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Activation of CB1 inhibits NGF-induced sensitization of TRPV1 in adult mouse afferent neurons

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Abstract

Transient receptor potential vanilloid 1 (TRPV1)-containing afferent neurons convey nociceptive signals and play an essential role in pain sensation. Exposure to nerve growth factor (NGF) rapidly increases TRPV1 activity (sensitization). In the present study, we investigated whether treatment with the selective cannabinoid receptor 1 (CB1) agonist arachidonyl-2'-chloroethylamide (ACEA) affects NGF-induced sensitization of TRPV1 in adult mouse dorsal root ganglion (DRG) afferent neurons. We found that CB1, NGF receptor tyrosine kinase A (trkA), and TRPV1 are present in cultured adult mouse small- to medium-sized afferent neurons and treatment with NGF (100 ng/ml) for 30 minutes significantly increased the number of neurons that responded to capsaicin (as indicated by increased intracellular Ca^{2+} concentration). Pretreatment with the CB1 agonist ACEA (10 nM) inhibited the NGF-induced response, and this effect of ACEA was reversed by a selective CB1 antagonist. Further, pretreatment with ACEA inhibited NGF-induced phosphorylation of AKT. Blocking PI3 kinase activity also attenuated the NGF-induced increase in the number of neurons that responded to capsaicin. Our results indicate that the analgesic effect of CB1 activation may in part be due to inhibition of NGF-induced sensitization of TRPV1 and also that the effect of CB1 activation is at least partly mediated by attenuation of NGF-induced increased PI3 signaling.

Keywords

Transient receptor potential vanilloid 1. Capsaicin; nerve growth factor; sensitization; cannabinoid receptor 1; phosphorylation of AKT; pain

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INTRODUCTION

Transient receptor potential vanilloid 1 (TRPV1) is a voltage-gated ion channel that allows influx of cations in response to noxious stimuli, including heat, acid (protons), and capsaicin (Caterina et al., 2000; Davis et al., 2000; White et al., 2011a; Sousa-Valente et al., 2014a). TRPV1 are expressed primarily by small to medium sized afferent neurons that are putative nociceptors (Immke and Gavva, 2006; Woolf and Ma, 2007; White et al., 2011b). The majority of TRPV1-expressing neurons contain nociceptive neuropeptides, such as calcitonin-gene related peptide (CGRP) and substance P, and also express the nerve growth factor (NGF) receptor, tyrosine kinase A (trkA) (Averill, et al., 1995; Guo et al., 1999; Immke and Gavva, 2006; Ernsberger, 2009). Expression of TRPV1 in afferent neurons is increased by inflammatory pain (Calton and Coggeshall, 2001; Ji et al., 2002; Luo et al., 2004; Schicho et al., 2004; Immke and Gavva, 2006; Miranda et al., 2007; Schwartz et al., 2013). Genetic deletion of TRPV1 (Caterina et al., 2000; Davis et al., 2000) and studies using selective TRPV1 antagonists have demonstrated that TRPV1 is essential for development of referred hyperalgesia associated with certain types of tissue injury and inflammation (Ji et al., 2002; Luo et al., 2004; Schicho et al., 2004; Immke and Gavva, 2006; Charrua et al., 2009; Sousa-Valente et al., 2014).

Tissue injury and inflammation generate an array of chemical mediators, including NGF, that activate and sensitize primary afferent neurons (Ji et al., 2002; Woolf and Ma, 2007; Stemkowski and Smith, 2012). NGF has been well-characterized as essential for development, growth, and function of afferent neurons (Woolf and Ma, 2007). Treatment with NGF (hours to days) increases expression of TRPV1 in cultured afferent neurons (Winston et al., 2001; Anand et al., 2006). Acute exposure to NGF has also been shown to rapidly increase TRPV1 activity (sensitization) (Winter et al., 1988; Shu and Mendell, 2001; Zhuang et al., 2004; Jankowski and Koerber, 2010). Multiple intracellular signaling pathways are involved in sensitization of TRPV1 by NGF, including ERK 1/2 (extracellular signal-regulated protein kinase, a member of the MAPK or mitogen-activated protein kinase family), and PI3 (phosphatidylinositol 3) kinase (Bonnington and McNaughton, 2003; Zhuang et al., 2004; Malik-Hall et al., 2005).

Cannabinoids have been shown to have analgesic and anti-inflammatory effects, and the effects of cannabinoids are mediated primarily by cannabinoid receptors 1 and 2 (CB1 and CB2), both coupled to inhibitory G proteins (Richardson et al., 1998; Clayton et al., 2002; Sagar et al., 2005; Demuth and Molleman, 2006; Khasabova et al., 2008). CB1 are predominantly present in neural tissues, including afferent neurons (Ross et al., 2001; Ahluwalia et al., 2000; Clayton et al., 2002; Demuth and Molleman, 2006; Agarwal et al., 2007; Hu et al., 2012). Conditional deletion of CB1 specifically in nociceptive afferent neurons in mice prevented cannabinoid-induced analgesia and exaggerated mechanical hyperalgesia induced by intraplantar injection of complete Freund's adjuvant (CFA) (inflammatory pain) or in the spared nerve injury model of neuropathic pain (axotomy and ligation of 2 of the 3 terminal branches of the sciatic nerve) (Agarwal et al., 2007). In the present study, we investigated whether treatment with the selective CB1 agonist arachidonyl-2'-chloroethylamide (ACEA) affects NGF-induced sensitization of TRPV1 in

adult mouse dorsal root ganglion (DRG) afferent neurons. We also investigated the effects of CB1 activation on intracellular signaling pathways involved in NGF-induced sensitization of TRPV1.

EXPERIMENTAL PROCEDURES

Animals

Forty-six male C57BL/NH mice (10-12 weeks old) were obtained from Harlan (Indianapolis, IN). Experiments were conducted in accordance with National Institutes of Health Guidelines, and all protocols were reviewed and approved by the Animal Care and Use Committee of the University of Wisconsin.

Culture of DRG neurons

Mice were deeply anesthetized with pentobarbital (50 mg/kg, ip) and perfused with saline (0.9 % NaCl) through a canula inserted into the left ventricle. Approximately 40–45 DRGs were removed from each individual mouse, and nerve trunks and connective tissue were dissected and discarded. DRGs were transferred to Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) and treated with trypsin (2.5 mg/ml) and collagenase (2 mg/ml) for 60 minutes at 37 °C. After enzyme treatment, DRG neurons were dissociated by trituration with fire-polished Pasteur pipettes in Dulbecco's Modified Eagle Medium containing fetal bovine serum (10 %), penicillin (50 U/ml), and streptomycin (50 µg/ml). The cell suspension was loaded on 30 % Percoll density solution (Sigma, St. Louis, MO), and DRG neurons were collected by low-speed (800× g) centrifugation for 10 minutes. Neurons were resuspended in Neurobasal medium containing B27 supplements (Life Technologies), penicillin (50 U/ml), and streptomycin (50 µg/ml), and allowed to settle on 35 mm glass-bottom dishes (MatTek, Ashland, MA, for Ca²⁺ imaging) or 18 mm cover slips (for immunoblotting), both coated with poly-D-lysine (Sigma). Cells from each animal were plated on 6-8 Ca²⁺ imaging dishes or cover slips and maintained at 37 °C in a humidified incubator with a gas mixture containing 5% CO₂. The neurons were used within 16-28 hours of isolation.

Immunohistochemistry

Neurons were cultured on poly-D-lysine-coated cover slips overnight. Cells were rinsed in phosphate-buffered saline (PBS), fixed with 2 % paraformaldehyde for 30 minutes, and permeabilized with cold methanol for 10 minutes. They were rinsed and blocked with 10 % normal donkey serum. Each specific antibody was then applied and incubated in a humid chamber overnight to 48 hours at 4° C. Staining was revealed using secondary donkey anti-rabbit IgG, conjugated with FITC or donkey anti-goat IgG, conjugated with rhodamine red (1:500, Jackson ImmunoResearch, West Grove, PA). Cover slips containing neurons were rinsed and mounted to slides with an anti-fading solution (Vector Labs, Burlingame, CA). Negative staining controls were prepared using normal rabbit or goat IgG instead of the specific antibody. Each staining was performed on 6 coverslips from 3 cultures. Staining was examined with a Nikon E600 microscope and photoimages from stained and control cells were acquired with the same acquisition set-up. For staining on each coverslip, 5-6 optical fields (18-37 neurons per field, about 120-200 neurons per coverslip) were randomly

selected. The staining intensities of outlined neurons from corresponding negative controls were measured as a grey level on a 0-255 scale, and the averaged value was used as the threshold to differentiate labeled neurons from negative ones (Peset et al., 2005; Baiou et al., 2007; Hong et al., 2011). The number of positively labeled neurons was normalized to the total number of neurons in the selected fields. The values from staining on 6 coverslips (n) of 3 cultures were averaged and presented as means $\pm$ SEM.

The primary antibodies used for immunohistochemistry included rabbit anti-CB1 (1:1500, EMD Millipore, Billerica, MA) (Coutts et al., 2001; Bouchard et al., 2003), rabbit anti-trkA (1:500, Abcam, Cambridge, MA) (Manca et al., 2009; Lu et al., 2010), and goat anti-TRPV1 (1:200, Santa Cruz Biotechnology, Inc., Dallas, TX) (Ma et al., 2009; Ferrini et al., 2010). The antibodies used have been validated in previous studies and by the manufacturer.

Triple-immunostaining was performed to study co-localization of TRPV1, trkA, and CB1 in DRG neurons. The staining was performed in sequence. Neurons were first incubated with rabbit anti-trkA (1:500, Abcam) overnight at 4° C. After rinsing, the neurons were visualized with a FITC-conjugated donkey anti-rabbit antibody (1:500, Jackson ImmunoResearch). The neurons were next incubated with goat anti-TRPV1 antibody (1:200, Santa Cruz) for 48 hours at 4° C. Staining with TRPV1 was revealed with a rhodamine red-conjugated donkey anti-goat antibody (1:500, Jackson ImmunoResearch). Staining of trkA and CB1 required using primary antibodies, both raised from rabbit hosts. The neurons were therefore blocked with unlabeled monovalent Fab fragments of goat anti-rabbit IgG (1:25, Jackson Immunochemical) for 3 hours at room temperature to block all possible remaining binding sites of the trkA primary antibody (Brouns et al., 2002). After blocking, the neurons were incubated with a rabbit anti-CB1 antibody (EMD Millipore) for 48 hours at 4° C, and staining was revealed using secondary goat anti-rabbit IgG, conjugated with FarRed (1:600, Life Technologies, Grand Island, NY). Images were acquired using a 40 $\times$ /1.45 NA objective lens on an Olympus Fluoview 500 laser-scanning confocal system mounted on an AX-70 upright microscope with green, red and blue filters in sequential scan mode to avoid bleed-through of fluorescence. Image processing was complete off-line using Image J (Kerstein et al., 2013). Staining was performed with neurons from 3 culture preparations. Control staining was performed using the same protocol except that each primary antibody was substituted with normal IgG from the same host. Intensity of control staining was determined on each channel and the value was used as the threshold to differentiate labeled neurons from negative ones.

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) Imaging

Neurons were loaded with Fura 2-AM (2 μM , Sigma) for 40 minutes at 37° C. Cells were rinsed and incubated in $[\text{Ca}^{2+}]_i$ imaging buffer (140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10mM Na-Hepes and 10 mM glucose, pH 7.4) for 30 minutes at room temperature to complete ester hydrolysis. $[\text{Ca}^{2+}]_i$ imaging was performed as previously described (Yi et al., 2010). Briefly, culture dishes were placed under an inverted Nikon epifluorescence microscope, and 40-50 neurons in the field of view were selected for recording. Fura-2 was excited alternately with ultraviolet light at 340 and 380 nm, and the fluorescence emission from individual neurons was monitored and recorded at 510 nm using

a computer-controlled digital CCD camera every 2 seconds. Wavelength selection, timing of excitation, and acquisition of images were controlled using the program MetaFluor (Molecular Devices, Sunnyvale, CA) running on a PC computer. Baseline $[Ca^{2+}]_i$ was determined from 8-10 measurements. Cells with changes in $[Ca^{2+}]_i$ that occurred immediately after drug application and in which the amplitude of changes was greater than two standard deviations of baseline were considered to respond to drug application positively (Lu and Gold, 2008).

Treatment of afferent neurons

Neurons were treated with murine NGF (100 ng/ml, Promega, Madison, WI) or vehicle (saline) for 30 minutes prior to application of capsaicin (300 nM, 2 minutes). The neurons were rinsed and exposed to KCl (60 mM), applied 30 minutes after capsaicin, to confirm viability of neurons. Only neurons that responded to KCl were included in the analysis. The number of neurons that responded to capsaicin was counted and expressed as percentage (%) of the total number of KCl-responsive neurons in the field of view. Neurons from 3-4 culture preparations were used in each group.

The selective CB1 agonist ACEA (10 nM) or vehicle (0.01 % ethanol) was applied 5 minutes prior to application of NGF in some experiments. ACEA is analogue of anandamide, an endogenous cannabinoid (endocannabinoid). In contrast to anandamide that binds to both the CB1 and CB2 receptors with moderate affinity, ACEA is a highly selective CB1 agonist with very low affinity to CB2 (Hillard et al., 1999). Like anandamide, ACEA has been reported to activate TRPV1 at concentrations greater than 1 μ M (Smart et al., 2000; Baker and McDougall, 2004; Price et al., 2004; 2005). We therefore tested ACEA at 10 nM to avoid potential non-CB1-mediated effects. In other experiments, the CB1 antagonist *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM 251, 100 nM) or vehicle (0.01 % ethanol) was added 5 minutes before ACEA.

In separate experiments, the PI3 kinase inhibitor wortmannin (10 nM) or vehicle (0.01 % ethanol) was added 30 minutes before NGF. Wortmannin at 10-20 nM is a highly selective inhibitor of PI3 kinase and affects other kinases only at much higher concentrations (Davies et al., 2000; Bonnington and McNaughton, 2003).

Immunoblotting analysis

Neurons were treated with the previously-described protocol. Neurons from 3-4 culture preparations were used in each group. After treatment, neurons were lysed with T-PER mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Supernatants were collected by centrifugation at 10,000 $\times$ g for 15 minutes at 4 $^{\circ}$ C. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific). Protein samples were mixed 1:1 with Laemmli Sample buffer (Bio-Rad, Hercules, CA), placed in boiling water for 5 minutes, and stored at -20 $^{\circ}$ C until analyzed.

Protein samples (~ 10 µg/lane) were resolved on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% dry fat-free milk in 1× TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20; pH 7.5). After rinsing, membranes were incubated at 4° C overnight with the specific primary antibody. Membranes were then washed free of primary antibody and incubated for 1 hour with appropriate secondary antibody conjugated to horseradish peroxidase at room temperature. Signals were revealed using a chemiluminescent detection reagent (Amersham, Arlington Heights, IL). Membranes were apposed to X-ray films, and films were developed. Membranes were then stripped and re-probed with a mouse anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as a loading control. Images were scanned, and protein abundance was estimated by optical density measurement with Image J software (NIH, Bethesda, MD, USA). Values were normalized to the loading control. To compare values between different treatment groups, the normalized values of each protein(s) were calculated as fold change relative to control samples (set equal to 1) (Wang and Bjorling, 2011). Primary antibodies used for immunoblotting were mouse monoclonal anti-GAPDH (1:5000) (Abcam), rabbit anti-phospho-ERK1/2 (1:3000), and phospho-AKT (1:3000) (Cell Signaling, Danvers, MA). Secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG (both conjugated to horseradish peroxidase and used at 1:20,000) (Santa Cruz, Biotechnology, Dallas, TX).

Reagents

The selective CB1 agonist (ACEA) and the selective CB1 antagonist (AM251) were obtained from Tocris (Bristol, UK). The K_i values for ACEA are 1.4 and 1,900 nM for CB1 and CB2, respectively. The K_i values of AM251 are 7.9 and 2,100 nM for CB1 and CB2, respectively (Tocris). Capsaicin was obtained from Sigma. NGF and wortmannin (inhibitor of PI3 kinase) were purchased from Promega. ACEA, AM 251, capsaicin, and wortmannin were dissolved in ethanol as stock solutions and diluted in saline to desired concentrations. NGF was prepared in saline.

Statistical analysis

Data are presented as arithmetic means $\pm$ SEM. Data were analyzed using oneway ANOVA followed by Bonferroni post hoc comparisons (GraphPad Prism, San Diego, CA) or Student's *t* test. *p* values < 0.05 were considered significant.

RESULTS

Presence of CB1, TRPV1 and trkA in adult mouse afferent neurons

Specific antibodies revealed positive immunostaining for trkA, TRPV1 and CB1 in small- to medium-sized afferent neurons (Figure 1). Cells were considered labeled with the specific antibody when the fluorescent intensity was distinctively higher than controls. Replacing specific antibodies with normal rabbit or goat IgG resulted in complete lack of specific staining (Figure 1, lower right panel). Under the experimental conditions used, $49.2 \pm 3.9 \%$, $53.9 \pm 4.3 \%$, and $62.1 \pm 3.8 \%$ neurons were positive for trkA, TRPV1 and CB1, respectively (*n* = 6). Triple co-localization staining revealed that $30.6 \pm 3.6 \%$ neurons expressed all three proteins (*n* = 6).

Effects of NGF on capsaicin-induced increase in $[Ca^{2+}]_i$

Exposure of neurons to capsaicin was generally characterized by a rapid increase in $[Ca^{2+}]_i$, and the amplitude and duration of capsaicin-induced responses varied considerably among neurons (Figure 2A). Exposure to capsaicin (300 nM) induced a rapid increase in $[Ca^{2+}]_i$ in about one-third of the neurons ($30.2 \pm 1.2\%$, $n = 8$, Figure 2B). Exposure to NGF (100 ng/ml) for 30 minutes did not affect basal $[Ca^{2+}]_i$ in neurons (not shown). Treatment with NGF significantly increased the number of neurons that responded to capsaicin ($41.4 \pm 1.8\%$, $n = 8$, $p < 0.01$ vs capsaicin-treated group; Figure 2B).

Effects of the selective CB1 agonist ACEA on NGF-induced responses

Exposure to ACEA (10 nM) did not affect basal $[Ca^{2+}]_i$ or the number of neurons that responded to capsaicin (Figure 2B). Treatment with ACEA abolished the NGF-induced increase in the number of neurons that responded to capsaicin ($30.1 \pm 1.3\%$, $n = 8$, $p < 0.01$ vs NGF-treated group), and this effect of ACEA was reversed by pretreatment with the selective CB1 antagonist AM251 (100 nM, $41.3 \pm 2.6\%$, $n = 8$, $p < 0.01$ vs ACEA+NGF-treated group; Figure 2B). Treatment with AM251 (100 nM) alone did not affect the NGF-induced increase in the number of neurons that responded to capsaicin ($42.1 \pm 4.3\%$, vs NGF-treated group, $n = 8$, $p > 0.05$).

Effects of the selective CB1 agonist ACEA on signaling pathways involved in NGF-induced responses

Immunoblotting demonstrated that exposure to capsaicin alone for 2 minutes did not alter abundance of phosphorylated AKT (Figure 3A, 0.93 ± 0.07 vs basal level 1 ± 0.02 , $n = 5$, $p > 0.05$) or ERK1/2 (Figure 3B, 1.12 ± 0.22 vs basal level 1 ± 0.21 , $n = 5$, $p > 0.05$). Treatment with NGF and capsaicin increased phosphorylation of AKT (Figure 3A, 3.1 ± 0.56 , $n = 5$, $p < 0.05$ vs basal level) and ERK1/2 (Figure 3B, 3.13 ± 0.28 , $n = 5$, $p < 0.05$ vs basal level). Treatment with ACEA attenuated the increase in phosphorylation of AKT (Figure 3A, 1.8 ± 0.32 , $n = 5$, $p < 0.05$ vs NGF and capsaicin-treated group) without affecting phosphorylation of ERK1/2 (Figure 3B) in neurons treated with NGF and capsaicin.

In neurons treated with the combination of NGF and capsaicin, the increase in phosphorylation of AKT was not different from those treated with NGF alone (Figure 3), indicating that the enhanced phosphorylation of AKT was primarily caused by NGF. In separate experiments, we evaluated the effects of ACEA on NGF-induced enhanced phosphorylation of AKT. NGF increased phosphorylation of AKT (3.06 ± 0.36 vs basal level 1 ± 0.28 , $n = 6$, $p < 0.01$). Treatment with ACEA (10 nM) attenuated NGF-induced enhanced phosphorylation of AKT (1.28 ± 0.23 vs NGF-treated group, $n = 6$, $p < 0.01$), and the inhibitory effects of ACEA were reversed by pretreatment with the selective CB1 antagonist AM251 (100 nM, 3.26 ± 0.27 , $n = 6$, $p > 0.05$ vs NGF-treated group; $p < 0.01$ vs ACEA+NGF-treated group).

Effects of the selective PI3 kinase inhibitor wortmannin on NGF-induced responses

These observations suggested that PI3 kinase could mediate the inhibitory effects of CB1. We therefore tested whether treatment with a selective PI3 kinase inhibitor affected NGF-

induced responses. In vehicle-treated neurons, NGF increased AKT phosphorylation (Figure 4A, 2.44 ± 0.23 vs basal level 1 ± 0.05 , $n = 5$, $p < 0.05$). Treatment with wortmannin (10 nM) abolished NGF-induced increased phosphorylation of AKT (Figure 4A, 1.06 ± 0.16 vs NGF-treated group, $n = 5$, $p < 0.05$). Similarly, treatment with wortmannin prevented the increase in phosphorylation of AKT in neurons treated with the combination of NGF and capsaicin (Figure 4A, 0.93 ± 0.15 , $n = 5$, $p < 0.05$ vs NGF and capsaicin-treated group). Treatment with wortmannin also attenuated the NGF-induced increase in the number of neurons responding to capsaicin (Figure 4C, $34.8 \pm 1.56\%$ vs vehicle-treated group $41 \pm 1.82\%$, $n = 8$, $p < 0.01$). Treatment with wortmannin did not affect NGF- or NGF and capsaicin-induced increased phosphorylation of ERK1/2 (Fig. 4B).

DISCUSSION

In the present study, we found that: 1) CB1, trkA, and TRPV1 are present in a subpopulation of cultured adult mouse small- to medium-sized afferent neurons; 2) treatment with NGF for 30 minutes significantly increased the number of neurons that responded to capsaicin; 3) treatment with the CB1 agonist ACEA inhibited the NGF-induced response, and this effect of ACEA was reversed by a selective CB1 antagonist; and 4) treatment with ACEA inhibited NGF-induced phosphorylation of AKT, and blocking PI3 kinase activity attenuated NGF-induced increase in the number of neurons that responded to capsaicin. To the best of our knowledge, this is the first report that activation of CB1 inhibits NGF-induced sensitization of TRPV1 and the inhibitory effects of CB1 are at least partly mediated by attenuating NGF-induced activation of PI3 kinase pathways. These findings reveal a novel mechanism that may contribute to the analgesic effects of cannabinoids.

A significant body of evidence indicates the presence of CB1 in afferent neurons (Ross et al., 2001; 2003; Ahluwalia et al., 2003a,b; Bridges et al., 2003; Khasabova et al., 2004; Agarwal et al., 2007; Hong et al., 2008). Analysis of CB1 localization in DRG afferent neurons yields variable results, partly dependent upon the sensitivity and specificity of reagents and techniques used. Several lines of evidence reveal that CB1 is present in small- and medium-sized nociceptive neurons containing CGRP (Khasabova et al., 2004; Agarwal et al., 2007; Veress et al., 2012). It has been shown previously that these peptidergic neurons frequently express TRPV1 (Amaya et al., 2006; Price and Flores, 2007; Ernsberger, 2009) and trkA (Averill et al., 1995; Dinh et al., 2004; Kobayashi et al., 2005). Moreover, TRPV1 mRNA and protein are detected in 90 % of NGF-responsive neurons (Winston et al., 2001). Ahluwalia et al. (2000; 2002) found that almost all TRPV1-positive neurons express CB1, and similar observations were reported by Binzin et al. (2006) and Hong et al. (2009), suggesting that a high proportion of TRPV1 positive afferent neurons express CB1. Interestingly, majority CB1 positive neurons (> 80 %) also express TRPV1 (Ahluwalia et al., 2002). We provide further evidence that that CB1, trkA, and TRPV1 are consistently observed in small- to medium-sized adult mouse afferent neurons, and about one third of neurons expressed all three proteins, suggesting the potential for functional interactions among these proteins in nociception.

The described effects of anandamide on afferent neurons are contradictory, partly due to the fact that anandamide exerts dual effects on afferent neurons, depending on the concentration

used (R Khasabova et al., 2002; Ross 2003; Evans et al., 2004; Kim et al., 2005; Sousa-Valente et al., 2014b). Specifically, anandamide at nM concentrations produces a CB1-mediated inhibitory effect, while at higher concentrations ($> \mu\text{M}$), it exerts a TRPV1-mediated stimulatory effect in afferent neurons (Tognetto et al., 2001; Roberts et al., 2002; Ahluwalia et al., 2003a; Ross 2003; Price et al., 2005; Fischbach et al., 2007). Millins et al. (2001) demonstrated that treatment with a selective CB1 agonist, HU210, inhibited the capsaicin-induced increase in intracellular calcium concentrations in afferent neurons. Mahmud et al. (2009) reported that treatment with anandamide (3-30 nM) and ACEA (500 nM) reduced the number of capsaicin responsive rat DRG neurons using TRPV1-mediated cobalt influx assay. Santha et al. (2010) also examined the effects of anandamide (10-30 nM) on capsaicin-induced whole-cell currents in rat DRG neurons, and these authors found that exposure to anandamide reduced the amplitude of capsaicin-induced currents in a subpopulation of afferent neurons. In the present study, ACEA (10 nM) did not affect the number of neurons that responded to capsaicin. We lack a satisfactory explanation for the discrepancy in findings, which may be attributable to different experimental protocols (e.g., neonatal or adult animals from which neurons are obtained, rats vs. mice, duration that neurons maintained in culture, etc.), concentrations of ACEA examined, or methods used to measure responses.

Exposure to NGF rapidly increased capsaicin-induced currents in cultured rat afferent neurons (Ji et al., 2002; Zhu and Oxford, 2007). Administration of capsaicin into hind paws induced thermal hypersensitivity in wild-type, but not in TRPV1 knock-out, mice (Caterina et al., 2000), and the effects of capsaicin were enhanced by pretreatment with NGF (Shu and Mendall, 2001). It is unlikely that sensitization of TRPV1 induced by NGF involves upregulation of gene transcription, because this occurs rapidly after application of NGF (Ji et al., 2002; Jankowski and Koerber, 2010). It has been suggested that exposure to NGF induces rapid phosphorylation of TRPV1 and translocation of TRPV1 into cell membranes, thereby enhancing TRPV1 activity (Zhang et al., 2005; Stein et al., 2006). However, the precise mechanisms underlying NGF-induced sensitization of TRPV1 remain to be elucidated (Jankowski and Koerber, 2010). Previous studies have revealed that NGF-induced sensitization of TRPV1 involves multiple signaling pathways, including ERK1/2 and PI3 kinase (Zhuang et al., 2004; Zhu and Oxford, 2007; Jankowski and Koerber, 2010). We found that NGF increased the number of afferent neurons that respond to capsaicin. We also demonstrated that NGF induced phosphorylation of AKT, and treatment with a PI3 kinase inhibitor attenuated NGF-induced increase in the number of capsaicin-responsive afferent neurons. Therefore, our results provide further evidence that NGF induces sensitization of TRPV1 of afferent neurons, and that this effect of NGF is at least partly mediated by PI3 kinase. Using a perforated patch clamp technique, Stein et al. (2006) reported similar findings that treatment with a PI3 kinase inhibitor abolished NGF-induced TRPV1 sensitization in acutely dissociated mouse afferent neurons.

Farquhar-Smith and Rice (2003) reported that intraplantar administration of NGF to the hind paw of rats induced thermal hyperalgesia, and treatment with anandamide inhibited the effects of NGF via activation of CB1 (Farquhar-Smith and Rice, 2003). These authors also demonstrated that intravesical installation of NGF replicates many features of visceral hyperalgesia, including bladder hyperreactivity and increased expression of the immediate

early gene c-fos in the spinal cord, and treatment with anandamide attenuated these responses via activating both CB1 and CB2 receptors (Farquhar-Smith et al., 2002). These studies clearly indicate that cannabinoids are capable of regulating NGF-induced responses. However, the mechanisms underlying the effects of cannabinoids on NGF-induced responses are largely unknown. In the present study, we demonstrated that treatment with the selective CB1 agonist ACEA prevented NGF-induced sensitization of TRPV1 in afferent neurons. Further, we found that ACEA selectively inhibited the NGF-induced phosphorylation of AKT without affecting NGF-induced phosphorylation of ERK1/2. It has been shown previously that activation of CB1 modifies PI3 kinase activity. Ibrahim and Abdel-Raham (2012) recently reported that activation of CB1 by WIN 55,212-2 reduced phosphorylation of Akt in rat brainstem. Similarly, treatment with WIN 55,212-2 down-regulated PI3 activity in C6 glioma cells (Ellert-Miklaszewska et al., 2005). The present study provides additional evidence that activation of CB1 exerts an inhibitory effect on enhanced PI3 kinase activity induced by NGF in afferent neurons.

NGF has been shown to induce phosphorylation of ERK in afferent neurons and our results are consistent with these findings. Further, we demonstrated that treatment with ACEA did not affect NGF-induced phosphorylation of ERK. In fact, we found that blocking the PI3 pathway abolished NGF-induced sensitization, suggesting that the ERK pathway is not crucial for NGF-induced acute sensitization of TRPV1 in isolated mouse adult afferent neurons. These findings are consistent with previous observations (Bonnington and McNaughton, 2003; Zhang et al., 2005). Zhuang et al. (2004) demonstrated that treatment with PI3 pathway inhibitor, but not an ERK pathway inhibitor, prevented the early phase of hyperalgesia induced by injection of NGF into hind paws of rats. These authors also reported that activation of the ERK pathway was important for maintenance of NGF-induced hyperalgesia (Zhuang et al., 2004). Thus, PI3 and ERK pathways are likely to play distinct roles during NGF-induced sensitization of TRPV1 in afferent neurons, particularly in the induction (PI3) vs maintenance (ERK) phases (Zhuang et al., 2004). Intraplantar injection of capsaicin induced phosphorylation of ERK rapidly in L4 DRG neurons in rats (Dai et al., 2002). We did not detect phosphorylation of ERK in cultured mouse neurons after 2 minutes exposure to a relatively low concentration of capsaicin. This discrepancy may be attributable to differences in experimental conditions (in vitro vs in vivo, concentrations of capsaicin examined, etc.).

CONCLUSION

In conclusion, we found that NGF induced sensitization of TRPV1 as indicated by increasing the number of afferent neurons that respond to capsaicin and that treatment with the selective CB1 agonist ACEA prevented this effect of NGF. Further, we demonstrated that ACEA selectively inhibited NGF-induced phosphorylation of AKT without affecting NGF-induced phosphorylation of ERK1/2. Moreover, treatment with a PI3 kinase inhibitor attenuated the NGF-induced increase in the number of capsaicin-responsive afferent neurons. Taken together, our results suggest that CB1 activation inhibited NGF-induced sensitization of TRPV1, and the effect of CB1 is at least partly mediated by attenuating the enhanced activity of PI3 signaling pathway-induced by NGF. Future studies will be focused

on the further investigation of signaling network involved in functional interactions among these receptors/proteins.

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REFERENCES

- Agarwal N, Pacher P, Tegeder I, Amaya F, Constantin CE, Brenner GJ, Rubino T, Michalski CW, Marsicano G, Monory K, Mackie K, Marian C, Batkai S, Parolaro D, Fischer MJ, Reeh P, Kunos G, Kress M, Lutz B, Woolf CJ, Kuner R. Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci.* 2007; 10:870–879. [PubMed: 17558404]
- Ahluwalia J, Urban L, Capogna M, Bevan S, Nagy I. Cannabinoid 1 receptors are expressed in nociceptive primary sensory neurons. *Neuroscience.* 2000; 100:685–688. [PubMed: 11036202]
- Ahluwalia J, Urban L, Bevan S, Capogna M, Nagy I. Cannabinoid 1 receptors are expressed by nerve growth factor- and glial cell-derived neurotrophic factor-responsive primary sensory neurones. *Neuroscience.* 2002; 110:747–753. [PubMed: 11934481]
- Ahluwalia J, Urban L, Bevan S, Nagy I. Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. *Eur J Neurosci.* 2003a; 17:2611–2618. [PubMed: 12823468]
- Ahluwalia J, Yaqoob M, Urban L, Bevan S, Nagy I. Activation of capsaicin-sensitive primary sensory neurones induces anandamide production and release. *J Neurochem.* 2003b; 84:585–591. [PubMed: 12558978]
- Amaya F, Shimosato G, Kawasaki Y, Hashimoto S, Tanaka Y, Ji RR, Tanaka M. Induction of CB1 cannabinoid receptor by inflammation in primary afferent neurons facilitates antihyperalgesic effect of peripheral CB1 agonist. *Pain.* 2006; 124:175–183. [PubMed: 16709443]
- Anand U, Otto WR, Casula MA, Day NC, Davis JB, Bountra C, Birch R, Anand P. The effect of neurotrophic factors on morphology, TRPV1 expression and capsaicin responses of cultured human DRG sensory neurons. *Neurosci Lett.* 2006; 399:51–56. [PubMed: 16481104]
- Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci.* 1995; 7:1484–1494. [PubMed: 7551174]
- Baiou D, Santha P, Avelino A, Charrua A, Bacskai T, Matesz K, Cruz F, Nagy I. Neurochemical characterization of insulin receptor-expressing primary sensory neurons in wild-type and vanilloid type 1 transient receptor potential receptor knockout mice. *J Comp Neurol.* 2007; 503:334–347. [PubMed: 17492627]
- Baker CL, McDougall JJ. The cannabinomimetic arachidonyl-2-chloroethylamide (ACEA) acts on capsaicin-sensitive TRPV1 receptors but not cannabinoid receptors in rat joints. *Br J Pharmacol.* 2004; 142:1361–1367. [PubMed: 15277316]
- Binzen U, Greffrath W, Hennessy S, Bausen M, Saaler-Reinhardt S, Treede RD. Co-expression of the voltage-gated potassium channel Kv1.4 with transient receptor potential channels (TRPV1 and TRPV2) and the cannabinoid receptor CB1 in rat dorsal root ganglion neurons. *Neuroscience.* 2006; 142:527–539. [PubMed: 16889902]
- Bonnington JK, McNaughton PA. Signalling pathways involved in the sensitisation of mouse nociceptive neurones by nerve growth factor. *J Physiol.* 2003; 551(Pt 2):433–446. [PubMed: 12815188]
- Bouchard JF, Lépiciér P, Lamontagne D. Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart. *Life Sci.* 2003; 72:1859–1870. [PubMed: 12586223]

- Bridges D, Rice AS, Egertová M, Elphick MR, Winter J, Michael GJ. Localisation of cannabinoid receptor 1 in rat dorsal root ganglion using in situ hybridisation and immunohistochemistry. *Neuroscience*. 2003; 119:803–812. [PubMed: 12809701]
- Brouns I, Van Nassauw L, Van Genechten J, Majewski M, Scheuermann DW, Timmermans JP, Adriaensen D. Triple immunofluorescence staining with antibodies raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. *J Histochem Cytochem*. 2002; 50:575–582. [PubMed: 11897811]
- Carlton SM, Coggeshall RE. Peripheral capsaicin receptors increase in the inflamed rat hindpaw: a possible mechanism for peripheral sensitization. *Neurosci Lett*. 2001; 310:53–56. [PubMed: 11524156]
- Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeit KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*. 2000; 288:306–313. [PubMed: 10764638]
- Charrua A, Cruz CD, Narayanan S, Gharat L, Gullapalli S, Cruz F, Avelino A. GRC-6211, a new oral specific TRPV1 antagonist, decreases bladder overactivity and noxious bladder input in cystitis animal models. *J Urol*. 2009; 181:379–386. [PubMed: 19010489]
- Clayton N, Marshall FH, Bountra C, O'Shaughnessy CT. CB1 and CB2 cannabinoid receptors are implicated in inflammatory pain. *Pain*. 2002; 96:253–260. [PubMed: 11972997]
- Coutts AA, Anavi-Goffer S, Ross RA, MacEwan DJ, Mackie K, Pertwee RG, Irving AJ. Agonist-induced internalization and trafficking of cannabinoid CB1 receptors in hippocampal neurons. *J Neurosci*. 2001; 21:2425–2433. [PubMed: 11264316]
- Dai Y, Iwata K, Fukuoka T, Kondo E, Tokunaga A, Yamanaka H, Tachibana T, Liu Y, Noguchi K. Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. *J Neurosci*. 2002; 22:7737–7745. [PubMed: 12196597]
- Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000; 351(Pt 1):95–105. [PubMed: 10998351]
- Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature*. 2000; 405:183–187. [PubMed: 10821274]
- Demuth DG, Molleman A. Cannabinoid signalling. *Life Sci*. 2006; 78:549–563. [PubMed: 16109430]
- Dinh QT, Groneberg DA, Peiser C, Mingomataj E, Joachim RA, Witt C, Arck PC, Klapp BF, Fischer A. Substance P expression in TRPV1 and trkA-positive dorsal root ganglion neurons innervating the mouse lung. *Respir Physiol Neurobiol*. 2004; 144:15–24. [PubMed: 15522699]
- Ellert-Miklaszewska A, Kaminska B, Konarska L. Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. *Cell Signal*. 2005; 17:25–37. [PubMed: 15451022]
- Ernsberger U. Role of neurotrophin signalling in the differentiation of neurons from dorsal root ganglia and sympathetic ganglia. *Cell Tissue Res*. 2009; 336:349–384. [PubMed: 19387688]
- Evans RM, Scott RH, Ross RA. Multiple actions of anandamide on neonatal rat cultured sensory neurones. *Br J Pharmacol*. 2004; 141:1223–1233. [PubMed: 15023857]
- Farquhar-Smith WP, Jaggar SI, Rice AS. Attenuation of nerve growth factor-induced visceral hyperalgesia via cannabinoid CB(1) and CB(2)-like receptors. *Pain*. 2002; 97:11–21. [PubMed: 12031775]
- Farquhar-Smith WP, Rice AS. A novel neuroimmune mechanism in cannabinoid-mediated attenuation of nerve growth factor-induced hyperalgesia. *Anesthesiology*. 2003; 99:1391–1401. [PubMed: 14639155]
- Ferrini F, Salio C, Lossi L, Gambino G, Merighi A. Modulation of inhibitory neurotransmission by the vanilloid receptor type 1 (TRPV1) in organotypically cultured mouse substantia gelatinosa neurons. *Pain*. 2010; 150:128–140. [PubMed: 20451324]
- Fischbach T, Greffrath W, Nawrath H, Treede RD. Effects of anandamide and noxious heat on intracellular calcium concentration in nociceptive drg neurons of rats. *J Neurophysiol*. 2007; 98:929–938. [PubMed: 17581853]

- Guo A, Vulchanova L, Wang J, Li X, Elde R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur J Neurosci.* 1999; 11:946–958. [PubMed: 10103088]
- Hillard CJ, Manna S, Greenberg MJ, DiCamelli R, Ross RA, Stevenson LA, Murphy V, Pertwee RG, Campbell WB. Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J Pharmacol Exp Ther.* 1999; 289:1427–1433. [PubMed: 10336536]
- Hong S, Fan J, Kemmerer ES, Evans S, Li Y, Wiley JW. Reciprocal changes in vanilloid (TRPV1) and endocannabinoid (CB1) receptors contribute to visceral hyperalgesia in the water avoidance stressed rat. *Gut.* 2009; 58:202–210. [PubMed: 18936104]
- Hong S, Zheng G, Wu X, Snider NT, Owyang C, Wiley JW. Corticosterone mediates reciprocal changes in CB 1 and TRPV1 receptors in primary sensory neurons in the chronically stressed rat. *Gastroenterology.* 2011; 140:627–637. [PubMed: 21070780]
- Hu H, Ho W, Mackie K, Pittman QJ, Sharkey KA. Brain CB₁ receptor expression following lipopolysaccharide-induced inflammation. *Neuroscience.* 2012; 227:211–222. [PubMed: 23041513]
- Ibrahim BM, Abdel-Rahman AA. Differential modulation of brainstem phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase 1/2 signaling underlies WIN55,212-2 centrally mediated pressor response in conscious rats. *J Pharmacol Exp Ther.* 2012; 340:11–18. [PubMed: 21946192]
- Immke DC, Gavva NR. The TRPV1 receptor and nociception. *Semin Cell Dev Biol.* 2006; 17:582–591. [PubMed: 17196854]
- Jankowski, MP.; Koerber, HR. Neurotrophic Factors and Nociceptor Sensitization.. In: Kruger, L.; Light, AR., editors. *Translational Pain Research: From Mouse to Man.* CRC Press; Boca Raton, FL: 2010. Chapter 2
- Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron.* 2002; 36:57–68. [PubMed: 12367506]
- Kerstein PC, Jacques-Fricke BT, Rengifo J, Mogen BJ, Williams JC, Gottlieb PA, Sachs F, Gomez TM. Mechanosensitive TRPC1 channels promote calpain proteolysis of talin to regulate spinal axon outgrowth. *J Neurosci.* 2013; 33:273–285. [PubMed: 23283340]
- Khasabova IA, Harding-Rose C, Simone DA, Seybold VS. Differential effects of CB1 and opioid agonists on two populations of adult rat dorsal root ganglion neurons. *J Neurosci.* 2004; 24:1744–53. [PubMed: 14973253]
- Khasabova IA, Simone DA, Seybold VS. Cannabinoids attenuate depolarization-dependent Ca²⁺ influx in intermediate-size primary afferent neurons of adult rats. *Neuroscience.* 2002; 115:613–625. [PubMed: 12421626]
- Kim HI, Kim TH, Shin YK, Lee CS, Park M, Song JH. Anandamide suppression of Na⁺ currents in rat dorsal root ganglion neurons. *Brain Res.* 2005; 1062:39–47. [PubMed: 16256960]
- Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K. Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with delta/c-fibers and colocalization with trk receptors. *J Comp Neurol.* 2005; 493:596–606. [PubMed: 16304633]
- Lu B, Luquetti AO, Rassi A, PereiraPerrin M. Autoantibodies to neurotrophic receptors TrkA, TrkB and TrkC in patients with acute Chagas' disease. *Scand J Immunol.* 2010; 71:220–225. [PubMed: 20415787]
- Lu SG, Gold MS. Inflammation-induced increase in evoked calcium transients in subpopulations of rat dorsal root ganglion neurons. *Neuroscience.* 2008; 153:279–288. [PubMed: 18367340]
- Luo H, Cheng J, Han JS, Wan Y. Change of vanilloid receptor 1 expression in dorsal root ganglion and spinal dorsal horn during inflammatory nociception induced by complete Freund's adjuvant in rats. *Neuroreport.* 2004; 15:655–658. [PubMed: 15094470]
- Ma F, Zhang L, Westlund KN. Reactive oxygen species mediate TNFR1 increase after TRPV1 activation in mouse DRG neurons. *Mol Pain.* 2009; 5:31. [PubMed: 19531269]
- Mahmud A, Santha P, Paule C, Nagy I. Cannabinoid 1 receptor activation inhibits transient receptor potential vanilloid type 1 receptor-mediated cationic influx into rat cultured primary sensory neurons. *Neuroscience.* 2009; 162:1202–1211. [PubMed: 19463903]

- Malik-Hall M, Dina OA, Levine JD. Primary afferent nociceptor mechanisms mediating NGF-induced mechanical hyperalgesia. *Eur J Neurosci*. 2005; 21:3387–3394. [PubMed: 16026476]
- Manca A, Capsoni S, Di Luzio A, Vignone D, Malerba F, Paoletti F, Brandi R, Arisi I, Cattaneo A, Levi-Montalcini R. Nerve growth factor regulates axial rotation during early stages of chick embryo development. *Proc Natl Acad Sci U S A*. 2012; 109:2009–2014. [PubMed: 22308471]
- Millns PJ, Chapman V, Kendall DA. Cannabinoid inhibition of the capsaicin-induced calcium response in rat dorsal root ganglion neurones. *Br J Pharmacol*. 2001; 132:969–971. [PubMed: 11226126]
- Miranda A, Nordstrom E, Mannem A, Smith C, Banerjee B, Sengupta JN. The role of transient receptor potential vanilloid 1 in mechanical and chemical visceral hyperalgesia following experimental colitis. *Neuroscience*. 2007; 148:1021–1032. [PubMed: 17719181]
- Ozaïta A, Puighermanal E, Maldonado R. Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. *J Neurochem*. 2007; 102:1105–1114. [PubMed: 17484726]
- Pezet S, Spyropoulos A, Williams RJ, McMahon SB. Activity-dependent phosphorylation of Akt/PKB in adult DRG neurons. *Eur J Neurosci*. 2005; 21:1785–1797. [PubMed: 15869474]
- Price TJ, Patwardhan A, Akopian AN, Hargreaves KM, Flores CM. Modulation of trigeminal sensory neuron activity by the dual cannabinoid-vanilloid agonists anandamide, N-arachidonoyl-dopamine and arachidonyl-2-chloroethylamide. *Br J Pharmacol*. 2004; 141:1118–1130. [PubMed: 15006899]
- Price TJ, Louria MD, Candelario-Soto D, Dussor GO, Jeske NA, Patwardhan AM, Diogenes A, Trotter AA, Hargreaves KM, Flores CM. Treatment of trigeminal ganglion neurons in vitro with NGF, GDNF or BDNF: effects on neuronal survival, neurochemical properties and TRPV1-mediated neuropeptide secretion. *BMC Neurosci*. 2005; 6:4. [PubMed: 15667652]
- Price TJ, Flores CM. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *J Pain*. 2007; 8:263–272. [PubMed: 17113352]
- Richardson JD, Kilo S, Hargreaves KM. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain*. 1998; 75:111–119. [PubMed: 9539680]
- Roberts LA, Christie MJ, Connor M. Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. *Br J Pharmacol*. 2002; 137:421–428. [PubMed: 12359623]
- Ross RA, Coutts AA, McFarlane SM, Anavi-Goffer S, Irving AJ, Pertwee RG, MacEwan DJ, Scott RH. Actions of cannabinoid receptor ligands on rat cultured sensory neurones: implications for antinociception. *Neuropharmacology*. 2001; 40:221–232. [PubMed: 11114401]
- Ross RA. Anandamide and vanilloid TRPV1 receptors. *Br J Pharmacol*. 2003; 140:790–801. [PubMed: 14517174]
- Sagar DR, Kelly S, Millns PJ, O'Shaughnessy CT, Kendall DA, Chapman V. Inhibitory effects of CB1 and CB2 receptor agonists on responses of DRG neurons and dorsal horn neurons in neuropathic rats. *Eur J Neurosci*. 2005; 22:371–379. [PubMed: 16045490]
- Sántha P, Jenes A, Somogyi C, Nagy I. The endogenous cannabinoid anandamide inhibits transient receptor potential vanilloid type 1 receptor-mediated currents in rat cultured primary sensory neurons. *Acta Physiol Hung*. 2010; 97:149–158. [PubMed: 20511124]
- Schicho R, Florian W, Liebmann I, Holzer P, Lippe IT. Increased expression of TRPV1 receptor in dorsal root ganglia by acid insult of the rat gastric mucosa. *Eur J Neurosci*. 2004; 19:1811–1818. [PubMed: 15078554]
- Schwartz ES, La JH, Scheff NN, Davis BM, Albers KM, Gebhart GF. TRPV1 and TRPA1 Antagonists Prevent the Transition of Acute to Chronic Inflammation and Pain in Chronic Pancreatitis. *J Neurosci*. 2013; 33:5603–5611. [PubMed: 23536075]
- Shu X, Mendell LM. Acute sensitization by NGF of the response of small-diameter sensory neurons to capsaicin. *J Neurophysiol*. 2001; 86:2931–2938. [PubMed: 11731549]
- Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, Davis JB. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol*. 2000; 129:227–230. [PubMed: 10694225]

- Sousa-Valente J, Andreou AP, Urban L, Nagy I. Transient receptor potential ion channels in primary sensory neurons as targets for novel analgesics. *Br J Pharmacol*. 2014a; 171:2508–2527. [PubMed: 24283624]
- Sousa-Valente J, Varga A, Ananthan K, Khajuria A, Nagy I. Anandamide in primary sensory neurons: too much of a good thing? *Eur J Neurosci*. 2014b; 39:409–418. [PubMed: 24494681]
- Stein AT, Ufret-Vincenty CA, Hua L, Santana LF, Gordon SE. Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. *J Gen Physiol*. 2006; 128:509–522. [PubMed: 17074976]
- Stemkowski PL, Smith PA. Sensory neurons, ion channels, inflammation and the onset of neuropathic pain. *Can J Neurol Sci*. 2012; 39:416–435. [PubMed: 22728849]
- Tognetto M, Amadesi S, Harrison S, Creminon C, Trevisani M, Carreras M, Matera M, Geppetti P, Bianchi A. Anandamide excites central terminals of dorsal root ganglion neurons via vanilloid receptor-1 activation. *J Neurosci*. 2001; 21:1104–1109. [PubMed: 11160380]
- Veress G, Meszar Z, Muszil D, Avelino A, Matesz K, Mackie K, Nagy I. Characterisation of cannabinoid 1 receptor expression in the perikarya, and peripheral and spinal processes of primary sensory neurons. *Brain Struct Funct*. 2013; 218:733–750. [PubMed: 22618437]
- Wang ZY, Bjorling DE. Tumor necrosis factor-induces expression and release of interleukin-6 by human urothelial cells. *Inflamm Res*. 2011; 60:525–532. [PubMed: 21191629]
- White JP, Cibelli M, Fidalgo AR, Nagy I. Extracellular signal-regulated kinases in pain of peripheral origin. *Eur J Pharmacol*. 2011a; 650:8–17. [PubMed: 20950608]
- White JP, Urban L, Nagy I. TRPV1 function in health and disease. *Curr Pharm Biotechnol*. 2011b; 12:130–144. [PubMed: 20932253]
- Winston J, Toma H, Shenoy M, Pasricha PJ. Nerve growth factor regulates VR-1 mRNA levels in cultures of adult dorsal root ganglion neurons. *Pain*. 2001; 89:181–186. [PubMed: 11166474]
- Winter J, Forbes CA, Sternberg J, Lindsay RM. Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. *Neuron*. 1988; 1:973–981. [PubMed: 3272159]
- Woolf CJ, Ma Q. Nociceptors--noxious stimulus detectors. *Neuron*. 2007; 55:353–364. [PubMed: 17678850]
- Yi FX, Boeldt DS, Gifford SM, Sullivan JA, Grummer MA, Magness RR, Bird IM. Pregnancy enhances sustained Ca²⁺ bursts and endothelial nitric oxide synthase activation in ovine uterine artery endothelial cells through increased connexin 43 function. *Biol Reprod*. 2010; 82:66–75. [PubMed: 19741206]
- Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J*. 2005; 24:4211–4223. [PubMed: 16319926]
- Zhu W, Oxford GS. Phosphoinositide-3-kinase and mitogen activated protein kinase signaling pathways mediate acute NGF sensitization of TRPV1. *Mol Cell Neurosci*. 2007; 34:689–700. [PubMed: 17324588]
- Zhuang ZY, Xu H, Clapham DE, Ji RR. Phosphatidylinositol 3-kinase activates ERK in primary sensory neurons and mediates inflammatory heat hyperalgesia through TRPV1 sensitization. *J Neurosci*. 2004; 24:8300–8309. [PubMed: 15385613]

Highlights

1. CB1, trkA, and TRPV1 are co-localized in a subpopulation of adult mouse afferent neurons.
2. NGF increased the number of neurons that responded to capsaicin.
3. CB1 agonist ACEA (10 nM) inhibited the NGF-induced responses.
4. ACEA inhibited NGF-induced phosphorylation of AKT.
5. Blocking PI3 kinase activity attenuated the NGF-induced responses.

Figure 1.

A: Representative photoimages showing localization of trkA, TRPV1, and CB1 in adult mouse DRG neurons (arrow heads). Neurons were considered labeled with the specific antibody when the fluorescent intensity was distinctively higher than background. Using IgG from normal, non-immunized rabbit (instead of specific antibodies) resulted in complete lack of specific staining (-). Scale bar indicates 50 μ m. B: Triple co-localization staining identified neurons that expressed all three proteins as indicated by arrows.

Figure 2.

A: Representative tracings illustrating that capsaicin (300 nM) induced a rapid increase in intracellular Ca^{2+} concentrations in about one third of the neurons and the amplitude and duration of capsaicin-induced responses varied considerably among neurons. B: Exposure to NGF (100 ng/ml) for 30 minutes significantly increased the number of neurons that responded to capsaicin. Pretreatment with the selective CB1 agonist ACEA (10 nM) abolished the NGF-induced increase in the number of neurons that responded to capsaicin, and this effect of ACEA was reversed by pretreatment with the selective CB1 antagonist AM251 (100 nM). $n = 8$. * $p < 0.01$ vs capsaicin-treated group. @: $p < 0.01$ vs NGF +capsaicin-treated group. # $p < 0.01$ vs ACEA+NGF+capsaicin-treated group.

Figure 3.

Immunoblotting analysis revealed that exposure to capsaicin alone did not alter the abundance of phosphorylated AKT (A) or ERK1/2 (B). Exposure to NGF (100 ng/ml) for 30 minutes increased phosphorylation of AKT (A) and ERK1/2 (B). The increase in phosphorylation of AKT or ERK1/2 in neurons treated with the combination of NGF and capsaicin was not different from those treated with NGF alone. Pretreatment with ACEA attenuated the increase in phosphorylation of AKT (A) without affecting phosphorylation of ERK1/2 (B) in neurons treated with NGF and capsaicin. $n = 5$. * $p < 0.05$ vs control group. @: $p < 0.05$ vs capsaicin-treated group. # $p < 0.05$ vs NGF+capsaicin-treated group.

Figure 4.

Immunoblotting analysis revealed that pretreatment with the PI3 kinase inhibitor wortmannin (wort, 10 nM) abolished increased phosphorylation of AKT in neurons treated with NGF or the combination of NGF and capsaicin (A). Treatment with wortmannin did not affect NGF- or NGF and capsaicin-induced increased phosphorylation of ERK1/2 (B). Pretreatment with wortmannin attenuated the NGF-induced increase in the number of

neurons responding to capsaicin (C). $n = 5-8$. * $p < 0.05$ vs control group. @: $p < 0.05$ vs capsaicin-treated group. #, ** $p < 0.05$, or 0.01 vs NGF+capsaicin-treated group.

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