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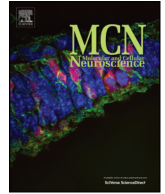

The importance of TRPV1-sensitisation factors for the development of neuropathic pain

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ABSTRACT

Transient receptor potential vanilloid type 1 (TRPV1), classically associated with transduction of high-temperature and low-pH pain, underlies pain hypersensitivity in neuropathic pain. The molecular regulation of TRPV1 channel activity is not yet fully understood. Therefore, we investigated factors regulating sensitisation of this receptor during development of neuropathic pain in a rat model of chronic construction injury (CCI) in the dorsal root ganglia (DRG).

In the rat CCI model, elevated levels of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) in DRG corresponded to development of neuropathic pain. We assessed the expression of known kinases influencing TRPV1 sensitisation at the mRNA and/or protein level. Protein kinase C ε (PKCε) showed the strongest upregulation at the mRNA and protein levels among all tested kinases. Co-expression of PKCε and TRPV1 in L5 DRG of CCI animals was high during the development of neuropathic pain. The number of neurons expressing PKCε increased throughout the experiment.

We provide complex data on the expression of a variety of factors involved in TRPV1 sensitisation in a CCI model of neuropathic pain. Our study supports evidence for involvement of TRPV1 in the development of neuropathic pain, by showing increased expression of interleukins and kinases responsible for the channel sensitisation. TNFα and NGF seem to play a role in the transition from acute to neuropathic pain, while PKCε in its maintenance. Further studies might confirm their significance as novel targets for the treatment of neuropathic pain.

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1. Background

The increasing knowledge on the molecular biology of transient receptor potential vanilloid type 1 (TRPV1), a non-selective cation channel, highlights its important role as a noxious signal integrator. It is expressed in both the peripheral and the central nervous system (CNS) and is involved in pain transduction, transmission, perception and modulation (Caterina et al., 2000). Dorsal root ganglia (DRG) contain TRPV1-expressing neurons (Hwang et al., 2005) that convey sensory information to the CNS. Activation of TRPV1, by external stimuli or endogenous agonists like anandamide (AEA), produces inward cation current, which may be involved in the sensitisation of nociceptive DRG neurons that causes hyperalgesia (Jara-Oseguera et al., 2008; Starowicz et al., 2008). Under pathological conditions, TRPV1 expression in nociceptive DRG neurons is altered in various models of peripheral neuropathy (Biggs et al., 2007; Hudson et al., 2001; Kim et al., 2008; Rashid et al., 2003). The association of TRPV1 expression level with pain perception remains unclear, so the importance of channel sensitisation, manifested by the reduced activation threshold was postulated (reviewed in Palazzo et al., 2012).

A number of modulators regulate TRPV1 activity and promote inflammatory or painful responses (Fig. 1). TRPV1 sensitisation is associated with phosphorylation controlled by at least four serine/threonine kinases, including protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent kinase 2 (CaMK 2), and cyclin-dependent kinase 5 (Cdk5) (reviewed in Suh and Oh, 2005). Among these, PKA- and PKC-mediated phosphorylation has been widely investigated in various pain models (reviewed in Palazzo et al., 2012). Ahern and Premkumar (2002) reported that activation of PKC strongly sensitises the nociceptive response, which can be attenuated by both PKC inhibitors and mutations in TRPV1 phosphorylation sites. Particularly, PKCε modulation of TRPV1 activity plays a critical role in pain perception (Pan et al., 2010; Vellani et al., 2010; Ferrari et al., 2014). In contrast, the neuronal phosphatase calcineurin plays a major role in dephosphorylation of TRPV1 and is primarily responsible for the decrease in channel activity (Por et al., 2010). Moreover, as demonstrated by Ji et al. (2002), p38 mitogen-activated protein kinase (P38-MAPK) activation, initiated by retrograde transport of nerve growth factor (NGF), acts to increase translocation of TRPV1 to the nociceptor membrane, thereby contributing to the maintenance of hypersensitivity. Indeed, the contribution of P38-MAPK to the progression of neuropathic pain has been demonstrated in preclinical models

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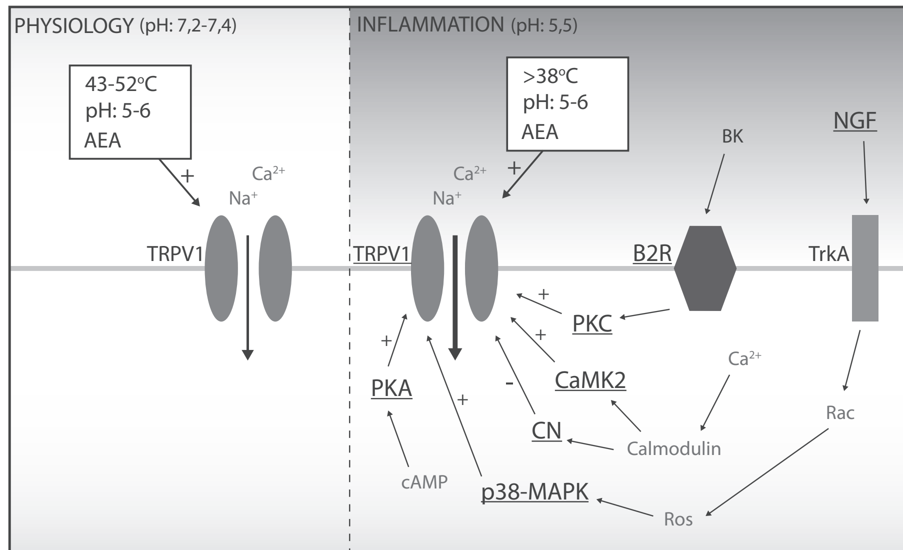

Fig. 1. Sensitization and activation of TRPV1 receptor. Under physiological conditions TRPV1 is activated by high temperature, low pH and endogenous AEA, leading to cation influx. During inflammation TRPV1 undergoes sensitization by numerous kinases, which may contribute to neuropathic pain development. Moreover inflammation lowers temperature threshold of TRPV1 activation. Underlined elements of TRPV1 sensitization pathways were investigated in present studies. AEA — anandamide; B2R — bradykinin receptor 2; BK — bradykinin; CaMK2 — Ca2+/calmodulin dependent kinase 2; CN — calcyneurin; NGF — nerve growth factor; p38-MAPK — p38 mitogen-activated protein kinases; PKA — protein kinase A; PKC — protein kinase C; Rac — subfamily of the Rho family of GTPases; Ros — reactive oxygen species; cAMP — cyclic adenosine monophosphate; TrkA — high affinity nerve growth factor receptor; TRPV1 — transient receptor potential vanilloid type 1.

(Jin et al., 2003; Schäfers et al., 2003b). TRPV1-evoked reactions are markedly enhanced by pro-inflammatory mediators, such as TNFα, IL-1β, IL-6 and bradykinin, all of which produce hypersensitivity to heat in vivo. Additionally, some of those factors activate PKA and PKC in primary sensory neurons (review in Ren and Dubner, 2010) and increase the efficacy of AEA to produce TRPV1-mediated excitation (Singh Tahim et al., 2005).

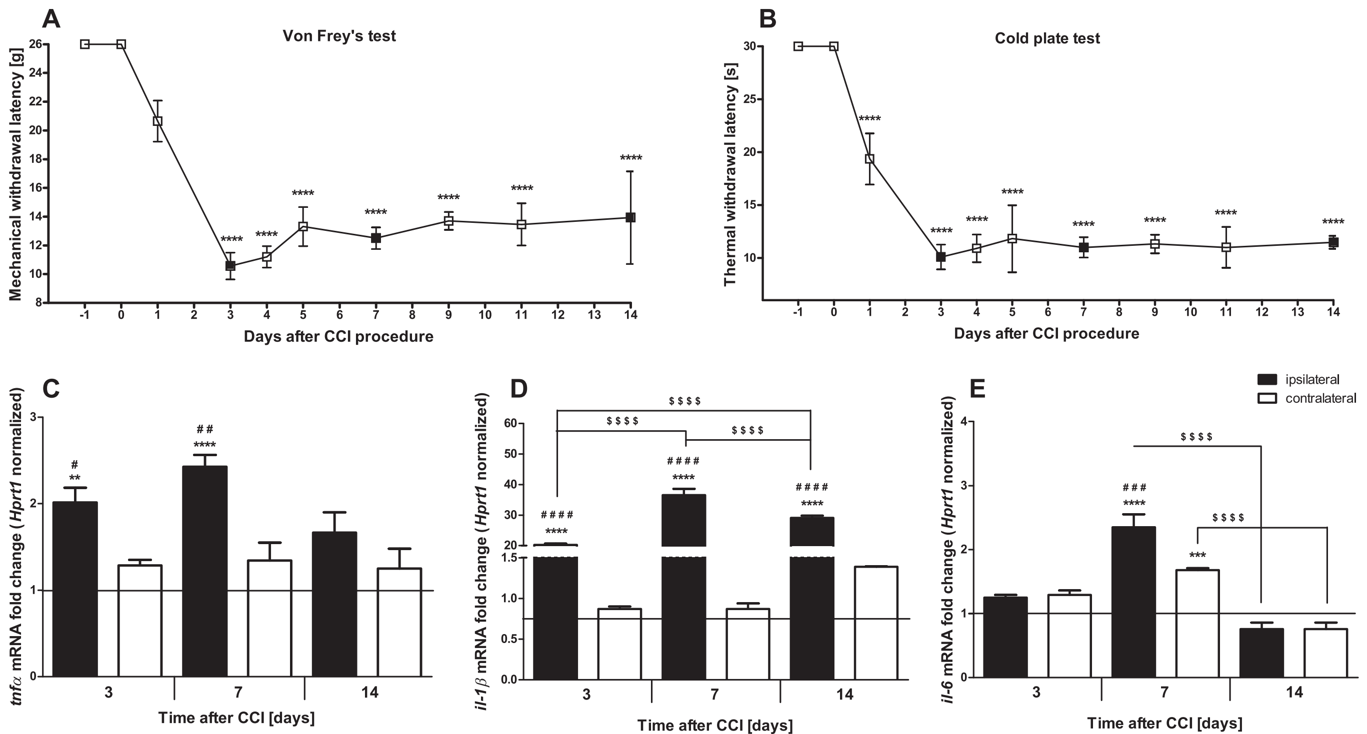

Fig. 2. Development of mechanical (A) and thermal (B) allodynia after sciatic nerve injury. Expression of *Tnf-α* (C), *Il-1β* (D) and *Il-6* (E) mRNA in the L4–L6 dorsal root ganglia 3, 7 and 14 days after CCI. Behavioural studies were performed before (−1, 0) and after CCI procedure (1–14). Mechanical allodynia (A) was measured as withdrawal latency in grammes and thermal allodynia (B) as thermal withdrawal latency in seconds. mRNA samples were collected 3, 7 and 14 days after CCI procedure (marked as ■ on behavioural graphs). Results were presented as fold change normalized to expression of reference gene *Hprt1* and were calculated by intact — on graphs presented as a line. Data are presented as mean ± SEM and represent normalized averages derived from 1012 animals for behavioural studies and 6–8 samples per each group for mRNA analysis. Statistical analysis of changes was performed using one-way ANOVA followed by Bonferroni post-hoc-test, values with $p < 0.05$ were considered significant. * denotes significant differences vs. intact, # vs. contralateral side, ^ vs. pre-operative values and $ vs. indicated bar.

Although the role of TRPV1 in neuropathic pain is well established, regulation of channel activity during neuropathic pain development remains unclear. Here we investigated factors regulating TRPV1 sensitisation in the rat model of chronic construction injury (CCI). We correlated changes in expression of TNFα, IL-1β, and IL-6 with the development of allodynia. Furthermore, we measured the expression of kinases influencing TRPV1 sensitisation at the mRNA and protein levels. Finally, we examined the co-localisation of PKCε and TRPV1 in nociceptive DRG neurons.

2. Results

2.1. Development of pain phenotype is accompanied with enhanced transcription of interleukins in DRG of rats who undergo CCI procedure

Pre-surgery thresholds reached the cut-off values for both mechanical and cold allodynia. Neuropathic animals showed significantly lower mechanical withdrawal latency starting at day 3 after the CCI procedure (Fig. 2A). Cold allodynia had developed at day 1 after sciatic nerve injury and progressed with time (Fig. 2B). Cut-off values were reached for intact animals at all tested time points (data not shown; for details see Malek et al., 2014). Behavioural changes were accompanied by elevated mRNA expression of *Tnfα*, *Il-1β* and *Il-6*. *Tnfα* showed significantly increased expression 3 and 7 days after CCI (approx. 2-fold change, Fig. 2C), exclusively ipsilateral. *Il-1β* transcript was upregulated ipsilateral to the injury at all tested time points (approx. 20-fold change, Fig. 2D). The level of *Il-1β* transcript on the contralateral side did not significantly change during the development of neuropathic pain. *Il-6* transcript increased only at day 7 after sciatic nerve injury (approx. 2.5 and 2-fold change ipsilateral and contralateral to the injury, respectively, Fig. 2E).

2.2. Upregulation of kinases involved in TRPV1 sensitisation as a consequence of neuropathic pain development

P38-mapk, *Camk2*, *Pka* and *Pkc* transcripts were detected in DRG dissected both from intact and CCI animals. At day 3 after CCI, significant upregulation of *P38-mapk* was observed ipsilateral to the injury (1.94 ± 0.12). Increased *P38-mapk* transcript persisted at day 7 on both the ipsilateral (5.01 ± 0.01) and contralateral sides (3.33 ± 0.30), but lateralisation was maintained (Fig. 3A). At day 14 after CCI, *P38-mapk* expression returned to that of intact animals. *Camk2* transcript was significantly elevated at days 3 and 7 after sciatic nerve injury (3.20 ± 0.32 and 3.34 ± 0.08, respectively) ipsilateral to the injury, as was *Camk2* contralateral to the injury on day 7 (2.56 ± 0.18) (Fig. 3B). *Pka* and *Pkc* expression was significantly upregulated 7 days after induction of neuropathic pain, but only for *Pkc* lateralisation was observed (1.63 ± 0.07 and 1.69 ± 0.11 for *Pka* and 6.70 ± 0.17 and 3.27 ± 0.41 for *Pkc*, ipsilateral and contralateral, respectively, Fig. 3C–D).

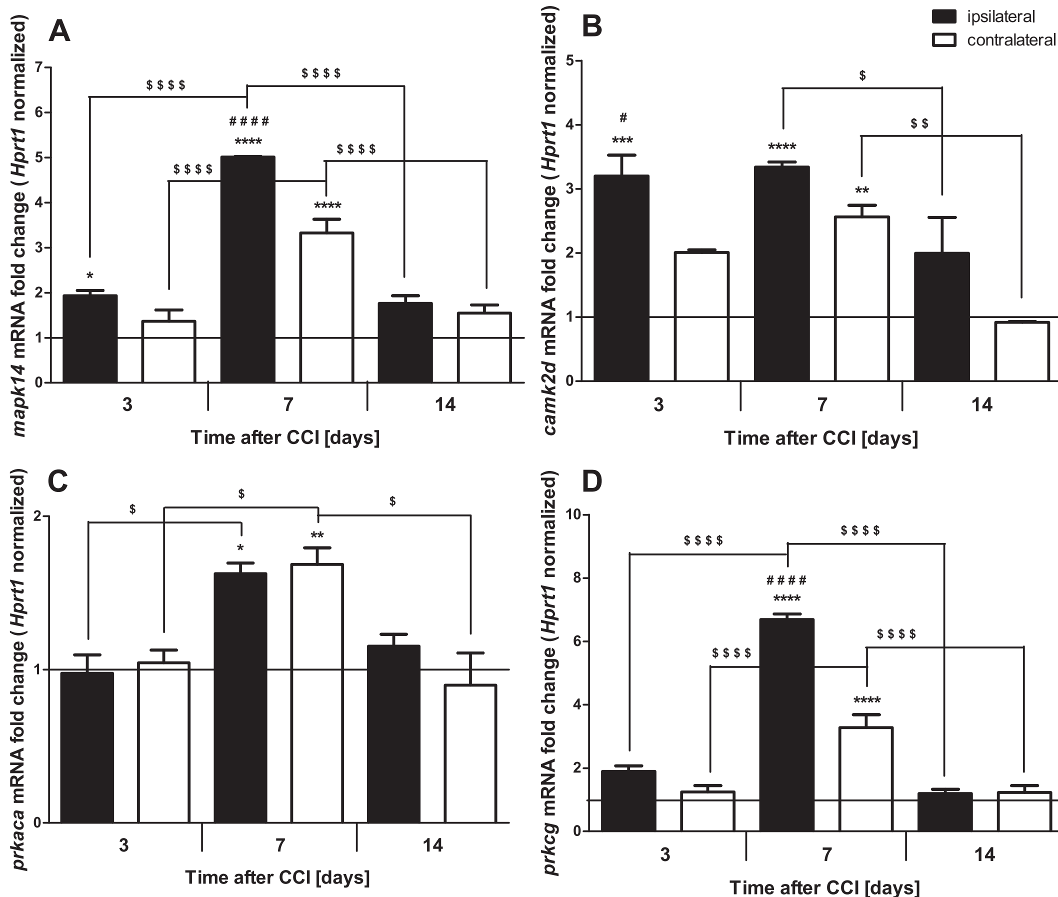

Fig. 3. Results of qPCR analysis of *Mapk14* (A), *Camk2d* (B), *Prkaca* (C) and *Prkc* (D) gene expression levels in the L4–L6 dorsal root ganglia during the development of neuropathic pain in CCI rats. Samples were collected 3, 7 and 14 days after CCI procedure. Data are presented as mean ± SEM and represent normalized averages derived from 6 to 8 samples per each group. Results are presented as fold change normalized to expression of reference gene *Hprt1* and were calculated by intact — on graphs presented as a line. Statistical analysis of changes was performed using one-way ANOVA followed by Bonferroni post-hoc-test, values with $p < 0.05$ were considered significant. * denotes significant differences vs. intact, # vs. contralateral side and $ vs. indicated bar.

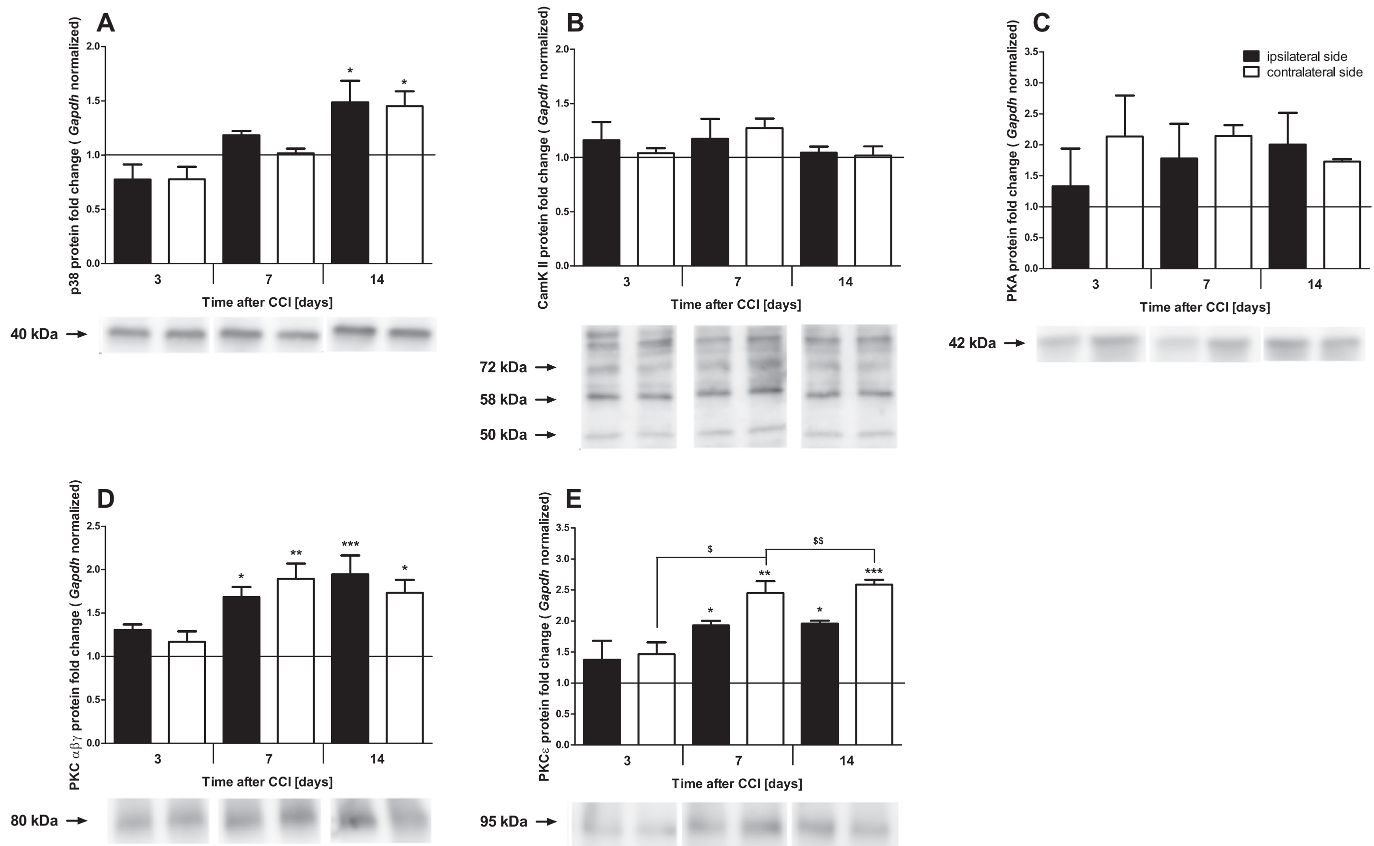

Fig. 4. Results of Western blot analysis of p38 (A), CaMKII (B), PKA (C), PKCαβγ (D) and PKCε (E) in L4–L6 dorsal root ganglia during the development of neuropathic pain in CCI rats. Samples were collected 3, 7 and 14 days after CCI procedure. Data are presented as mean ± SEM and represent normalized averages derived from 4 to 6 samples per each group. Results are presented as fold change normalized to expression of reference protein GAPDH and were calculated by intact — on graphs presented as a line. Each graph is accompanied by picture of membrane with group representative. Statistical analysis of changes was performed using one-way ANOVA followed by Bonferroni post-hoc-test, values with $p < 0.05$ were considered significant. * denotes significant differences vs. intact, # vs. contralateral side and $ vs. indicated bar.

2.3. Isoforms of PKC show strongest upregulation in DRG after sciatic nerve injury at protein level

Western blot analysis showed significantly elevated P38-MAPK both ipsilaterally and contralaterally 14 days after CCI (1.77 ± 0.17 and 1.55 ± 0.18, respectively, Fig. 4A). CaMK2 and PKA showed no significant alterations in L4–L5 DRG of rats in the model of neuropathic pain (Fig. 4B–C). PKCαβγ showed significant upregulation without lateralisation on day 7 (1.68 ± 0.12, 1.89 ± 0.18 ipsi- and contralateral, respectively) and day 14 (1.95 ± 0.22, 1.73 ± 0.15 ipsi- and contralateral, respectively) after sciatic nerve injury (Fig. 4D). The same pattern of expression was observed for the ε isoform of PKC (Fig. 4E).

2.4. No changes in co-localisation of TRPV1 and PKCε in rat L5 DRG after CCI, although the population of TRPV1$^-$/PKCε$^+$ neurons increases after sciatic nerve injury

Immunohistochemical localisation of TRPV1 and PKCε in rat L5 DRG was determined by immunofluorescence 3, 7 and 14 days after CCI surgery (Figs. 5 and 6). Only NeuN-positive cells that exhibited expression of at least one protein of interest (TRPV1 or/and PKCε) were analysed (Fig. 5A1–D1). In accordance with the results obtained by single staining, we identified a large number of TRPV1-positive (Fig. 5B1–B2) and PKCε-positive cells (Fig. 5C1–C2) in the DRG at all examined time points regardless of the presence of sciatic nerve injury. The staining profile of DRG on the contralateral side was similar (data not shown). By double immunofluorescence, we found that TRPV1 receptor and PKCε were co-localised in a high percentage (approx. 80%) of analysed cells, irrespective of the side, at all examined time points (Fig. 6A). TRPV1$^+$/PKCε$^-$ neurons reached 15% of examined DRG neurons, although significant elevation of TRPV1$^+$/PKCε$^-$ cell number was observed ipsilateral to the injury at day 3 after CCI in comparison to day 7 and day 14 after sciatic nerve injury (Fig. 6B). The population of TRPV1$^-$/PKCε$^+$ neurons was significantly increased at day 3 and day 14 after CCI ipsilateral and contralateral to the injury and at day 7 only ipsilateral, in comparison to the DRG of intact animals (Fig. 6C).

3. Discussion

TRPV1 is an important contributor to pain, although its role in the development of neuropathic pain is more complex than initially thought, as even TRPV1 knock-out mice develop the behavioural signs of hypersensitivity to noxious stimuli (Caterina et al., 2000; Davis et al., 2000). TRPV1 functions as an integrator of pain signals in nociceptive primary afferents, which in the presence of nerve injury display enhanced excitation. It is believed to be the major cause of neuropathic pain (Sukhotinsky et al., 2004). In these studies we identified factors responsible for TRPV1 sensitisation during development of pain in a model of sciatic nerve injury. Our studies performed at 3, 7 and 14 days after CCI procedure are illustrating gradual development of neuropathic pain. As molecular changes at day 3 might be still affected by performed procedure itself, we rather focus on days 7 and 14 as representatives of developed neuropathic pain.

TRPV1 expression in nociceptive DRG neurons has been widely studied and displays altered expression under pathological conditions in some animal models of inflammatory or neuropathic pain. Depending

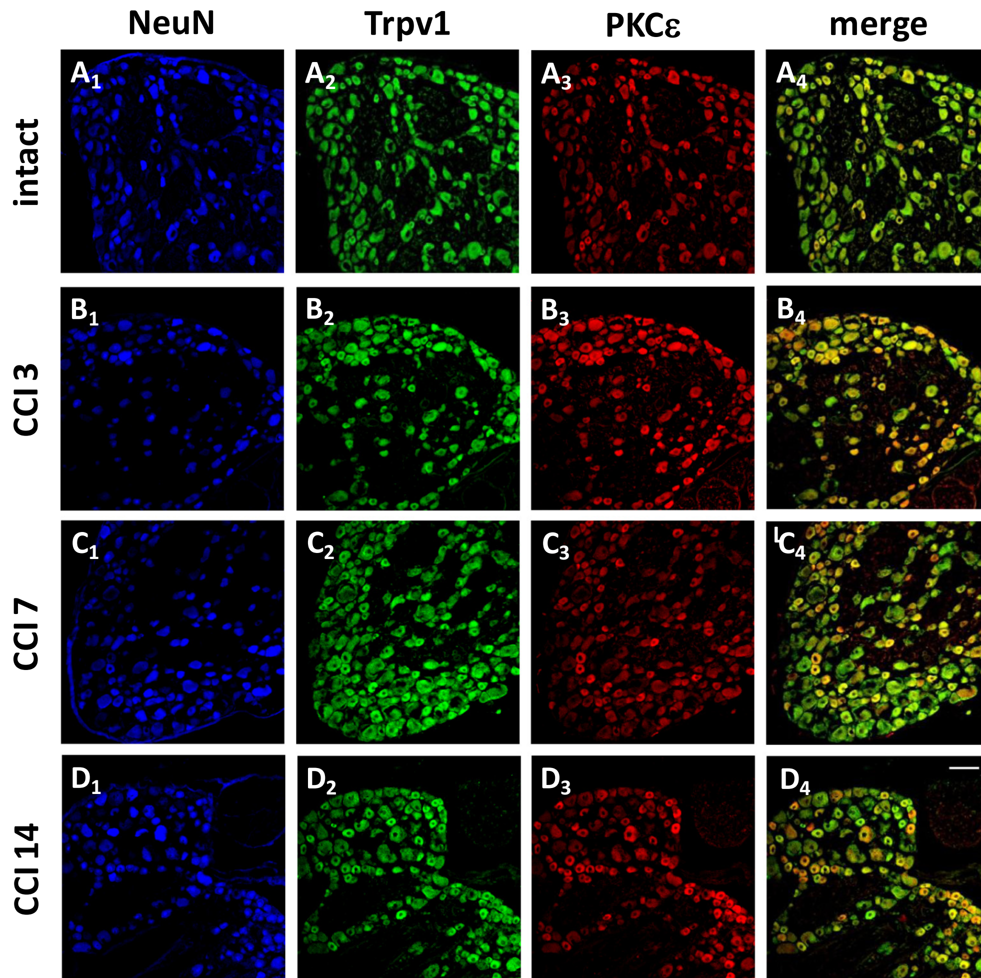

Fig. 5. Photomicrographs of L5 DRG immunolabelled for NeuN (A_1–D_1), TRPV1 receptor (A_2–D_2) and PKCε (A_3–D_3) in the rat model of neuropathic pain. Samples were collected 3, 7 and 14 days after CCI. Micrographs represents control (A_1–A_4) and neuropathic rat L5 DRG section at 3 (B_1–B_4), 7 (C_1–C_4) and 14 (D_1–D_4) days after CCI. Last panel (A_4–D_4) shows merged images for TRPV1 (green) and PKCε (red) immunoreactivity. Scale bar = 100 μm, applies to all photos. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on the model and strain of animal used, TRPV1 expression in nociceptive DRG neurons was upregulated at the mRNA or protein level when its total expression level was measured (Ji et al., 2002; Wu et al., 2013), or it tends to decrease (Rasband et al., 2001; Schäfers et al., 2003a). Even the genotype of the animal strain affects the regulation of TRPV1 (Persson et al., 2009). Wistar rats, which show no alteration in TRPV1 expression in pulled samples of L4–L6 DRG in the CCI model of neuropathic pain (Malek et al., 2014), were chosen for the present study. In this model we additionally performed immunofluorescence staining for TRPV1 protein in L5 DRG. We observed a time-dependent decrease in the number of TRPV1-expressing neurons after injury of the sciatic nerve (Supplementary Fig. A). This result is consistent with

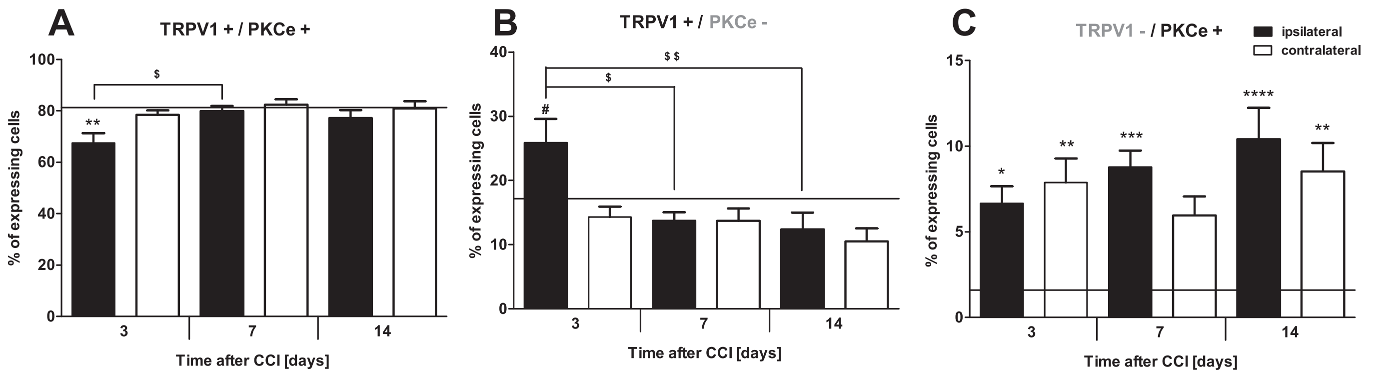

Fig. 6. Immunohistochemical localization of TRPV1 and PKCε in rat L5 DRGs neurons at different time points after CCI. Samples were collected 3, 7 and 14 days after CCI procedure. Data are presented as mean ± SEM. Results are presented as % of analysed cells. Expression in intact is presented as a line on graphs. Each column represents average of 8–12 areas counted by 2 observers blinded to the study. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc-test, values with $p < 0.05$ were considered significant. * denotes significant differences vs. intact, $^{\#}$ vs. contralateral side and $^{\$}$ vs. indicated bar.

reports showing that an increase in TRPV1 protein level in undamaged DRG is accompanied by a decrease in DRG effected by nerve injury (Fukuoka et al., 2002; Hudson et al., 2001). In addition, downregulation of TRPV1 mRNA has previously been reported in the L5 ganglion in axotomy model of neuropathic pain (Michael and Priestley, 1999).

Regardless of increased or decreased TRPV1 expression in nociceptive DRG neurons, behavioural signs of developing neuropathic pain have been present in all studies, so upregulated function and sensitisation of TRPV1 is believed to mediate the development of both inflammatory and neuropathic pain (Patapoutian et al., 2009). In the present study we evaluated pain behaviour in the Von Frey's test and the cold plate test, which measure development of mechanical and thermal allodynia (Fig. 2A, B), common symptoms of neuropathic pain (reviewed in Starowicz et al., 2013b). At the same time, we determined that mechanical and thermal allodynia in neuropathic animals is accompanied by elevated mRNA expression of the proinflammatory cytokines TNF-α, IL-1β and IL-6 (Fig. 2C–E), confirming ongoing inflammation in the DRG after CCI. Nerve injury is almost always associated with a strong immune response, although the literature focuses on the consequences of neuronal damage. The association of mechanical and thermal hypersensitivity with an upregulation of proinflammatory cytokines in DRG neurons is consistent with studies on different models of pain (Cunha et al., 2000; Samad et al., 2001).

IL-1β showed elevated expression at all tested time points, which might suggest its involvement in the development and maintenance of neuropathic pain (Fig. 2). IL-1β is involved in TRPV1 receptor sensitisation (Obreja et al., 2002; Piper et al., 1999), so its elevated expression may be involved in the transition from acute to chronic pain and maintaining the pain through a peripheral mechanism in the model of CCI. Unlike IL-1β, TNFα transcript was elevated only at the beginning of neuropathic pain development (Fig. 2). Upregulation of TNFα transcription in DRG neurons is in the line with studies by Jancálek et al. (2010) in experimental models of peripheral neuropathy. Furthermore, TRPV1 receptor sensitivity to endogenous and exogenous ligands may be modulated by TNFα pretreatment, resulting in increased nociceptive signal transmission (Khan et al., 2008; Spicarova and Palecek, 2009). Therefore, TNFα might be one of the factors responsible for enhanced TRPV1 sensibility during the transition from acute to neuropathic pain, although additional experiments are needed to explain the specific mechanisms involved. Expression of IL-6 mRNA was elevated only after the development of the pain phenotype, which might be a consequence of TRPV1 action, as TRPV1 mediates the increase in IL-6 production, not vice versa (Geppetti et al., 2008; Lin et al., 2007; Terenzi et al., 2013). The underlying mechanisms of this process need further study. Additionally, the ongoing inflammation can reduce the pH of the extracellular environment, which activates and sensitises TRPV1 (Jones et al., 2004; Tominaga et al., 1998), and the threshold temperature is lowered when receptor is phosphorylated (Sugiura et al., 2002; Vellani et al., 2001).

Sensitisation of TRPV1 receptor by proinflammatory agents involves PKA- and PKC-dependent phosphorylation (reviewed in Palazzo et al., 2012; Suh and Oh, 2005). Our data show that mRNA expression of both kinases was significantly elevated on day 7 after CCI (Fig. 3C,D), but only PKC showed upregulation at the protein level 7 and 14 days after CCI (Fig. 4D,E). These data suggest greater involvement of PKC-mediated phosphorylation, especially in maintaining of neuropathic pain in CCI model, rather than a transition from an acute to a chronic pain state. Supportive of this hypothesis are studies showing that PKC is particularly involved in later-stage inflammation rather than early (Bonnington and McNaughton, 2003). Other studies have shown reduced neuropathic pain symptoms in mice lacking one of the PKC isoforms (γ or ε), supporting a role for PKC in sustaining chronic pain (Khasar et al., 1999; Malmberg et al., 1997). Although we showed upregulation of different isoforms of PKC (αβγ and ε), we focused on the ε isoform based on previous reports showing co-localisation of this form with TRPV1 receptor in DRG during inflammation (Zhou et al., 2003). Additionally, while PKCε-mediated activation of TRPV1 is involved in pain development (Pan et al., 2010; Vellani et al., 2010), it was never studied in a model of peripheral neuropathy. Our immunofluorescence data show similar results for a neuropathic pain model, where high co-localisation of PKCε and TRPV1 was present (Figs. 5 and 6). Moreover, we confirmed an elevated number of neurons expressing PKCε in DRG at all tested time points, which further supports the hypothesis of its involvement in neuropathic pain maintenance. Additionally, as PKCε is responsible for bradykinin-induced TRPV1 sensitisation to heat (Numazaki et al., 2002; Spicarova and Palecek, 2009), we analysed bradykinin B1 (BDKRB1) and B2 (BDKRB2) receptors at the mRNA level in DRG neurons and observed elevated transcription of BDKRB2, but not BDKRB1 (Supplementary Fig. B). Because activation of bradykinin BDKRB2 receptor promotes the formation of lipoxygenase metabolites (Petcu et al., 2008), which act as agonists of TRPV1 (Starowicz et al., 2013a), we believe that the above result might imply a contribution of bradykinin to development of pain in this CCI model of neuropathy.

In addition to PKA and PKC, other kinases modulate TRPV1 channel activity (reviewed in Palazzo et al., 2012). Among them, CaMK2 alters TRPV1 function (Jung et al., 2004; Rosenbaum et al., 2004). Although upregulation of CaMK2 mRNA occurred as early as day 3 after CCI (Fig. 3B), there was no alteration at the protein level (Fig. 4B). Therefore, we concluded that the action of this particular kinase might not be involved in the development of hyperalgesia in the CCI model of neuropathic pain. Similarly, no changes were observed in the mRNA levels of Cdk5 and calcineurin, so we excluded these kinases from further analysis (Supplementary Fig. C). Phosphorylation not only sensitises TRPV1 but also promotes translocation of the receptor to the plasma membrane. PKC- (Morenilla-Palao et al., 2004) and p38-MAPK-mediated mechanisms are responsible for this effect (Ji et al., 2002). We showed delayed activation of p38-MAPK transcription (Fig. 3A) and protein expression (Fig. 4A) in DRG neurons after CCI, so it is likely that p38-MAPK expression is influenced by nerve inflammation and degeneration rather than injury itself. Moreover, NGF mRNA was significantly upregulated at day 3 after CCI (Supplementary Fig. B), which is consistent with previous data (Herzberg et al., 1997) and also indicates the involvement of this molecule in the transition from acute to chronic pain. Our data show the complexity of networks involved in TRPV1 expression, sensitisation and activation during neuropathic pain development. Although changes in mRNA and protein expression levels in pulled DRG might provide valuable information on the functions of genes of interest, studies on various subpopulations of DRG neurons would provide additional data.

4. Conclusions

We provide complex data on the expression of a variety of factors involved in TRPV1 sensitisation in a CCI model of neuropathic pain. Although the number of TRPV1-expressing neurons decreases with time, the expression of TRPV1 sensitisation factors is elevated, allowing for development of pain behaviour. We showed that maintenance of TRPV1 sensitisation in this CCI model of neuropathic pain is mainly due to PKCε-mediated phosphorylation. Moreover, in a time course study, we identified factors (TNFα and NGF) that might be responsible for the acute-to-chronic transition and that might be responsible for maintenance of pain, thus suggesting new targets for the therapy of neuropathic pain.

5. Methods

5.1. Animals

Male Wistar rats (Charles River, Hamburg, Germany), initially weighing 225–250 g, were used for all experiments. Animals were housed five per cage under a standard 12 h/12 h light/dark cycle (lights

on at 06:00 h) with food and water available ad libitum. All experiments followed the recommendations of the International Association of Studies on Pain (Zimmermann, 1983) and were approved by the Local Bioethics Committee of the Institute of Pharmacology (Cracow, Poland). Results obtained in our research group (Osikowicz et al., 2008) as well as those reported by others (Paszcuk et al., 2011) showed no significant differences between sham operated group and intact animals in allodynia and hyperalgesia thresholds in development of neuropathic pain. Moreover Paszcuk et al. reported no significant differences in expression of EC system components in sham vs. intact animals. Therefore, respecting 3R policy in laboratory animal use, we decided to compare only intact and neuropathic pain groups in our behavioural and biochemical experiments.

5.2. Sciatic nerve surgery

Peripheral neuropathy was induced by chronic constriction injury (CCI) as described (Bennett and Xie, 1988). The sciatic nerve injury was performed under sodium pentobarbital anaesthesia (60 mg/kg, intraperitoneal). The biceps femoris and the gluteus superficialis were separated, and the right sciatic nerve was exposed. Proximal to the sciatic trifurcation, approximately 7 mm of nerve was freed from the adhering tissue, and the injury was produced by tying four loose ligatures (4/0 silk, 1 mm spacing) around the sciatic nerve. The twitch of muscles was a sign of proper tightness and prevented too strong ligation of the nerve. The total length of nerve affected was 5–6 mm. No procedure was conducted on the control animals.

5.3. Nociceptive behaviour

All experiments were conducted 3, 7 and 14 days after the sciatic nerve injury to determine thermal and mechanical withdrawal thresholds during the development of neuropathic pain. The cold allodynia was assessed using the cold plate test (Cold/Hot Plate Analgesia Meter No. 05044 Columbus Instruments, USA). The temperature of the cold plate was kept at 5 °C, and the cut-off latency was 30 s. The rats were placed on the cold plate, and the time when the hind paw was lifted was read. The injured paw exhibited a lower reaction latency. For the assessment of mechanical allodynia, rats were tested for their foot withdrawal threshold in response to an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer Cat. No.37400, Ugo Basile Italy). Rats were placed in plastic cages with a wire net floor 5 min before the experiment. The von Frey filament was applied to the midplantar surface of the ipsilateral hind paw, and the measurements of applied mechanical force were taken automatically. The strength of the von Frey stimuli in our experiments ranged from 0.5 to 26 g.

5.4. RNA preparation and quantitative real-time PCR

Animals were sacrificed 3, 7 or 14 days after the CCI procedure. A group of naive animals was used as a reference. The L4–L6 DRG were collected both ipsilateral and contralateral with respect to the side of injury. DRG L4–L6 were pooled, placed in tubes with the tissue storage reagent RNAlater (Qiagen Inc., Valencia, CA, USA), frozen and stored at −80 °C until RNA isolation. Samples were thawed at room temperature (RT) and homogenised in Trizol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was isolated according to Chomczynski's method (Chomczynski and Sacchi, 1987). The total RNA concentration was measured using a NanoDrop ND-1000 Spectrometer (Nano-Drop Technologies, Wilmington, DE, USA). Reverse transcription of total RNA (1 μg per sample) was performed using Omniscript reverse transcriptase (Qiagen Inc., Valencia, CA, USA) at 37 °C for 60 min. The reaction was carried out in the presence of the RNase inhibitor rRNAsin (Promega, Madison, WI, USA), and an oligo(dT_{16}) primer (Qiagen) was used to selectively amplify mRNA. For quantitative PCR, 45 ng of cDNA was used as a template. Reactions were performed using Assay-On-Demand TaqMan probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA, USA) according to the manufacturer's protocol. The following assays were used: Rn01527840_m1 (*Hprt1*), Rn00578842_m1 (*Mapk14*), Rn01432300_g1 (*Prkaca*), Rn00440861_m1 (*Prkcg*), Rn04219635_m1 (*Cdk5*), Rn00711106_m1 (*Chp2*), Rn00560913_m1 (*Camk2d*), Rn00580432_m1 (*Il1b*), Rn00561420_m1 (*Il6*), Rn01533872 (*Ngf*), Rn02064589 (*Bdkrb1*), Rn04338900 (*Bdkrb2*), and Rn01525859 (*Tnf*). Reactions were run on a Real-Time PCR iCycler IQ (Bio-Rad, Hercules, CA, USA) with the 3.0 software version. Cycle threshold values (Ct) were calculated automatically. Expression of the *Hprt1* (hypoxanthine phosphoribosyltransferase 1) transcript, with stable levels between the control and CCI groups (Table 1), was quantified to control for variation in cDNA amounts. The abundance of RNA was calculated as $2^{-(\text{normalized threshold cycle})}$.

5.5. Western blot

Animals were sacrificed 3, 7 or 14 days after CCI. A group of naive animals was used as a reference. The L4–L6 dorsal root ganglia (DRG) were collected both ipsilateral and contralateral with respect to the side of injury. DRG were pooled, placed in individual tubes, frozen on dry ice and stored at −80 °C until protein isolation. Tissue samples were homogenised in RIPA buffer with protease and phospatase inhibitors (Sigma-Aldrich, Irvine, UK) and cleared by centrifugation (10,000 ×g, 4 °C, 30 min). The protein concentration in the supernatant was determined using the BCA Protein Assay Kit (Sigma-Aldrich, Irvine, UK). Samples containing 30 mg of protein were heated for 5 min at 96 °C in loading buffer (50 mM Tris–HCl, 2% SDS, 2% β-mercaptoethanol, 8% glycerol and 0.1% bromophenol blue) and resolved by SDS-PAGE on 10% or 12% polyacrylamide gels. After gel electrophoresis, proteins were electrophoretically transferred to PVDF membranes (Trans-Blot Turbo; Bio-Rad, Hercules, CA, USA). The blots were blocked using 5% blocking buffer (5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20) for 1 h. Blots were incubated overnight at 4 °C with primary antibodies (Table 2) and then incubated at RT with a peroxidase-conjugated secondary antibody (Table 2) for 1 h. Immunocomplexes were detected using a Immuno-Star HPR kit (BioRad, Hercules, CA, USA) and visualised using a Fujifilm LAS-1000 System (Fuji Film, Tokyo, Japan). The blots were stripped and reprobed with a mouse polyclonal anti-GAPDH antibody as a loading control. Relative levels of immunoreactivity were quantified using the Fujifilm Image Gauge software (Fuji Film, Tokyo, Japan).

5.6. Immunofluorescence microscopy

The animals were deeply anaesthetised with pentobarbital (pentobarbital, 60 mg/kg i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer

Table 1
Threshold cycle of *Hprt1* expression in different experimental groups.

Experimental group	Spinal cord control	Spinal cord CCI day 3	Spinal cord CCI day 7	Spinal cord CCI day 14	DRG control	DRG CCI day 3	DRG CCI day 7	DRG CCI day 14
Ct	22.08 ± 0.10	22.03 ± 0.08	22.25 ± 0.05	22.31 ± 0.10	22.11 ± 0.14	21.97 ± 0.20	22.17 ± 0.11	22.14 ± 0.06

Table 2
List of antibodies used during experiments.

	Used concentration
Primary antibody	
Anti-PKC(αβγ), Clone M110, Millipore, Cat# 05-983	1:1000
Anti-PKCε, Millipore, Cat#06-991	1:2000
Anti-CaM Kinase II, Millipore, Cat#07-1496	1:1000
Anti-p38-MAPK, Sigma-Aldrich, Cat#M0800	1:5000
PKA C-α, Cell Signaling, Cat#4782	1:1000
Anti-GAPDH, Millipore, Cat#MAB374	1:1000
Secondary antibody	
Goat anti-rabbit IgG HRP, Bio-Rad, Cat#166-2408EDU	1:1000
Goat anti-mouse IgG HRP, Bio-Rad, Cat#170-6516	1:1000

(PB), pH 7.4. DRG were dissected, postfixed for 2 h, then washed and soaked for cryoprotection in sucrose solutions of increasing concentration (10–30%) at 4 °C until immersion. Samples were frozen and stored at −80 °C until sections were cut at 12 μm thick and collected onto gelatin-coated slides (Menzel GmbH, Braunschweig, Germany). For immunofluorescence labelling, serial sections of DRG were hydrated in PB buffer (pH = 7.4) for 15 min, then cell membranes were permeabilised in PB containing 0.1% Triton X-100 and afterwards incubated for 1 h in 10% normal goat serum (NGS, Jackson ImmunoResearch Laboratories, West Grove, USA) in PB containing 0.4% Triton X-100 (blocking solution). Subsequently, the sections were incubated for 48 h at 4 °C in a humid chamber with the respective primary antibodies (all diluted in blocking solution; Table 3). After three washes in PB, triple immunofluorescence was revealed by incubation at 4 °C in for 24 h in the dark with the appropriate fluorochrome-conjugated secondary antibody (Table 3). Thereafter, sections were washed 3 times for 15 min with PB and coverslipped with Aquatex mounting medium (Merck, Darmstadt, Germany). Controls for immunostaining included omission of either the primary antisera or the secondary antibodies. These control experiments did not show notable staining. The sections processed for immunofluorescence were studied with a fluorescence microscope (DM RXA2) with confocal scanner (TCS SL) (Leica Microsystems GmbH, Manheim, Germany) equipped with the following lasers: Ar 488, He–Ne 543, He–Ne 633. Images were acquired at depth (thickness) intervals of 4 μm, and 8 cross-sections were scanned in steps of 0,5 μm using the image acquisition software Leica Microsystem Software Ver. 2.5 (Leica Microsystems GmbH, Manheim, Germany). Digital images were stacked with average fluorescence intensity and processed in the image analysis program ImageJ (NIH, Maryland, USA) with brightness and contrast being the only adjustments made.

Table 3
List of antibodies used for immunofluorescence experiment.

	Used concentration
Primary antibody	
Guinea pig polyclonal anti-TRPV1, Cat.# GP14100, Neuromics, Edina, USA	1:200
Rabbit polyclonal anti-PKCε, Cat.# 06–991, Millipore, Massachusetts, USA	1:200
Mouse monoclonal anti-NeuN, clone A60, Cat.# MAB377, Millipore, Massachusetts, USA	1:100
Secondary antibody	
Goat anti-guinea pig conjugated with Alexa 488, Cat.# A11073, Invitrogen, Carlsbad, USA	1:400
Goat anti-rabbit conjugated with Alexa 555, Cat.# A21428, Invitrogen, Carlsbad, USA	1:400
Goat anti-mouse conjugated with Alexa 633, Cat.# A21050, Invitrogen, Carlsbad, USA	1:200

5.7. Statistical analyses

All data are presented as the mean ± S.E.M. The results of qPCR and Western blot were evaluated by the analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Groups of 4–6 animals were used for molecular experiments. A value of $p < 0.05$ was considered statistically significant. The mean percentage of the number of neurons labelled with TRPV1 and PKCε was quantified by 2 independent observers blinded to the experimental protocols. All neurons (NeuN$^+$ cells) in the field of view were identified, but only cells that exhibited expression of at least one protein of interest (TRPV1 or/and PKCε) were taken under consideration and analysed in a total of 8–12 randomly selected sections per group (4–6 sections per animal, 2 animals per group). The results were evaluated by ANOVA followed by Bonferroni's post-hoc test. A value of $p < 0.05$ was considered statistically significant.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2015.02.001.

List of abbreviations

AEA	anandamide
BDKRB1	bradykinin receptor 1
BDKRB2	bradykinin receptor 2
CaMK2	Ca^{2+}/calmodulin-dependent protein kinase
CCI	chronic constriction injury
Cdk5	cyclin-dependent kinase 5
DRG	dorsal root ganglia
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPRT1	hypoxanthine–guanine phosphoribosyltransferase 1
IL-1β	interleukin 1β
IL-6	interleukin 6
NGF	nerve growth factor
P38-MAPK	p38 mitogen-activated protein kinase
PKA	protein kinase A
PKC	protein kinase C (isoform αβγ and ε)
TNFα	tumour necrosis factor α
TRPV1	transient receptor potential vanilloid 1

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NM: experimental design, acquisition of mRNA data, analysis and interpretation of data, writing of the manuscript. AP: acquisition of immunohistochemical data, analysis and interpretation of data, writing of the manuscript. NK: acquisition of Western blot data, analysis and interpretation of data. MK: analysis and interpretation of data. KS: study conception, experimental design, analysis and interpretation of data, writing of manuscript. All authors read and approved the final manuscript.

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