunction pivotally contributes to pain signalling (see for reviews: Ferrer-Montiel *et al.*, 2012; Julius & Nathans, 2012; Nilius *et al.*, 2012). The activity of these ion channels may be greatly modified under pathological conditions giving rise to abnormal thermoreception (Cortright & Szallasi, 2009; Planells-Cases *et al.*, 2011). For instance, TRPV1, TRPA1 and TRPM8 channel responses in nociceptors are enhanced by pro-inflammatory mediators or cytotoxic compounds, leading to thermal hyperalgesia, as well as contributing to mechanical allodynia, pruritus and sunburn pain (Obata *et al.*, 2005; Diogenes *et al.*, 2007; Cortright & Szallasi, 2009; Planells-Cases *et al.*, 2011; Belghiti *et al.*, 2013; Brederson *et al.*, 2013; Lippoldt *et al.*, 2013; Liu *et al.*, 2013; Moore *et al.*, 2013; Schwartz *et al.*, 2013; Wilson *et al.*, 2013; Zheng, 2013). In addition, an activity which is independent of ion conduction of these channels and which directly modulates signalling in nociceptors, such as TRPV1-mediated regulation of cytoskeleton dynamics (Goswami *et al.*, 2011), may also contribute to pain transduction in some peripheral neuropathies.

The molecular mechanisms involved in thermoTRP inflammatory sensitisation are still under intense scrutiny for several pain disorders. Progress in this field has identified two main modulation strategies involved in receptor sensitisation, namely an enhancement of channel gating due to posttranslational protein modification and an augmentation of channel expression in the neuronal surface. The channel activity of thermoTRPs such as TRPV1, TRPA1 and TRPM8 is modified by protein kinases and phosphatases that are activated by signalling pathways stimulated by algogens released upon tissue damage. Protein phosphorylation or dephosphorylation of these receptors may lead to up-regulation of channel gating by decreasing the threshold of activation, by releasing tonically-inhibited channels and/or by modulating the extent of receptor desensitisation. Indeed, all these modalities probably act synergistically to ensure a significant increment of channel activity under pathological conditions. The signalling routes that contribute to the modulation of thermoTRPs function in nociceptors involve the protein kinase C (PKC), protein kinase A (PKA), Src, calcium–calmodulin kinase II (CAMKII), calcineurin, phosphatidylinositide-3 kinase (PI3K), mitogen-activated protein kinase (MAPK), phospholipase A_2 (PLA_2), and phosphatidylinositol 4,5 bisphosphate (PIP_2) cascades (see for reviews Planells-Cases *et al.*, 2011; Yudin & Rohacs, 2012; Zheng, 2013). Interestingly, sensory neurons appear to have a PLC-dependent, Ca^{2+}/CAMKII-based molecular switch that swaps their signalling form pro- to anti-algesic (Hucho *et al.*, 2012), most likely by modulating thermoTRP channel activity.

Pathological potentiation of thermoTRP function is further established by stimulation of receptor trafficking to, and expression into, the plasma membrane. Algogens increase the channel recruitment to the cell surface through both fast (acute) and slow (chronic) means. Exposure of primary nociceptors in culture to pro-algesic substances such as nerve-growth factor (NGF) or ATP induces a rapid recruitment of TRPV1 channels to the plasma membrane by a mechanism that involves Ca^{2+}- and SNARE-dependent neuronal exocytosis (Morenilla-Palao *et al.*, 2004; Zhang *et al.*, 2005; Stein *et al.*, 2006; Camprubi-Robles *et al.*, 2009; Schmidt *et al.*, 2009). Blockade of neuronal exocytosis of thermoTRPs in primary afferent nociceptors produces anti-nociceptive activity *in vivo* in models of inflammatory, cancer and neuropathic pain (Ponsati *et al.*, 2012). This observation is consistent with the analgesic activity displayed by botulinum neurotoxin A, which also reduces the expression level of TRPV1 at the nociceptor surface (Gazerani *et al.*, 2009; Giannantoni *et al.*, 2013; Xiao *et al.*, 2013).

In chronic pain syndromes, the levels of thermoTRP channels in primary sensory neurons are also augmented (Chan *et al.*, 2003; Matthews *et al.*, 2004; Facer *et al.*, 2007; Yilmaz *et al.*, 2007; Akbar *et al.*, 2008; Anand *et al.*, 2008; Belghiti *et al.*, 2013), and blockade of their membrane recruitment induces long-lasting anti-nociception (Ponsati *et al.*, 2012). At variance with acute channel recruitment, the increase in receptor expression seen in chronic conditions may involve both transcriptional and translational mechanisms, with the subsequent trafficking of the channels to the plasma membrane (Ji *et al.*, 2002; Amaya *et al.*, 2004; Diogenes *et al.*, 2007). In support of this tenet, analysis of the TRPV1 interactome using a yeast two-hybrid screen revealed the interaction of the channel with a plethora of proteins, most of them implicated in protein trafficking from the endoplasmic reticulum to the peripheral terminal (Stein *et al.*, 2006; Planells-Cases *et al.*, 2011), as well as with the SNARE protein complex (Morenilla-Palao *et al.*, 2004) and tubulin (Laínez *et al.*, 2010; Goswami *et al.*, 2011). An interesting TRPV1-interacting protein is the signalling molecule PI3K that mediates NGF-stimulated TRPV1 trafficking to the plasma membrane (Stein *et al.*, 2006), which may be driven by Src-induced TRPV1 phosphorylation (Zhang *et al.*, 2005). Taken together, all this evidence suggests that up-regulation of thermoTRP activity and biogenesis is a critical step in the onset, maintenance and chronification of pain signals. Indeed, attenuation of thermoTRP activity in inflammatory conditions induces significant anti-nociception (Ferrer-Montiel *et al.*, 2012).

Endocannabinoid signalling

Cannabinoids (including endocannabinoids) in general display antinociceptive activity in a plethora of inflammatory and neuropathic animal pain models (Walker & Hohmann, 2005; Guindon & Hohmann, 2009; Uhelski *et al.*, 2013; Zogopoulos *et al.*, 2013). The molecular mechanisms implicated in cannabinoid-induced pain suppression are still under debate regarding the site of action, the receptor and the signalling pathways involved (Akopian *et al.*, 2009; Kress & Kuner, 2009), as well as the dual activity of endocannabinoids, as they are also ligands of thermoTRP channels. Hence, the cannabinoid signalling system is composed of metabotropic (CB1, CB2 and GPR55) and ionotropic (thermoTRPs) membrane receptors that are located in the central and peripheral nervous system. However, it appears that most of the anti-hyperalgesic efficacy of cannabinoids, including endocannabinoids, is exerted through peripherally

expressed CB1 receptors (Agarwal *et al.*, 2007). Nonetheless, participation of centrally expressed receptors in cannabinoid-induced analgesia cannot be ruled out, although this activity has been mostly associated with psychotropic side effects, temporary memory impairment and dependence, which is limiting the use of brain-permeating cannabinoids as analgesic drugs (Pacher *et al.*, 2006; Di Marzo, 2008; Pacher & Kunos, 2013). Here, we will focus briefly on describing the composition and function of the cannabinoid system in primary afferent neurons and the spinal cord. An analgesic activity has been largely attributed to endocannabinoid signalling through CB receptors by 'quenching' the activation of TRPV1 and TRPA1 induced by pro-inflammatory neurotrophins (Sharkey *et al.*, 2007; Engel *et al.*, 2011; Starowicz *et al.*, 2012; McDowell *et al.*, 2013). Peripheral metabotropic CB receptors are primarily composed of CB1 and CB2, and a possible contribution, although not well characterised, of GPR55 receptors (Kress & Kuner, 2009). CB receptors are widely expressed in neuronal and non-neuronal cells in the periphery where they can be found on nerve fibres, mast cells, epidermal keratinocytes and cells of the adnexal tissues (Bíró *et al.*, 2009). Metabotropic CB receptors are 7-TM integral membrane proteins coupled to trimeric G-proteins of the $G_{i/0}$ family (Kress & Kuner, 2009), although an association with the $G_q/_{11}$ family has also been reported in experiments using the agonist WIN55,212-2 (Lauckner *et al.*, 2005). Activation of $G_{i/o}$ proteins leads to inhibition of adenylate cyclase by the α-subunit that in turn prevents the activation of PKA signalling (De Petrocellis & Di Marzo, 2009). CB1 agonists also activate the MAPK and PI3K pathways and promote the hydrolysis of PIP_2 via activation of the PLC cascade (De Petrocellis & Di Marzo, 2009). Interestingly, most of these signalling pathways are shared by neurotrophins and converge onto thermoTRPs (Fig. 2), implying an intimate crosstalk of these two signalling systems in the setting, maintenance and modulation of chronic pain and/or analgesia. An additional pivotal element in this interplay is the modulation of the intracellular Ca^{2+} level, as the invoked signalling routes may modulate it either by affecting the activity of Ca^{2+}-permeable receptors in the cell surface or by regulating its release from intracellular stores. Intracellular Ca^{2+}, in turn, will activate cascades such as PKC, calcineurin and calmodulin which also act on thermoTRPs, increasing the complexity of CB1 and CB2 signalling in nociception and pain transduction.

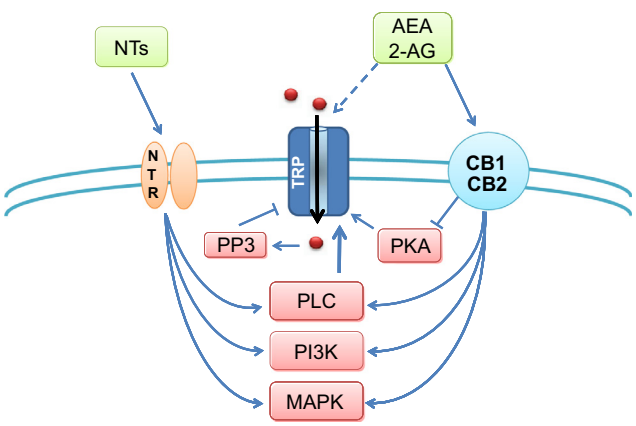

FIG. 2. Major pathways involved in endocannabinoids and neurotrophic factors signalling to thermoTRPs. PLC, PI3K and MAPK are three cascades shared by endocannabinoids and neurotrophins. The dynamics and interplay of these pathways that lead to nociception or anti-nociception under inflammatory conditions are as yet largely unknown, although they will critically depend on the molecular and cellular context. PP3, calcineurin phosphatase; AEA, anandamide; NTR, neurotrophin; PLC, phospholipase C; red balls are Ca^{2+} ions. Dashed line denotes lower potency.

Endocannabinoids can also signal through an ionotropic mechanism, as several TRP receptors, such as TRPV1, TRPV2, TRPV4, TRPA1 and TRPM8, can be directly gated by endocannabinoids (Akopian *et al.*, 2009). Indeed, endocannabinoids produced under inflammation, such as anandamide and 2-arachidonyl glycerol, may act as activators of TRPV1 channels (Singh *et al.*, 2005; Clapper *et al.*, 2010; Schreiber *et al.*, 2012; Khasabova *et al.*, 2013). However, their lower potency for TRPV1 as compared to CB receptors implies that a direct action on the thermoTRP channel would require micromolar amounts of the endocannabinoids, a concentration that may not be reached even in pathological conditions. Nonetheless, because inflammatory mediators sensitise TRPV1 receptors, they may contribute to the conversion of pathologically-produced levels of endocannabinoids into activators of TRPV1 (Singh *et al.*, 2005). Therefore, endocannabinoids could generate pain rather than analgesia or, akin to capsaicin, could produce pain and anti-nociception by inducing TRPV1 activation and desensitisation. Paradoxically, even though activation of thermoTRPs is associated with nociception and pain (Ferrer-Montiel *et al.*, 2012), it appears that their stimulation by endocannabinoids leads to anti-nociception (Akopian *et al.*, 2009; Andersson *et al.*, 2011; Starowicz & Przewlocka, 2012; Maione *et al.*, 2013). The probable mechanism underlying endocannabinoid-induced TRP-mediated anti-nociception is receptor desensitisation via Ca^{2+}-dependent dephosphorylation by calcineurin (Patwardhan *et al.*, 2006; Akopian *et al.*, 2008; Ruparel *et al.*, 2011). An argument in favour of a prevalent desensitizing mechanism induced by endocannabinoids is that they act as partial agonists of thermoTRP channels, resulting in low Ca^{2+} entry, which may not suffice to excite nociceptors. Furthermore, because TRP channels are allosteric proteins, prolonged exposure to endocannabinoids could desensitise channel gating by inducing a high-affinity closed conformation and/or by favouring receptor internalisation, similar to that produced by prolonged exposure of nociceptors to vanilloids (Sanz-Salvador *et al.*, 2012). In contrast, endocannabinoids could produce nociceptor sensitisation by the PLC-mediated activation of the Ca^{2+}/CAMKII-based molecular switch (Hucho *et al.*, 2012), which could up-regulate the gating of thermoTRP channels. Additionally, spinal endocannabinoid mediation of activity-dependent pain sensitisation in dorsal horn neuronal circuits has been ascribed to cannabinoid-induced disinhibition of afferent synaptic inputs to nociceptive circuits (Pernía-Andrade *et al.*, 2009). This disinhibition was initially attributed to modulation of spinal CB1 receptors (Pernía-Andrade *et al.*, 2009), although it appears mediated, or at least contributed to, by TRPV1 channels located in GABAergic interneurons (Kim *et al.*, 2012; Higgins *et al.*, 2013).

An additional component of the endocannabinoid signalling pathway is provided by the dynamic changes in endocannabinoid levels in chronic pain sates (Rani Sagar *et al.*, 2012; Maione *et al.*, 2013). The amount of endocannabinoids is dependent on the rate of synthesis and degradation by a complex network of enzymes that are co-localised with metabotropic and ionotropic CB receptors (De Petrocellis & Di Marzo, 2009). The enzymes that degrade endocannabinoids include fatty-acid amide hydrolase and monoacylglycerol lipase. Inhibition of these enzymes maintains the high levels of endocannabinoids, and induces anti-hyperalgesia and anti-nociception (Petrosino & Di Marzo, 2010; Starowicz *et al.*, 2013). Conversely, the levels of endocannabinoids may be kept high by inhibiting the membrane transporters that facilitate their cellular uptake for degradation (Maione *et al.*, 2013). Because metabotropic CB1 and CB2 receptors are probably desensitised and/or internalised by prolonged

exposure to endocannabinoids (Wu *et al.*, 2008; Smith *et al.*, 2010), their anti-nociceptive effect in the periphery and spinal cord could be produced through desensitisation of thermoTRPs (Engel *et al.*, 2011), and/or their endocytosis as reported for TRPV1 upon the prolonged exposure to vanilloids (Sanz-Salvador *et al.*, 2012). Taking together, available data from several laboratories have attributed the main anti-nociceptive activity to peripheral cannabinoid signalling, although some studies are revealing that, under certain conditions yet unclear, this system may promote nociception and contribute to pain signalling.

Neurotrophin signalling

Neurotrophic factors and their receptors are essential components for the development of the peripheral nervous system (Klein, 1994; Heppenstall & Lewin, 2000; Luo *et al.*, 2007; Valdés-Sánchez *et al.*, 2010). In addition, neurotrophins are pivotally involved in the pathophysiology of human sensory neuropathies (Anand, 2004). They are well known for their sensitizing effect on nociceptor function, which leads to an enhancement of nociceptor excitability (Jankowski & Koerber, 2010). The main neurotrophins involved in the aetiology of chronic pain are NGF, glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), neurturin and artemin (Anand, 2004; Allen & Dawbarn, 2006). These factors act on specific nociceptor subpopulations through specific receptors. For instance, NGF appear to primarily sensitise small and medium peptidergic, isolectin B-negative ($IB4^-$) nociceptors that heavily express the tyrosine kinase A (TrkA) receptor, while GDNF and neurturin appear to act on non-peptidergic $IB4^+$ sensory neurons that largely express the Ret receptor and the GFRα1 and GFRα2 co-receptors (Bespalov & Saarma, 2007; Paratcha & Ledda, 2008). NT-3 binds to the TrkC receptor that is mainly located in large-diameter DRG neurons, and plays a key role in sympathetic neuron survival (Anand, 2004). Furthermore, artemin binds highly selectively to the GFRα3 receptor that co-localises with TRPV1 channels in peptidergic nociceptors (Allen & Dawbarn, 2006).

Nerve-growth factor is perhaps the most studied neurotrophic factor in chronic pain. The presence of this neurotrophin is necessary during nociceptor development for securing the survival of sensory neurons expressing the TrkA receptor (Price *et al.*, 2005; Luo *et al.*, 2007). The role of NGF in pain transduction has been substantiated by: (i) the observation that peripheral injection of NGF in animals and humans induces thermal and mechanical sensitisation (Jankowski & Koerber, 2010; McKelvey *et al.*, 2013); (ii) the high levels of the neurotrophin found in painful conditions such as bone cancer and joint immobilisation (Jimenez-Andrade *et al.*, 2010; Ye *et al.*, 2011; Nishigami *et al.*, 2013); (iii) the anti-nociceptive effect of anti-NGF antibodies and blockers of NGF signalling (Hefti *et al.*, 2006; Hill, 2011; Jimenez-Andrade *et al.*, 2011; Mantyh *et al.*, 2011; Matsuura *et al.*, 2013); (iv) inhibition of NGF degradation induces mechanical allodynia and thermal hyperalgesia (Osikowicz *et al.*, 2013); and (v) the genetic linkage of congenital insensitivity to pain with anhidrosis to mutations in the TrkA receptor (Indo, 2001). In pathological conditions, NGF is released by immune cells and keratinocytes, and sensitises peripheral afferent neurons by activating the TrkA and TrkA/$p75^{NTR}$ signalling pathways. As a consequence, NGF increases the expression of pro-inflammatory neuropeptides calcitonin gene-related peptide alpha (α-CGRP) and substance P in peptidergic sensory neurons, and augments nociceptor excitability by potentiating the activity of the thermoTRP channels TRPV1 and TRPA1, along with the increase in the expression of Nav1.8 channels (Amaya *et al.*, 2004; Price *et al.*, 2005; Zhang *et al.*, 2005; Diogenes *et al.*, 2007; Camprubi-Robles *et al.*, 2009). In addition, NGF provokes the direct and indirect release of other algogens from mast cells and this further enhances nociceptor sensitisation under inflammatory conditions. The signalling pathway underlying the nociceptive effect of NGF involves activation of the MAPK kinase, the PI3K and the PLC cascades, resulting in an increase in thermoTRP expression in the neuronal surface and a stimulation of their channel activity by modulating their interaction with PIP_2, and phosphorylation of the channels by protein kinases (Ji *et al.*, 2002; Zhang *et al.*, 2005; Stein *et al.*, 2006; Zhu & Oxford, 2007; Camprubi-Robles *et al.*, 2009; Ha *et al.*, 2011).

Neurotrophic factors NT-3, GDNF, neurturin and artemin have been associated with nociception in a variety of pain models (Elitt *et al.*, 2006; Malin *et al.*, 2006; McIlwrath *et al.*, 2007; Bogen *et al.*, 2008; Alvarez *et al.*, 2012). Constitutive overexpression of NT-3 in the skin enhances the response of mechanosensory nociceptors that are immunoreactive to TrkC and acid-sensitive ion channel 3 (McIlwrath *et al.*, 2007). Paradoxically, NT-3 has been associated with anti-nociceptive activity in some pain syndromes (Wilson-Gerwing & Verge, 2006; Wilson-Gerwing *et al.*, 2008; Hubbard *et al.*, 2009; Takasu *et al.*, 2011; Tender *et al.*, 2011; Xu *et al.*, 2013). The molecular mechanism underlying this contradictory effect of the neurotrophin is still uncertain, although NT-3 anti-nociception may be primarily through its interaction with TrkC receptors located in medium and large DRG neurons (Tender *et al.*, 2011). Alternatively, a contribution of TrkA receptors to the actions of NT-3 has also been proposed to account for its activity on small diameter nociceptors that do not express the TrkC receptor. These effects include a decrease in the expression of sodium channels Nav1.8 and Nav1.9 and the thermoTRP channel TRPV1 (Wilson-Gerwing *et al.*, 2005, 2008), though the detailed signalling pathway remains elusive.

Glial cell line-derived neurotrophic factor also display opposite effects in nociception that are poorly understood in terms of molecular and cellular mechanisms. GDNF acts primarily on non-peptidergic, $IB4^+$ nociceptors through heterodimers of Ret, GFRα1 and GFRα2, although Ret-independent pathways could be also involved (Sariola & Saarma, 2003; Paratcha & Ledda, 2008; Sakai & Suzuki, 2008). In this regard, increased levels of GDNF in the skin have been shown to lower the mechanical threshold of mechanically sensitive C-fibres, probably by increasing the expression of putative mechanosensitive channels (Albers *et al.*, 2006), thus leading to mechanical hypersensitivity. Alternatively, local administration of GDNF produces thermal hyperalgesia (Malin *et al.*, 2006). This thermosensitive effect has been associated with stimulation of all signalling pathways activated by GDNF in non-peptidergic, $IB4^+$ nociceptors, namely PLCγ, MAPK/ERK, PI3K, cell division kinase 5 and Src (Bogen *et al.*, 2008). GDNF could also contribute to nociception by sensitisation of peptidergic nociceptors through Src signalling (Schmutzler *et al.*, 2009). As for NT-3, GDNF also displays anti-nociception in neuropathic pain models, where intrathecal administration of the neurotrophin eliminated the mechanical allodynia via a somatostaminergic mechanism (Adler *et al.*, 2009), though a detailed molecular mechanism indicating the signalling route is still missing. Nonetheless, it may involve a Ret-independent signalling cascade through the interaction of GFRα1 and GFRα2 with neuronal cell adhesion molecules (NCAMs; Sakai & Suzuki, 2008), and/or heparin sulphate proteoglycan syndecan-3 (Bespalov *et al.*, 2011).

A similar nociceptive mechanism may be used by neurturin, which signals through the Ret–GFRα2 and/or –GFRα1 heterodimer receptor systems (Malin *et al.*, 2006). However, akin to GDNF,

a Ret-independent pathway also appears to be involved for the induction of nociceptor sensitisation (Schmutzler *et al.*, 2011). Ret-independent neurturin signalling in small-diameter sensory neurons involves the interaction of GFRα1,2 receptors with NCAM and/or integrins that lead to the activation of the PI3K pathway which, in turn, produces nociceptor sensitisation acting on thermoTRPs (Schmutzler *et al.*, 2011).

Artemin is another member of the GDNF family of ligands. This neurotrophic factor modulates sensory neurons through GFRα3 receptors. As mentioned, these receptors are widely expressed in peptidergic and non-peptidergic nociceptors, including a subset of neurons distinct from GDNF-responsive neurons. An interesting observation is that GFRα3 receptors largely co-localise (99%) with TRPV1 channels (Orozco *et al.*, 2001). The *in vivo* activity of artemin has been mainly associated with nociceptive actions (Elitt *et al.*, 2006; Malin *et al.*, 2006; Ceyhan *et al.*, 2010; Murota *et al.*, 2012; Lippoldt *et al.*, 2013; Thornton *et al.*, 2013). Akin to other neurotrophins, artemin induces thermal hyperalgesia *in vivo* and *in vitro* by sensitizing thermoTRP receptors (Elitt *et al.*, 2006, 2008; Malin *et al.*, 2006; Lippoldt *et al.*, 2013), most likely through Ret-dependent and -independent mechanisms. Indeed, in peptidergic nociceptors Ret-dependent signalling appears to activate Src kinase leading to enhanced stimulation of capsaicin-evoked α-CGRP release (Schmutzler *et al.*, 2011). Alternatively, Ret-independent signalling appears mediated by NCAM/GFRα3 through activation of Fyn kinase, which sensitises small-diameter sensory neurons by potentiating TRPV1 activity (Bespalov *et al.*, 2011; Schmutzler *et al.*, 2011). Additionally, artemin has also been shown to display anti-nociceptive activity in neuropathic pain models (Gardell *et al.*, 2003; Asano *et al.*, 2006; Yoshida *et al.*, 2011). It has been reported that artemin may produce anti-nociception, at least in part by attenuating the channel activity of the TRPA1 channel (Yoshida *et al.*, 2011). The molecular mechanism implicated in this contrasting activity is under scrutiny, and will probably also involve Ret-dependent and -independent activities of this neurotrophic factor in different subpopulations of primary sensory neurons. All these findings further illustrate the complex role of neurotrophic factors in pain signalling and emphasise a tissue-, cell- and modality-dependent sensory activity that encompasses from the development and survival of nociceptors to their phenotypic regulation in pain syndromes.

The thermoTRP–cannabinoid–neurotrophin threesome in pain signalling

From the above sections, it can be readily concluded that the thermoTRP, endocannabinoid and neurotrophin systems are pivotal transducers of pain critically implicated in the aetiology of several painful syndromes. Apparently, neurotrophins such as NGF are major drivers of thermoTRP sensitisation, which leads to pain transduction, while agonists of peripheral CB1 receptors act as inhibitors of NGF-induced thermoTRP sensitization (McDowell *et al.*, 2013). However, we have shown that in this system things are not as simple as they seem, and that the interaction of this threesome in pain signalling is more complex than previously imagined. Neurotrophin and cannabinoid systems appear to have a dual opposing activity on thermoTRP receptors, acting as sensitisers or desensitisers depending on the cellular and molecular context, and the intensity of their signalling. In this regard, the wide cellular distribution in the highly heterogeneous population of nociceptors adds more complexity to their participation in pain signalling.

At a molecular level, it is remarkable that CB and neurotrophin receptors share quite a few of intracellular signalling routes that converge onto thermoTRPs, particularly onto TRPV1, which is the most studied thermosensory channel. These signalling pathways include the PLC, MAPK and PI3K and they lead to TRPV1 potentiation (Fig. 2), and yet cannabinoid agonists, including endocannabinoids, mostly display anti-nociceptive activity under inflammatory conditions (Agarwal *et al.*, 2007; Walczak & Cervero, 2011). The analgesic activity of CB1 receptors has been primarily linked to the inhibition of the PKA pathway which potentiates TRPV1 channels by direct phosphorylation (Hermann *et al.*, 2003; Jeske *et al.*, 2008) and by activation of calcineurin, which promotes TRPV1 desensitisation and tachyphylaxia (Patwardhan *et al.*, 2006). A TRPA1-mediated mechanism for cannabinoid-induced desensitisation of TRPV1 channel activity has also been described (Akopian *et al.*, 2008). However, cannabinoid agonists may also activate the sensitizing routes leading to potentiation of TRPV1 channels. Indeed, it has been proposed that CB1 receptors may be tonically active in nociceptors, presumably due to the presence of endocannabinoids, thus contributing to pain sensitisation (Fioravanti *et al.*, 2008). Blockade of these constitutively active CB1 receptors leads to anti-nociception. Although the molecular mechanisms involved in CB1-induced sensitisation of TRPV1 are as yet unknown, it is reasonable to assume that they probably involve the activation of PLC, MAPK and/or PI3K pathways (Hermann *et al.*, 2003). Interestingly, chronic exposure of sensory neurons in culture to increased levels of NGF alters the CB1-mediated modulation of TRPV1 (Evans *et al.*, 2007). This study found that CB1 agonists blocked capsaicin-stimulated release of pro-inflammatory peptides in sensory neurons chronically exposed to a low NGF concentration, while significantly potentiating their vanilloid-induced secretion in nociceptors exposed to a high concentration of the neurotrophin. This result further suggests the existence of a significant crosstalk between the CB1 and TrkA or TrkA/p57^{NTR} to modulate TRPV1 channel activity, and probably other thermoTRPs, under inflammatory conditions, and imply that disrupting this interaction in peripheral terminals may lead to novel therapeutic approaches.

A question that emerges from this puzzle of signalling networks is how does this threesome really work in generating and preventing pain transduction? Thus far, we have commented it on rather fragmented information derived from different studies that have been carried out in dissimilar experimental conditions and have primarily focused on one signalling route in a subpopulation of nociceptors. However, we cannot neglect a critical contribution of the different subpopulations of nociceptors as the final response will depend on the balance of intracellularly activated signalling cascades in the diverse population of targeted sensory neurons by environmental pro- and anti-inflammatory cues. Consequently, it follows that a complex communication network such as this threesome requires analysis in a system-based strategy that allows monitoring of the contributions of all implicated signalling pathways, along with the specific populations of nociceptors (Andres *et al.*, 2010, 2012). A systems biology approach that favours 'looking at the forest instead of centring all the efforts in individual trees' will undoubtedly shed light on the dynamics of complex networks such as neurotrophins–cannabinoids–thermoTRPs under inflammatory conditions. Indeed, Andres *et al.* (2010) have successfully used quantitative automated microscopy to analyse the responses of nociceptors in culture to NGF. This methodology is based on monitoring the activation of intracellular signalling cascades such as PKA, ERK1/2 or CREB activation by different environmental signals that activate distinct receptors. Data collected from these measurements are related to nociceptor populations, and used to build models of signalling. For instance, a seminal study found that larger DRG neurons respond

more strongly to NGF stimulation than do smaller neurons (Andres *et al.*, 2012). Although still in its infancy, it can be anticipated that a systems-based approach will render fundamental information on the dynamics and interplay of inter- and intracellular signalling in pain transduction and chronification.

Outlook

Here, we have attempted to briefly expose how the complex and intricate signalling networks of neurotrophins and endocannabinoids may communicate to modulate thermoTRP activity in nociceptors and lead to their sensitisation or desensitisation. This crosstalk appears to rely on the activation of a limited set of intracellular, interconnected signalling pathways that convey the information to the thermoTRPs and other sensory channels. The specific mechanisms defining the final nociceptor response evoked by neurotrophins and endocannabinoids is as yet elusive, although continuous advances in this field, along with the incorporation of 'pain systems' approaches, will provide pivotal information on the dynamics and intercommunication of these networks favouring the design of better pain therapies. There is no doubt that the threesome consisting of neurotrophins–cannabinoid–thermoTRPs is a pivotal network in pain transduction, but we should not forget the implication of other sensory components that will also influence and will be affected by the dynamics of this triad. Surely, we are looking at a very complex constellation of inter- and intracellular network interactions which underlie modality-specific pain transduction.

Conflict of interest statement

Authors declare no conflict of interest.

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Abbreviations

CB, cannabinoid (receptor); DRG, dorsal root ganglion; GDNF, glial cell line-derived neurotrophic factor; MAPK, mitogen-activated protein kinase; NCAM, neuronal cell adhesion molecule; NGF, nerve-growth factor; NT-3, neurotrophin-3; PI3K, phosphatidylinositide-3 kinase; PKA, protein kinase A; TrkA, tyrosine kinase A; TRP, transient receptor potential; TRPA1, TRP ankyrin; TRPV1, TRP vanilloid.

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