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Presynaptic inhibition of transient receptor potential vanilloid type 1 (TRPV1) receptors by noradrenaline in nociceptive neurons

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Key points

- The transient receptor potential vanilloid type 1 (TRPV1) receptor is a polymodal molecular integrator in the pain pathway expressed in Aδ- and C-fibre nociceptors and is responsible for the thermal hyperalgesia associated with inflammatory pain.
- Noradrenaline strongly inhibited the activity of TRPV1 channels in dorsal root ganglia neurons. The effect of noradrenaline was reproduced by clonidine and antagonized by yohimbine, consistent with contribution of α2 adrenergic receptors.
- The inhibitory effect of noradrenaline on TRPV1 channels was dependent on calcium influx and linked to calcium/calmodulin-dependent protein kinase II.
- In spinal cord slices, clonidine reduced the frequency of capsaicin-induced miniature EPSCs in the presence of tetrodotoxin and ω-conotoxin-MVIIC, consistent with inhibition of presynaptic TRPV1 channels by α2 adrenergic receptors.
- We suggest that modulation of presynaptic TRPV1 channels in nociceptive neurons by descending noradrenergic inputs may constitute a mechanism for noradrenaline to modulate incoming noxious stimuli in the dorsal horn of the spinal cord.

Abstract The transient receptor potential vanilloid type 1 (TRPV1) receptor is a well-known contributor to nociceptor excitability. To address whether noradrenaline can down-regulate TRPV1 channel activity in nociceptors and reduce their synaptic transmission, the effects of noradrenaline and clonidine were tested on the capsaicin-activated current recorded from acutely dissociated small diameter (<27 μm) dorsal root ganglia (DRG) neurons and on miniature (m)EPSCs recorded from large lamina I neurons in horizontal spinal cord slices. Noradrenaline or clonidine inhibited the capsaicin-activated current by ~60%, and the effect was reversed by yohimbine, confirming that it was mediated by activation of α2 adrenergic receptors. Similarly, clonidine reduced the frequency of capsaicin-induced mEPSCs by ~60%. Inhibition of capsaicin-activated current by noradrenaline was mediated by GTP binding proteins, and was highly dependent on calcium influx. The inhibitory effect of noradrenaline on the capsaicin-activated current was not affected either by blocking the activity of protein kinase A with H89, or by blocking the activity of protein kinase C with bisindolylmaleimide II. In contrast, when the calcium/calmodulin-dependent protein kinase II (CaMKII) was blocked with KN-93, the inhibitory effect of noradrenaline on the capsaicin-activated current was greatly reduced, suggesting that activation of adrenergic receptors in DRG neurons is preferentially linked to CaMKII activity. We suggest that modulation of TRPV1 channels by noradrenaline in nociceptive neurons is a mechanism whereby noradrenaline may suppress incoming noxious stimuli at the primary synaptic afferents in the dorsal horn of the spinal cord.

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Abbreviations BIM, bisindolylmaleimide II; CaMKII, calcium/calmodulin-dependent protein kinase II; CMF, Ca^{2+}-, Mg^{2+}-free; DRG, dorsal root ganglia; GPCR, G-protein-coupled receptor; mEPSCs, miniature EPSCs; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TRPV1, transient receptor potential vanilloid type 1.

Introduction

Nociceptive signals relayed to the dorsal horn of the spinal cord by nociceptors are subject to modulation by supraspinal centres (Fields *et al.* 1991; Millan, 2002; Ossipov *et al.* 2014). Behavioural studies support the anti-nociceptive effects of noradrenaline mediated by spinal α2 adrenergic receptors (Millan, 1992; Takano & Yaksh, 1992, 1993; Millan *et al.* 1994). Potentiation of descending noradrenergic inputs may be particularly significant during sustained nociceptive signals associated with nerve injury or inflammation (Cho *et al.* 1995; Tsuruoka & Willis, 1996; Green *et al.* 1998; Martin *et al.* 1999; Stone *et al.* 1999; Xu *et al.* 1999).

Noradrenergic fibres from A5, A6 and A7 regions provide the main source of spinal noradrenaline (Clark & Proudfit, 1991*b*, *a*; Kwiat & Basbaum, 1992; Sluka & Westlund, 1992), and establish axo-somatic and axo-dendritic synaptic contacts with projection neurons and interneurons (Hagihira *et al.* 1990; Nicholas *et al.* 1993; Rosin *et al.* 1993; Stone *et al.* 1998; Shi *et al.* 1999; Riedl *et al.* 2009), supporting postsynaptic effects of noradrenaline. However, noradrenergic fibres, similar to other monoaminergic inputs, also possess varicosities with non-synaptic terminations (Hagihira *et al.* 1990; Ridet *et al.* 1992, 1993), suggesting the possibility of volume transmission and presynaptic effects. Possible presynaptic effects of noradrenaline on primary sensory neurons are supported by expression of adrenergic receptors both in the cell body and in central terminals of dorsal root ganglia (DRG) neurons (Nicholas *et al.* 1993; Cho *et al.* 1997; Gold *et al.* 1997; Stone *et al.* 1998; Birder & Perl, 1999). Consistent with this pattern of expression, noradrenaline modulates calcium channels (Dunlap & Fischbach, 1981; Marchetti *et al.* 1986; Bean, 1989), ATP-sensitive channels (Maruo *et al.* 2006), sodium channels (Khasar *et al.* 1999; Oda *et al.* 2007) and TRPM8 channels (Bavencoffe *et al.* 2010) in DRG neurons.

The transient receptor potential vanilloid type 1 (TRPV1) receptor is a polymodal molecular integrator in the pain pathway expressed in Aδ- and C-fibre nociceptors (Szallasi *et al.* 1995; Caterina *et al.* 1997; Cavanaugh *et al.* 2011), and can be activated by vanilloids (capsaicin and resineferatoxin) (Caterina *et al.* 1997), endogenous lipids (anandamide) (Zygmunt *et al.* 1999), protons (Ahern *et al.* 2005; Dhaka *et al.* 2009), polyamines (Ahern *et al.* 2006) and noxious heat (Caterina *et al.* 1997). Pharmacological and genetic studies support the contribution of TRPV1 channels to the development of different pain conditions, including thermal hyperalgesia associated with inflammatory pain (Caterina *et al.* 2000; Davis *et al.* 2000), bone cancer pain (Ghilardi *et al.* 2005), migraine (Akerman *et al.* 2004), irritable bowel syndrome (Jones *et al.* 2005) and arthritis (Szabo *et al.* 2005).

TRPV1 channels are expressed on the soma, peripheral and central terminals of nociceptors which make synaptic contacts with second-order neurons in the dorsal horn of the spinal cord (Holzer, 1991; Winter *et al.* 1993; Szallasi *et al.* 1995; Caterina *et al.* 1997; Tominaga *et al.* 1998; Guo *et al.* 1999; Hwang *et al.* 2004). Activation of presynaptic TRPV1 channels by endovanilloids or capsaicin triggers the release of peptides and modulates glutamatergic transmission in the dorsal horn of the spinal cord by increasing the frequency of miniature (m)EPSCs and by inhibiting evoked EPSCs (Yang *et al.* 1998, 1999; Tognetto *et al.* 2001; Nakatsuka *et al.* 2002; Baccei *et al.* 2003; Labrakakis & MacDermott, 2003; Tong & MacDermott, 2006; Medvedeva *et al.* 2008). The presynaptic localization of TRPV1 channels on the central terminals of nociceptors suggests that they are well positioned to interact with neuromodulators released in the dorsal horn of the spinal cord by descending fibres (Kim *et al.* 2014; Chakraborty *et al.* 2016), and point to a possible mechanism by which supraspinal centres may modulate the excitability of nociceptors.

Here, using acutely dissociated DRG neurons and horizontal spinal cord slices *in vitro*, we show that noradrenaline down-regulates the activity of presynaptic TRPV1 channels expressed in nociceptors, and reduces the frequency of capsaicin-induced mEPSCs recorded from large lamina I neurons in the dorsal horn of the spinal cord. Pharmacological studies indicate that the effect of noradrenaline is mediated by activation of α2 adrenergic receptors, is dependent on calcium influx and is coupled to calcium/calmodulin-dependent protein kinase II (CaMKII).

Methods

Animals

Sprague–Dawley rats (both male and female) of postnatal days 18–28 were used in this study. This postnatal age was chosen because the postnatal development of dorsal

horn sensory processing is mostly complete (Fitzgerald, 2005), although afferent synaptic input is further refined into adulthood (Park *et al.* 1999; Nakatsuka *et al.* 2000). Furthermore, in horizontal spinal cord slices, it is possible to record from large lamina I neurons at these ages, which becomes increasingly difficult with heavier laminar myelination at older postnatal ages. All procedures were performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by Stony Brook University Institutional Animal Care and Use Committee.

Dissociated DRG neurons

Rats were deeply anaesthetized with isoflurane and decapitated. Both thoracic and lumbar segments of the spinal cord were removed and placed in a cold Ca^{2+}-, Mg^{2+}-free (CMF) Hank's solution containing (in mM): 137 NaCl, 5.3 KCl, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 Hepes and 5.5 glucose (pH 7.4 with NaOH). The bone surrounding the spinal cord was removed and DRG were exposed and pulled out. After removing the roots, ganglia were chopped in half and incubated for 20 min at 34°C in CMF Hank's solution containing 20 U ml^{-1} papain (Worthington Biochemical, Lakewood, NJ, USA) and 5 mM DL-cysteine. Ganglia were then treated for 20 min at 34°C with 3 mg ml^{-1} collagenase (Type I, Sigma-Aldrich, St. Louis, MO, USA) and 4 mg ml^{-1} Dispase II (Boehringer Mannheim, Indianapolis, IN, USA) in CMF Hank's solution. Ganglia were then washed with Leibovitz's L-15 medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal calf serum and 5 mM Hepes. Individual cells were dispersed by mechanical trituration using fire-polished Pasteur pipettes with decreasing bore size and plated on glass coverslips treated with 30 μg ml^{-1} poly-D-lysine. Cells were incubated in the supplemented L-15 solution at 34°C (in 5% CO_2) for 2 h, and then stored at room temperature in Neurobasal medium (Gibco) and used over the next 4–6 h. This protocol yields spherical cell bodies without neurites. The cells can be lifted from the cover slip after establishing the whole-cell configuration in order to facilitate rapid solution changes using flow pipes. Small DRG neurons (diameters <27 μm) sensitive to capsaicin were chosen for recording. Small DRG neurons were initially selected by measuring the diameter from images captured to a computer by a CCD camera (Oly-150, Olympus Imaging America Inc., Center Valley, PA, USA) using a video acquisition card (dP dPict Imaging, Inc., Indianapolis, IN, USA). A more accurate measurement of cell diameter was obtained from measurements of whole-cell capacitance assuming a membrane capacitance of 1 μF cm^{-2} and spherical shape. Cell capacitance was measured by integrating the average of 10 current responses to a –5 mV step from −80 mV filtered at 10 kHz and acquired at 50 kHz. DRG neurons were first tested for capsaicin sensitivity, and only those responding to capsaicin (75% of those tested), corresponding to a subset of nociceptors (Cardenas *et al.* 1995; Caterina *et al.* 1997; Petruska *et al.* 2000), were used for the experiments.

Horizontal spinal cord slices

Rats were deeply anaesthetized with isoflurane and decapitated. After decapitation, the ventral aspect of the vertebral column was exposed and immersed in ice-cold dissecting solution (in mM): 87 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4.H_2O$, 26 $NaHCO_3$, 6 $MgCl_2$, 0.5 $CaCl_2$, 20 glucose, 77 sucrose, 1 kynurenic acid, oxygenated with 95/5% O_2/CO_2. The lumbar part of the spinal cord was exposed and carefully removed with L4 and L5 dorsal roots attached. Horizontal slices were made manually. First, the spinal cord was cut in half with a razor blade through the parasagittal plane to produce a hemisected spinal cord. With a second cut, about 45 degree to the parasagittal plane, the ventral part of the hemisected cord was removed such that the result was a horizontal slice (400–500 μm thick) with the L4–L5 dorsal roots attached. Horizontal slices were then immersed in oxygenated recovery solution (same as dissecting solution, but without kynurenic acid) at 35°C and allowed to recover for 1 h. After 1 h the slices were transferred to a storage solution (same as recovery solution, but at room temperature) and kept for the next 4–5 h. Horizontal spinal cord slices, in contrast to coronal or parasagittal slices, offer the unique advantage that afferent fibres and large lamina I neurons in the dorsal horn of the spinal cord are preserved virtually intact. In horizontal slices, large lamina I neurons were visualized through the white matter using an infrared light-emitting diode (IR-LED) illumination (Safronov *et al.* 2007; Szucs *et al.* 2009; Li *et al.* 2015) and a CCD video camera (Oly 150, Olympus). The IR-LED is attached to a 40× water immersion objective mounted on an upright microscope (BX51WI, Olympus). Large lamina I neurons (cross-sectional soma area > 220 μm^2), consistent with projection neurons (Al Ghamdi *et al.* 2009), located 20–25 μm below the white matter were selected for recording.

Electrophysiology in isolated DRG neurons

Whole-cell recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were pulled from borosilicate glass (A-M Systems, Sequim, WA, USA) using a Sutter P97 puller (Sutter Instrument, Novato, CA, USA). The resistance of the patch pipette was 1.3–1.8 MΩ when filled with the standard internal CsCl-based solution. The shank of the patch pipette was wrapped with parafilm to reduce pipette capacitance. In whole-cell mode, the capacity current was

reduced by using the amplifier circuitry. To reduce voltage errors, 70–80% of series resistance compensation was applied. After the whole cell configuration was established, the cell was lifted up and placed in front of an array of quartz fibre flow pipes (320 μm internal diameter) containing the test solutions. Solutions were changed in ∼1 s by moving the cell from one pipe to another. All experiments with isolated DRGs were done at room temperature (22 ± 1°C), with the exception of those in Fig. 6 that were carried out at 35 ± 1°C. Solutions were heated with a temperature controller (Warner TC-344B, Warner Instruments, Hamden, CT, USA). In voltage clamp experiments a Cs-based internal solution was used to block outward currents through potassium channels. This solution contained (in mM): 125 CsCl, 10 NaCl, 2 $MgCl_2$, 10 EGTA, 10 Hepes, 14 Tris-creatine phosphate, 4 Mg-ATP and 0.3 Na-GTP (pH 7.2 with CsOH). The standard external solution was a modified Tyrode's solution containing (in mM): 151 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 2.5 KCl, 10 Hepes and 13 glucose (pH 7.4 with NaOH).

Voltage clamp protocols for isolated DRG neurons

TRPV1 current was determined as the capsaicin-activated current by subtracting currents before and after application of 1 μM capsaicin. The *I–V* relationship for the TRPV1 current was determined using voltage ramps (1.6 mV ms^{-1}). Using fast ramp commands rather than obtaining the time courses of inward/outwards currents activated by puffs of capsaicin offers two main advantages. First, it is possible to study the *I–V* relationship of the capsaicin-activated current on a broad range of voltages (from −100 to +100 mV) during a single sweep. Second, ramps are delivered in the same sweep from −100 to +100 mV and then from +100 to −100 mV (Fig. 1*A*,

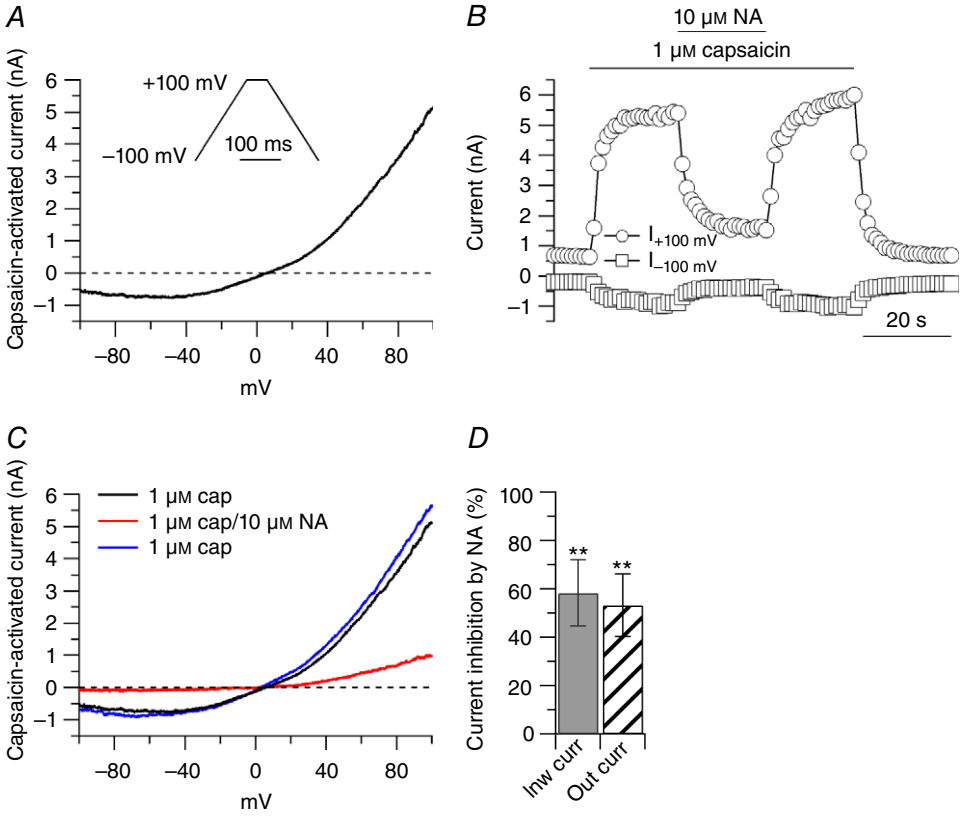

Figure 1. Inhibition of capsaicin-activated current by noradrenaline
A, ionic currents through TRPV1 channels were determined by subtracting currents before and after application of 1 μM capsaicin. Representative capsaicin-activated current recorded during the 'down ramp'. *Inset*: voltage clamp protocol. Ramps (1.6 mV ms^{-1}) were delivered in the same sweep from −100 to + 100 mV and from + 100 to −100 mV with the 'down ramp' preceded by 50–200 ms at + 100 mV to inactivate voltage-dependent sodium and calcium channels. *B*, effects of 10 μM noradrenaline (NA) on capsaicin-activated current. Inward and outward currents were measured at −100 and +100 mV, respectively. *C*, capsaicin-activated currents from the cell in *B*: 1 μM capsaicin (black trace), 10 μM noradrenaline on top of capsaicin (red trace), 1 μM capsaicin upon washing out noradrenaline (blue trace). *D*, in collected results, 10 μM noradrenaline, applied on top of capsaicin, inhibited the inward current by 58 ± 13% ($n = 6$, $^{**}P < 0.01$, paired *t* test), and the outward current by 53 ± 13% ($n = 6$, $^{**}P < 0.01$, paired *t* test), with respect to control.

inset), with the 'down ramp' preceded by 50–200 ms at +100 mV. With this procedure, currents carried by voltage-dependent sodium and calcium channels are largely inactivated before the 'down ramp', which allows more precise determination of TRPV1 currents by eliminating overlapping voltage-dependent sodium and calcium currents (Puopolo *et al.* 2013; Chakraborty *et al.* 2016). *I–V* relationships for TRPV1 channels were therefore determined by using the 'down ramp'. TRPV1 channels show some degree of desensitization upon repeated or sustained application of the agonist capsaicin (Caterina *et al.* 1997). To allow reliable comparison between capsaicin-activated currents before and after drug treatment, the effect of drugs on the capsaicin-activated current was measured as soon as the capsaicin-activated current reached a stable value (usually 15–20 s, Fig. 1*B*). The typical experiment consisted of 20 s of perfusion with capsaicin, followed by 20 s of the test drug in combination with capsaicin, and 20 s of washout in capsaicin.

Electrophysiology in spinal cord slices

Whole-cell voltage clamp recordings were made with a Multiclamp 700B amplifier (Molecular Devices). Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL, USA) using a Sutter P97 puller (Sutter Instrument). The resistance of the patch pipette was 1.3–1.8 MΩ when filled with the standard internal Cs-methanesulfonate-based solution. The shank of the patch pipette was wrapped with parafilm to reduce pipette capacitance. In whole-cell mode, the capacity current was further reduced by using the amplifier circuitry. To reduce voltage errors, 40–50% of series resistance compensation was applied. EPSCs were elicited by electrical stimulation of the dorsal root (L4 or L5) attached to the spinal cord slice. The dorsal root was included in a glass suction electrode and electrically stimulated at 0.016 Hz with 500 μA of constant current (100 μs duration) delivered with a constant current stimulus isolation unit (ISO-flex, AMPI, Jerusalem, Israel). Spontaneous mEPSCs and evoked EPSCs were recorded in voltage clamp at a holding potential of −70 mV. For evoked EPSCs (Fig. 10), the external solution was (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4.H_2O$, 20 glucose, 0.01 bicuculline and 0.005 strychnine, oxygenated with 95/5% O_2/CO_2; the internal solution was (in mM): 125 caesium methanesulfonate, 10 NaCl, 2 $MgCl_2$, 14 phosphocreatine, 4 Mg-ATP, 0.3 GTP, 10 EGTA, 10

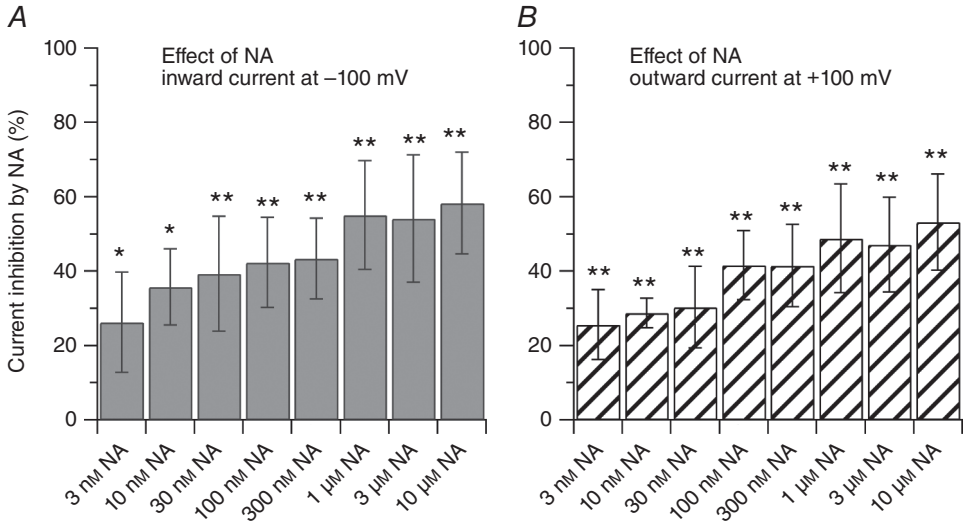

Figure 2. Dose-dependent inhibition of capsaicin-activated current by noradrenaline
Dose-dependent inhibition of capsaicin-activated currents by noradrenaline showing similar effects on inward and outward currents. Each bar represents an independent experiment. For each concentration, statistical significance was assessed with a paired *t* test by comparing the effect of the drug to its own control. The IC_{50} was determined using a Log inhibitor *versus* normalized response equation: $y = 100/(1 + 10^{\wedge}((x\text{-Log}IC_{50})))$. *A*, the inward current was reduced by 26 ± 13% ($n = 8$, $^{*}P < 0.05$) by 3 nM; by 36 ± 10% ($n = 6$, $^{*}P < 0.05$) by 10 nM; by 39 ± 15% ($n = 11$, $^{**}P < 0.01$) by 30 nM; by 42 ± 12% ($n = 8$, $^{**}P < 0.01$) by 100 nM; by 43 ± 11% ($n = 7$, $^{**}P < 0.01$) by 300 nM; by 55 ± 14% ($n = 17$, $^{**}P < 0.01$) by 1 μM; by 54 ± 17% ($n = 9$, $^{**}P < 0.01$) by 3 μM; by 58 ± 13% ($n = 6$, $^{**}P < 0.01$) by 10 μM noradrenaline. The IC_{50} for the inward current was estimated to be 7.1 nM (2.1–22.7 nM, 95% confidence interval). *B*, the outward current was reduced by 26 ± 9% ($n = 8$, $^{**}P < 0.01$) by 3 nM; by 29 ± 4% ($n = 6$, $^{**}P < 0.01$) by 10 nM; by 30 ± 11% ($n = 11$, $^{**}P < 0.01$) by 30 nM; by 42 ± 9% ($n = 8$, $^{**}P < 0.01$) by 100 nM; by 42 ± 11% ($n = 7$, $^{**}P < 0.01$) by 300 nM; by 49 ± 15% ($n = 17$, $^{**}P < 0.01$) by 1 μM; by 47 ± 13% ($n = 9$, $^{**}P < 0.01$) by 3 μM; by 53 ± 13% ($n = 6$, $^{**}P < 0.01$) by 10 μM noradrenaline. The IC_{50} for the outward current was estimated to be 9.6 nM (2.5–34.2 nM, 95% confidence interval).

Hepes and 5 mM QX-314 (pH 7.2 with CsOH). For mEPSCs, the external solution was (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4.H_2O$, 20 glucose, 0.01 bicuculline, 0.005 strychnine, 300 nM tetrodotoxin (Fig. 9) and 200 nM ω-conotoxin-MVIIC (Figs 11 and 12), oxygenated with 95/5% O_2/CO_2; the internal solution was (in mM): 125 caesium methanesulfonate, 10 NaCl, 2 $MgCl_2$, 14 phosphocreatine, 4 Mg-ATP, 0.6 GDP-β-S, 10 EGTA, 10 Hepes and 5 mM QX-314 (pH 7.2 with CsOH). Recordings of evoked EPSCs and mEPSCs were made at 35 ± 1°C by heating the solutions with a temperature controller (Warner TC-344B, Warner Instruments).

Data acquisition and analysis

Currents and voltages were controlled and sampled using a Digidata 1440A interface and pCLAMP 10.4 software (Molecular Devices). For isolated DRG neurons, current or voltage signals were filtered at 10 kHz (3 dB, 4-pole Bessel) and digitized at 50 kHz. Capsaicin-activated inward and outward currents were measured at −100 and +100 mV, respectively, during the "down ramp". Spontaneous and evoked EPSCs were filtered at 1 kHz (3 dB, 4-pole Bessel) and digitized at 50 kHz. Analysis was performed using Clampfit 10.4 and Igor Pro (version 6.2; WaveMetrics, Lake Oswego, OR, USA) using DataAccess (Bruxton, Seattle, WA) to import pCLAMP files into

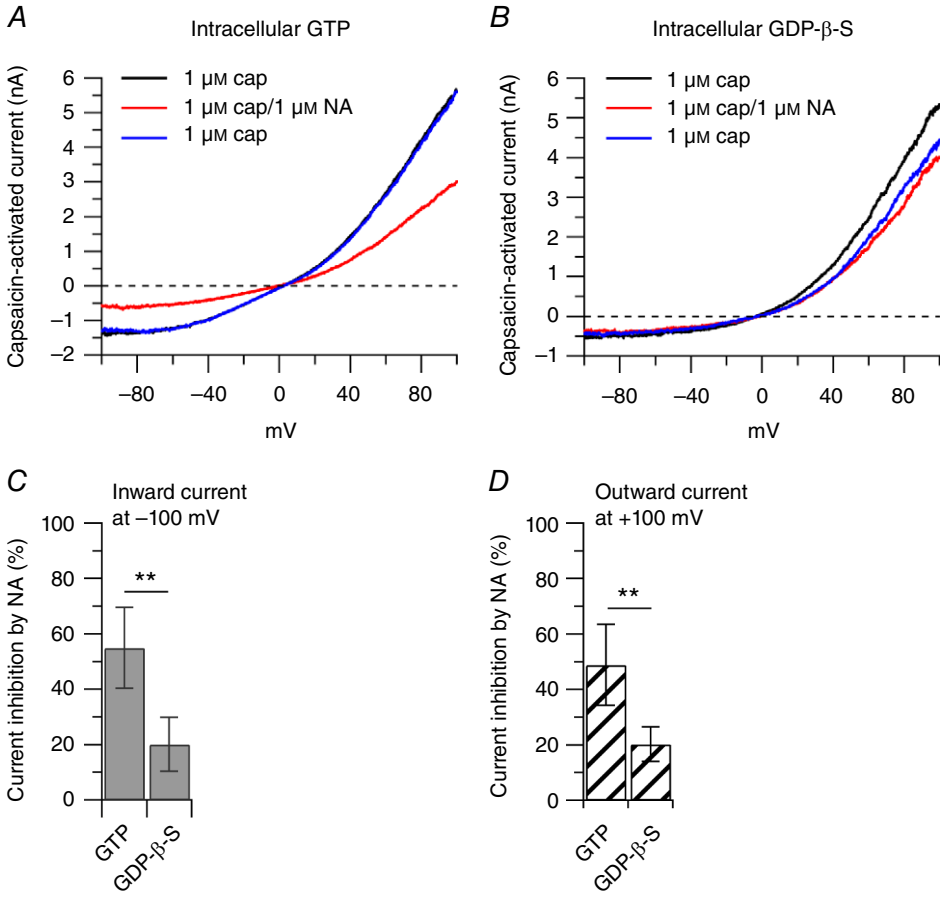

Figure 3. The effect of noradrenaline on the capsaicin-activated current is mediated by G proteins
Collected results show reduction of the effect of 1 μM noradrenaline on the capsaicin-activated inward and outward currents when the usual 0.3 mM GTP was replaced by 0.6 mM of the non-hydrolysable analogue GDP-β-S. *A*, representative capsaicin-activated currents recorded with 0.3 mM GTP in the intracellular solution: 1 μM capsaicin (black trace), after application of 1 μM noradrenaline on top of capsaicin (red trace) and upon removal of noradrenaline (blue trace). *B*, representative capsaicin-activated currents recorded with 0.6 mM GDP-β-S in the intracellular solution: 1 μM capsaicin (black trace), after application of 1 μM noradrenaline on top of capsaicin (red trace) and upon removal of noradrenaline (blue trace). *C*, the inward current was reduced by 55 ± 14% ($n = 17$) with GTP included in the intracellular solution, and by 20 ± 10% when GTP was replaced by GDP-β-S ($n = 10$), unpaired *t* test, $^{**}P < 0.01$. *D*, the outward current was reduced by 49 ± 15% ($n = 17$) with GTP included in the intracellular solution, and by 20 ± 6% when GTP was replaced by GDP-β-S ($n = 10$), unpaired *t* test, $^{**}P < 0.01$.

Igor. For mEPSCs analysis, the threshold was set at twice the average noise. Frequency and peak of mEPSCs were determined during a 1 min period in each condition. Reported voltages for spontaneous and evoked EPSCs were corrected for junction potential (−8 mV) between the internal solution and the extracellular solution measured using a flowing 3 M KCl electrode (Neher, 1992). Data are reported as mean ± SD.

Statistics

Statistical differences between data sets were analysed using Student's *t* test, or one-way ANOVA, or Wilcoxon matched-pairs test, or Kolmogorov–Smirnov test. Differences were considered significant at $^{*}P < 0.05$.

Results

Ionic currents through TRPV1 channels were activated by challenging small DRG neurons with 1 μM capsaicin, and isolated by subtracting currents before and after application of capsaicin (Fig. 1*A*). The capsaicin-activated current showed the typical outward rectification and reversal potential close to 0 mV, as expected for a TRPV1 current (Caterina *et al.* 1997). To test whether activation of adrenergic receptors could modulate currents through TRPV1 channels, the effect of noradrenaline was tested on the capsaicin-activated current. When applied on top of capsaicin, 10 μM noradrenaline caused substantial inhibition of the capsaicin-activated current (Fig. 1*B* and *C*), suggesting strong modulation of TRPV1 channels by adrenergic receptors. The effects of noradrenaline reached a maximum in 15–20 s and reversed completely with a similar time course. Noradrenaline inhibited the inward and outward currents (measured at −100 and + 100 mV, respectively) to a similar extent (Fig. 1*D*), in a dose-dependent manner. In collected results (Fig. 2), the capsaicin-activated inward current was reduced by 43 ± 11% ($n = 7$) by 300 nM noradrenaline, by 55 ± 14 % ($n = 17$) by 1 μM noradrenaline, with little or no additional effects at 3 μM (reduction by 54 ± 17%, $n = 9$) and 10 μM (reduction by 58 ± 14%, $n = 6$).

Our standard intracellular solution contained 0.3 mM GTP (see Methods). To confirm that the effect of noradrenaline was mediated by G-protein-coupled receptors (GPCRs) and dependent on GTP, we tested an intracellular solution in which GTP was replaced by the non-hydrolysable analogue GDP-β-S. Replacing the intracellular GTP with GDP-β-S caused a small reduction in the current density of the capsaicin-activated current. The inward current was reduced from 34 ± 20 to 25 ± 26 pA pF^{-1}, and the outward current was reduced from 267 ± 120 to 204 ± 212 pA pF^{-1} ($n = 10$). The inhibitory effect of 1 μM noradrenaline on the capsaicin-activated

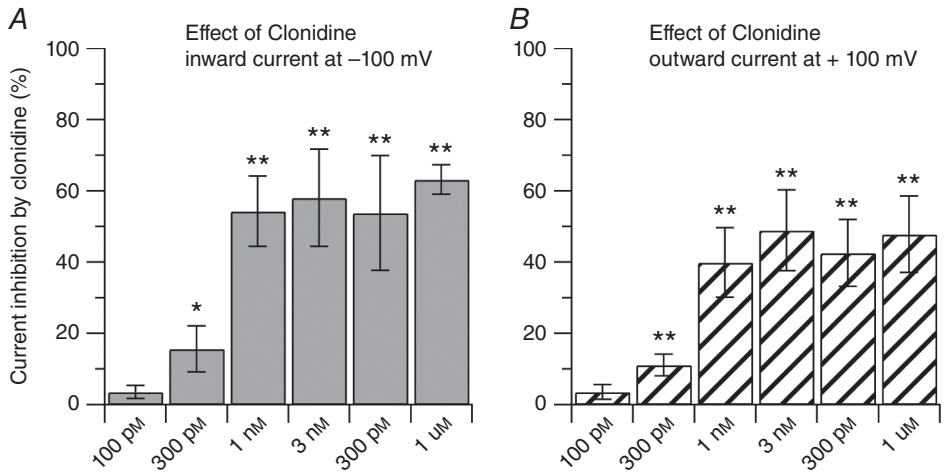

Figure 4. Dose-dependent inhibition of capsaicin-activated current by clonidine
Dose-dependent inhibition of capsaicin-activated currents by the $\alpha 2$ agonist clonidine, showing similar effects on inward and outward currents. Each bar represents an independent experiment. For each concentration, statistical significance was assessed with a paired *t* test by comparing the effect of the drug to its own control. The IC_{50} was determined using a Log inhibitor *versus* normalized response equation: $y = 100/(1 + 10\hat{}((x - LogIC_{50})))$. *A*, the inward current was reduced by 4 ± 2% ($n = 6$, $P = 0.116$) by 100 pM; by 16 ± 6% ($n = 7$, $^{*}P < 0.05$) by 300 pM; by 54 ± 10% ($n = 6$, $^{**}P < 0.01$) by 1 nM; by 58 ± 14% ($n = 6$, $^{**}P < 0.01$) by 3 nM; by 54 ± 16% ($n = 10$, $^{**}P < 0.01$) by 300 nM; by 63 ± 4% ($n = 7$, $^{**}P < 0.01$) by 1 μM clonidine. The IC_{50} for the inward current was estimated to be 487.7 pM (209.9–1130 pM, 95% confidence interval). *B*, the outward current was reduced by 4 ± 2% ($n = 6$, $P = 0.068$) by 100 pM; by 11 ± 3% ($n = 7$, $^{**}P < 0.01$) by 300 pM; by 40 ± 10% ($n = 6$, $^{**}P < 0.01$) by 1 nM; by 49 ± 11% ($n = 6$, $^{**}P < 0.01$) by 3 nM; by 43 ± 9% ($n = 10$, $^{**}P < 0.01$) by 300 nM; by 48 ± 11% ($n = 7$, $^{**}P < 0.01$) by 1 μM clonidine. The IC_{50} for the outward current was estimated to be 470.1 pM (199.6–1089 pM, 95% confidence interval).

inward current was reduced from 55 ± 14 % (n = 17, with intracellular GTP) to 20 ± 10 % (n = 10, with intracellular GDP-β-S) (Fig. 3). Taken together, these results are consistent with coupling between adrenergic receptors and TRPV1 channels that is dependent on GTP acting via G-proteins.

The next step was to identify which adrenergic receptors mediate the effect of noradrenaline. The α2 adrenergic receptor agonist clonidine potently inhibited the capsaicin-activated current similar to noradrenaline, in a dose-dependent manner (Fig. 4). The capsaicin-activated inward current was reduced by 4 ± 2% by 100 pM clonidine (n = 6), by 16 ± 6% by 300 pM clonidine (n = 7), by 54 ± 10% (n = 6) by 1 nM clonidine, with little or no additional effects at 3 nM (reduction by 58 ± 14 %, n = 6), 300 nM (reduction 54 ± 17 %, n = 7) and 1 μM (reduction by 63 ± 4 %, n = 7). The potency and saturation of the effects of clonidine suggest mediation by a receptor rather than a direct blocking effect on the channel. Mediation by a receptor was further tested by applying clonidine in combination with yohimbine, a selective antagonist at α2 adrenergic receptors. In this series of experiments, the inhibitory effect of 1 nM clonidine on the capsaicin-activated inward current was reduced from 54 ± 10% (n = 6) when used alone to 8 ± 6% (n = 7) when applied in combination with 1 nM yohimbine (Fig. 5).

A change in temperature can affect the response of TRPV1 channels to agonists (Neelands *et al.* 2008), its modulation by protons and the open probability (Neelands *et al.* 2010; Jara-Oseguera *et al.* 2016), and the voltage dependence of activation (Voets *et al.* 2004). We therefore determined whether the inhibitory effect of clonidine on the capsaicin-activated current observed at room temperature was unchanged at a more physiological temperature. For this, we tested the effect of 1 μM clonidine on the capsaicin-activated current at 35 ± 1°C. As shown in Fig. 6, 1μM clonidine inhibited the inward current by 56 ± 19% and the outward current by 48 ± 12% (n = 8), which were not different from the inhibitory effects observed at room temperature.

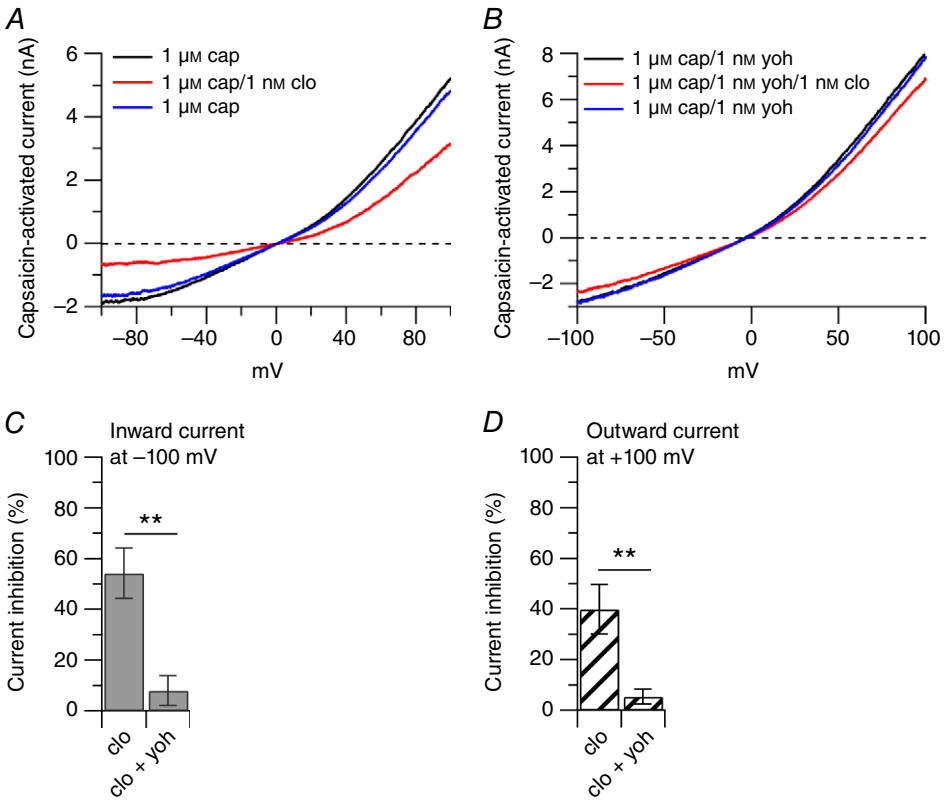

Figure 5. The effect of clonidine is reversed by the α2 antagonist yohimbine
Collected results show the effect of yohimbine (α2 antagonist) on the capsaicin-activated inward and outward currents. *A*, representative capsaicin-activated currents recorded in the presence of 1 μM capsaicin (cap, black trace), after application of 1 nM clonidine (clo) on top of capsaicin (red trace), and upon removal of clonidine (blue trace). *B*, representative capsaicin-activated currents recorded in the presence of 1 μM capsaicin + 1 nM yohimbine (yoh, black trace), after application of 1 nM clonidine on top of capsaicin and yohimbine (red trace), and upon removal of clonidine (blue trace). *C*, the inward current was reduced by 54 ± 10% (n = 6) by 1 nM clonidine and by 8 ± 5% by 1 nM clonidine + 1 nM yohimbine (n = 7), $^{**}P < 0.01$, unpaired t test. *D*, the outward current was reduced by 40 ± 10% (n = 6) by 1 nM clonidine and by 5 ± 3% by 1 nM clonidine + 1 nM yohimbine (n = 7), $^{**}P < 0.01$, unpaired t test.

Protein phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and CaMKII has been shown to play prominent roles in the modulation of TRPV1 channels (Vellani *et al.* 2001; Bhave *et al.* 2002; Jung *et al.* 2004; Bangaru *et al.* 2015). Therefore, we tested whether PKA, PKC or CaMKII activity was linked to inhibition of TRPV1 channels by adrenergic receptor activation in DRG neurons. When PKA activity was blocked by pre-incubating DRG neurons with 1 μM H89 for 30 min, the inhibitory effect of 1 μM noradrenaline on the capsaicin-activated current remained unchanged: 1 μM noradrenaline inhibited the capsaicin-activated inward current by 55 ± 10% (n = 9) in control and by 51 ± 13% (n = 11) in H89 (Fig. 7*A*). The inhibitory effect of 1 μM noradrenaline on the capsaicin-activated current remained unchanged also when PKC activity was blocked by pre-incubating DRG neurons with 1 μM bisindolylmaleimide II (BIM) for 30 min: 1 μM noradrenaline inhibited the capsaicin-activated inward current by 59 ± 10% (n = 7) in control and by 63 ± 4% (n = 7) in BMI (Fig. 7*C*). Results were very similar when inhibitors of PKA or PKC were directly included in the patch pipette. When 1 μM H89 was included in the patch pipette, noradrenaline inhibited the inward current by 47 ± 10% and the outward current by 50 ± 10% (n = 6). When 1 μM BIM was included in the patch pipette, noradrenaline inhibited the inward current by 44 ± 14% and the outward current by 43 ± 9% (n = 6). In contrast, when CaMKII activity was blocked by including 10 μM KN-93 in the patch pipette, the inhibitory effect of 1 μM noradrenaline on the capsaicin-activated current was significantly affected: 1 μM noradrenaline inhibited the capsaicin-activated inward current by 55 ± 14% (n = 17) in control and by 11 ± 9% (n = 12) with intracellular KN-93 (Fig. 7*E*). When KN-93 was replaced by the inactive analogue KN-92 (10 μM), 1 μM noradrenaline inhibited the capsaicin-activated inward current by 52 ± 14% (n = 8) (Fig. 7*E*). Taken together, these results suggest that activation of adrenergic receptors is linked to CaMKII activity, and point to a calcium-dependent mechanism.

A rise in cytoplasmic Ca^{2+} to activate CaMKII may reflect influx of external Ca^{2+}, release of Ca^{2+} from internal stores or both. To test these possibilities, we carried out a series of experiments to manipulate the external concentration of calcium, to manipulate the calcium buffer capacity of the intracellular solution and to deplete the internal calcium stores (Fig. 8*A*). When the external Ca^{2+} (2 mM) was replaced by an equimolar concentration of Mg^{2+}, such that the external solution contained a final concentration of 0 mM Ca^{2+} and 3 mM Mg^{2+}, using 10 mM EGTA as the intracellular calcium chelator, the inhibitory effect of 1 μM noradrenaline on the capsaicin-activated inward current was reduced from 55 ± 14% (n = 17) in 2 mM Ca^{2+} to 15 ± 10% (n = 12) in 0 mM Ca^{2+}, suggesting a strong contribution of calcium influx. The residual inhibitory effect of noradrenaline could reflect the contribution of calcium released from internal stores, or a calcium-independent effect. To test these possibilities, first, the slow calcium buffer EGTA (10 mM) used in our experimental condition was replaced by 10 mM of the fast calcium buffer BAPTA. The inhibitory effect of 1 μM noradrenaline on the capsaicin-activated inward current was reduced to 18 ± 15% (n = 6) in 2 mM Ca^{2+} external solution with BAPTA internally. Second, the external solution with 0 mM Ca^{2+} and 3 mM Mg^{2+} was tested with an internal solution containing 10 mM BAPTA. The inhibitory effect of 1 μM noradrenaline on the capsaicin-activated inward current was reduced to 8 ± 3% (n = 10) in 0 mM Ca^{2+} external solution

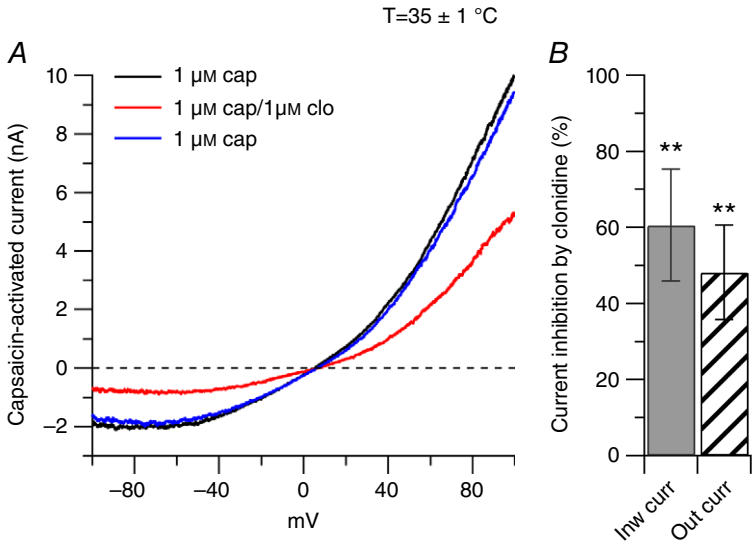

Figure 6. Effect of clonidine on the capsaicin-activated current at physiological temperature
A, representative capsaicin-activated currents recorded at physiological temperature (35 ± 1°C) in control (black trace), in the presence of 1 μM clonidine (clo, red trace) and upon washing out clonidine (blue trace). *B*, in collected results, 1 μM clonidine, applied on top of capsaicin, inhibited the inward current by 61 ± 15% (n = 8), $^{**}P < 0.01$, paired t test, and the outward current by 48 ± 12% (n = 8), $^{**}P < 0.01$, paired t test.

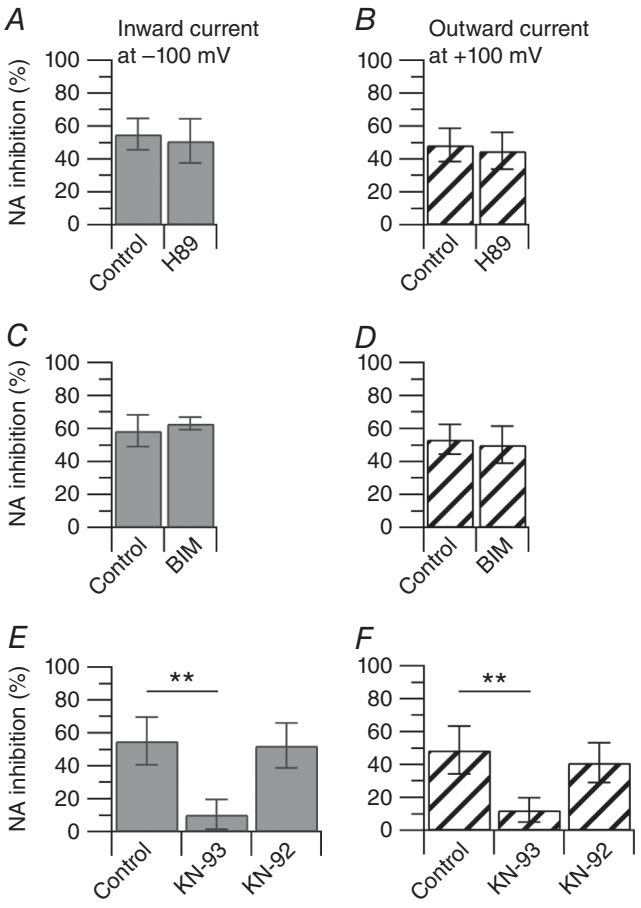

Figure 7. Intracellular pathways linked to α2 adrenergic receptors activation
Collected results show the effect of 1 μM noradrenaline on the capsaicin-activated inward and outward currents with manipulation of intracellular protein kinases. *A*, the inward current was reduced by 55 ± 10% ($n = 9$) in 1 μM noradrenaline alone, and by 51 ± 13% ($n = 11$) following incubation (30 min) with 1 μM H89, $P = 0.420$, unpaired *t* test. *B*, the outward current was reduced by 48 ± 10% ($n = 9$) in 1 μM noradrenaline alone, and by 45 ± 11% ($n = 11$) following incubation with 1 μM H89 (30 min), $P = 0.474$, unpaired *t* test. *C*, the inward current was reduced by 59 ± 10% ($n = 7$) in 1 μM noradrenaline alone, and by 63 ± 4% ($n = 7$) following incubation with 1 μM BIM (30 min), $P = 0.362$, unpaired *t* test. *D*, the outward current was reduced by 53 ± 9% ($n = 7$) in 1 μM noradrenaline alone, and by 50 ± 9% ($n = 7$) following incubation with 1 μM BIM (30 min), $P = 0.609$, unpaired *t* test. *E*, the inward current was reduced by 55 ± 14% ($n = 17$) in 1 μM noradrenaline alone, by 11 ± 9% ($n = 12$) with intracellular 10 μM KN-93 and by 52 ± 14% ($n = 8$) with intracellular 10 μM KN-92. Control *versus* KN-93, $^{**}P < 0.01$; control *versus* KN-92, $P = 0.845$, one way ANOVA followed by Dunnett's *post hoc* comparison test. *F*, the outward current was reduced by 49 ± 15% ($n = 17$) by 1 μM noradrenaline alone, by 12 ± 7% ($n = 12$) with intracellular 10 μM KN-93 and by 41 ± 12% ($n = 8$) with intracellular 10 μM KN-92. Control *versus* KN-93, $^{**}P < 0.01$; control *versus* KN-92, $P = 0.364$, one-way ANOVA followed by Dunnett's *post hoc* comparison test.

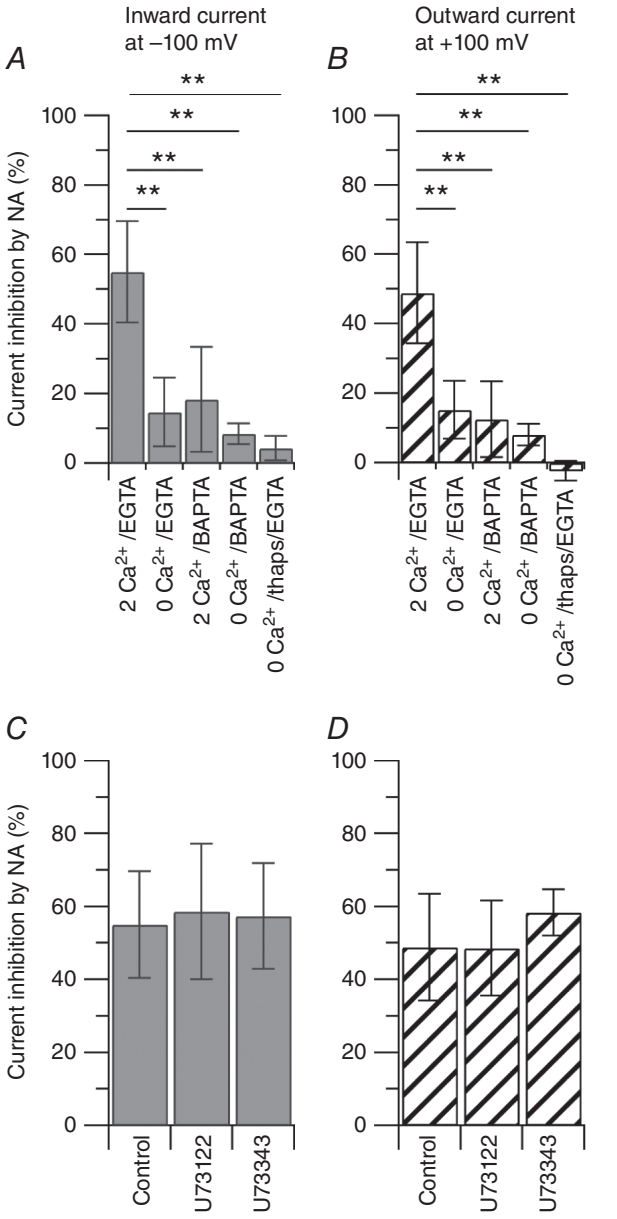

Figure 8. The effects of noradrenaline on the capsaicin-activated current are dependent on calcium
Collected results show the effect of 1 μM noradrenaline on the capsaicin-activated inward and outward currents with manipulation of external and/or internal calcium. The effect of 1 μM noradrenaline was determined with the following conditions: (1) 2 mM Ca^{2+} in the external solution and 10 mM EGTA in the internal solution (2 Ca^{2+}/EGTA); (2) 2 mM Ca^{2+} in the external solution was replaced by 2 mM Mg^{2+} (0 Ca^{2+}) and tested with 10 mM EGTA in the internal solution (0 Ca^{2+}/EGTA); (3) 2 mM Ca^{2+} in the external solution and 10 mM BAPTA in the internal solution (2 Ca^{2+}/BAPTA); (4) 2 mM Ca^{2+} in the external solution was replaced by 2 mM Mg^{2+} (0 Ca^{2+}) and tested with 10 mM BAPTA in the internal solution (0 Ca^{2+}/BAPTA); (5) 2 mM Ca^{2+} in the external solution was replaced by 2 mM Mg^{2+} (0 Ca^{2+}) and tested with 10 mM EGTA in the internal solution after depleting the internal calcium stores by pre-incubating the cells with 1 μM thapsigargin for 15 min (0 Ca^{2+}/thaps/EGTA). *A*, the inward current was reduced by 55 ± 14% ($n = 17$) in 2 Ca^{2+}/EGTA; by 15 ± 10% ($n = 12$) in 0 Ca^{2+}/EGTA; by 18 ± 15%

and internal BAPTA. Third, the 0 mM Ca^{2+} external solution with 10 mM internal EGTA was tested after depleting the internal calcium stores by pre-incubating the cells with 1 μM thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), for 15 min. The inhibitory effect of 1 μM noradrenaline on the capsaicin-activated inward current was reduced to 4 ± 3% ($n = 6$) under these conditions, consistent with a contribution of Ca^{2+} released from internal stores.

Release of Ca^{2+} from internal stores may be triggered by activation of phospholipase C (PLC) and a subsequent increase in inositol 1,4,5-trisphosphate ($InsP_3$). When DRG neurons were pre-incubated with 5 μM U73122 (a non-selective inhibitor of PLC) or with 5 μM U73343 (inactive control) for 30 min, the inhibitory effects of 1 μM noradrenaline on the capsaicin-activated inward current were 58 ± 18% ($n = 6$) and 57 ± 14% ($n = 5$), respectively, and were not different from those observed in control (55 ± 14%, $n = 17$), arguing against a contribution by U73122-sensitive PLC. Taken together, the data suggest that the inhibitory effects of noradrenaline on the capsaicin-activated current are strongly Ca^{2+}-dependent, mediated by CaMKII, and that both calcium influx and calcium released from internal stores contribute to the rise in cytoplasmic calcium activating CaMKII.

Activation of presynaptic TRPV1 channels by endovanilloids or capsaicin has been shown to trigger the release of peptides and modulate glutamatergic transmission in the dorsal horn of the spinal cord (Yang *et al.* 1998, 1999; Tognetto *et al.* 2001; Nakatsuka *et al.* 2002; Baccei *et al.* 2003; Labrakakis & MacDermott, 2003; Tong & MacDermott, 2006; Medvedeva *et al.* 2008). Based on these observations, our data predict that activation of presynaptic α2 adrenergic receptors could interact with presynaptic TRPV1 channels to modulate glutamatergic transmission between nociceptors and second-order neurons in the dorsal horn of the spinal cord. To test this possibility, we recorded mEPSCs from large lamina I neurons in horizontal spinal cord slices. To restrict the effects of adrenergic agonists to presynaptic targets, mEPSCs were recorded in the presence of 300 nM TTX, and with an intracellular solution in which Na-GTP (0.3 mM) was replaced by the non-hydrolysable analogue GDP-β-S (0.6 mM) to inhibit GPCRs signalling in the postsynaptic neuron. Capsaicin (500 nM) increased the median frequency of mEPSCs by 963%, consistent with activation of presynaptic TRPV1 channels. Clonidine (1 μM), applied on top of capsaicin, strongly reduced the increased median frequency of mEPSCs to 137% ($n = 7$, Fig. 9*E*), suggesting that activation of presynaptic α2 adrenergic receptors down-regulates the capsaicin-induced neurotransmitter release from nociceptors to lamina I neurons. Although clonidine also reduced the median of mEPSCs peak (Fig. 9*D*, left panel), analysis of the distribution of events (Fig. 9, middle panel) indicated that the reduction of mEPSCs peak was restricted to events of larger size (probably reflecting multiple vesicles release), while smaller events (probably reflecting single vesicle release) remained largely unchanged (see also Fig. 11*E*, middle panel), supporting our interpretation that the main effects of clonidine are on capsaicin-induced presynaptic events and not inhibition of synaptic vesicle release *per se* or changes in postsynaptic excitability.

Neurotransmitter release from Aδ- and C-fibre nociceptors to lamina I neurons is dependent on presynaptic N- and P/Q-type calcium channels (Bao *et al.* 1998; Heinke *et al.* 2004), and noradrenaline is well known to inhibit presynaptic calcium channels and neurotransmitter release (Dunlap & Fischbach, 1981; Bean, 1989; Lipscombe *et al.* 1989; Pollo *et al.* 1992; Boehm & Huck, 1996; Ikeda, 1996; Li & Horn, 2008). These observations raise the possibility that the reduced frequency of capsaicin-induced mEPSCs observed with clonidine (Fig. 9) could be mediated also by inhibition of calcium channels upon activation of presynaptic α2 adrenergic receptors. To exclude this possibility, the effects of clonidine on capsaicin-induced mEPSCs recorded from large lamina I neurons were determined in the presence of ω-conotoxin-MVIIC, a selective blocker of both N- and P/Q-type calcium channels (Hillyard *et al.* 1992; McDonough *et al.* 1996). Initial control experiments were carried out to confirm the contribution of presynaptic N- and P/Q-type calcium channels to neurotransmitter release from primary afferent fibres to lamina I neurons. Evoked EPSCs, elicited by electrical stimulation of the

($n = 6$) in 2 Ca^{2+}/BAPTA; by 8 ± 3% ($n = 10$) in 0 Ca^{2+}/BAPTA; by 4 ± 3% ($n = 6$) in 0 Ca^{2+}/thaps/EGTA. 2 Ca^{2+}/EGTA *versus* 0 Ca^{2+}/EGTA, 2 Ca^{2+}/BAPTA, 0 Ca^{2+}/BAPTA and 0 Ca^{2+}/thaps/EGTA, $^{**}P < 0.01$, one-way ANOVA followed by Dunnett's *post hoc* comparison test. *B*, the outward current was reduced by 49 ± 15% ($n = 17$) in 2 Ca^{2+}/EGTA; by 15 ± 8% ($n = 12$) in 0 Ca^{2+}/EGTA; by 13 ± 11% ($n = 6$) in 2 Ca^{2+}/BAPTA; by 8 ± 3% ($n = 10$) in 0 Ca^{2+}/BAPTA; by −2 ± 3% ($n = 6$) in 0 Ca^{2+}/thaps/EGTA. 2 Ca^{2+}/EGTA *versus* 0 Ca^{2+}/EGTA, 2 Ca^{2+}/BAPTA, 0 Ca^{2+}/BAPTA and 0 Ca^{2+}/thaps/EGTA, $^{**}P < 0.01$, one-way ANOVA followed by Dunnett's *post hoc* comparison test. *C*, the capsaicin-activated inward current was reduced by 55 ± 14% ($n = 17$) in 1 μM noradrenaline alone, by 59 ± 19% ($n = 6$) following incubation with 5 μM U73122 (PLC inhibitor) and by 57 ± 14% ($n = 6$) following incubation with 5 μM U73343 (inactive control). Control *versus* U73122, $P = 0.862$; control *versus* U73343, $P = 0.953$, one-way ANOVA followed by Dunnett's *post hoc* comparison test. *D*, the capsaicin-activated outward current was reduced by 49 ± 15% ($n = 17$) in 1 μM noradrenaline alone, by 49 ± 13% ($n = 6$) following incubation with 5 μM U73122 and by 58 ± 6% ($n = 6$) following incubation with 5 μM U73343. Control *versus* U73122, $P = 0.998$; control *versus* U73343, $P = 0.352$, one-way ANOVA followed by Dunnett's *post hoc* comparison test.

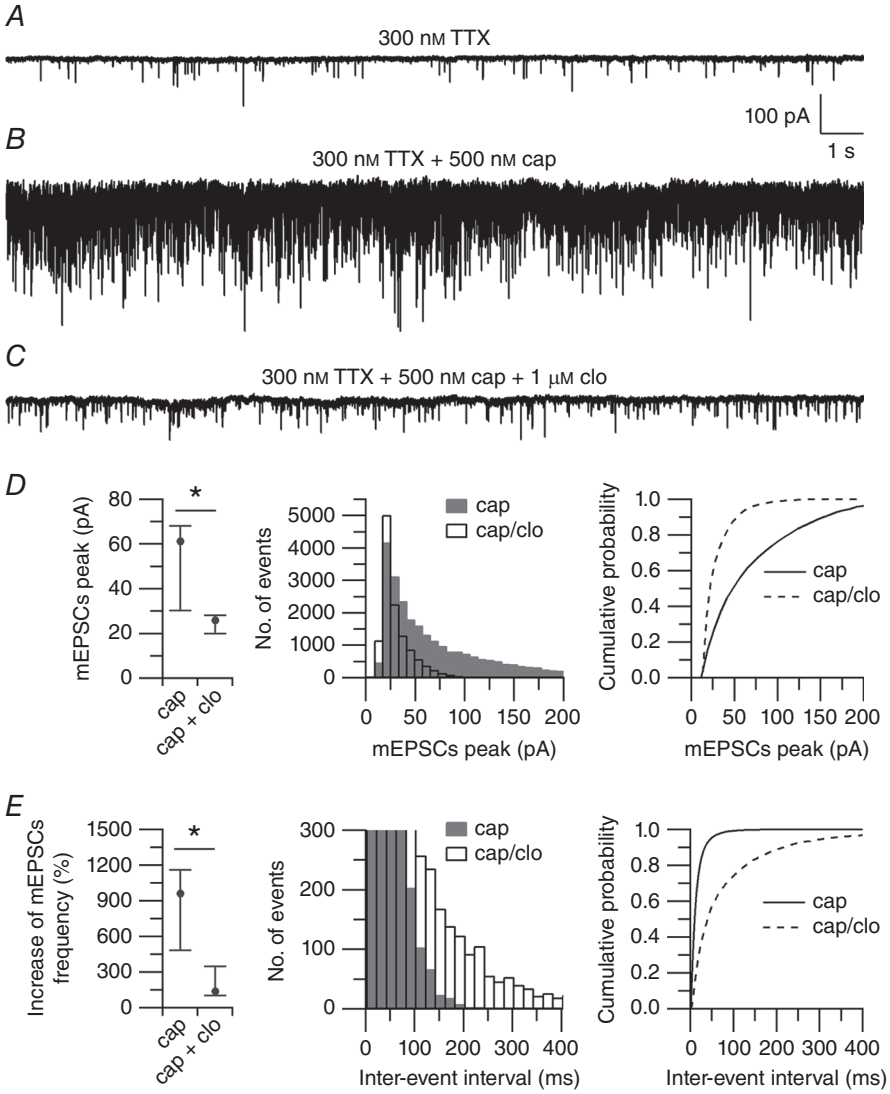

Figure 9. Effect of clonidine on capsaicin-induced mEPSCs
A, spontaneous mEPSCs recorded from a large lamina I neuron in a horizontal spinal cord slice. mEPSCS were recorded at a holding potential (V_h) = −70 mV in the presence of 300 nM TTX, and with an intracellular solution in which Na-GTP (0.3 mM) was replaced by the non-hydrolysable analogue GDP-β-S (0.6 mM). *B*, capsaicin (500 nM) increased the median frequency of mEPSCs from 12 to 116 Hz. *C*, clonidine (1 μM), applied on top of capsaicin, reduced the median frequency of mEPSCs to 43 Hz. *D*, collected results showing the effects of clonidine on the peak of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile), and third quartile (75th percentile). Capsaicin (cap): median = 61.2, 25th percentile = 30.1, 75th percentile = 68.1. Capsaicin + clonidine (cap + clo): median = 25.9, 25th percentile = 19.9, 75th percentile = 28.1 (n = 7). *P < 0.05, Wilcoxon matched-pairs test. *Middle*: distribution of mEPSC peaks recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (open bars) on top of capsaicin (n = 7). *Right*: cumulative probabilities of mEPSC peaks recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin (n = 7). ****P < 0.0001, Kolmogorov–Smirnov test. *E*, collected results showing the effects of clonidine on the frequency of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile) and third quartile (75th percentile). Capsaicin (cap): median = 963%, 25th percentile = 483%, 75th percentile = 1160%. Capsaicin + clonidine (cap + clo): median = 137%, 25th percentile = 101%, 75th percentile = 346% (n = 7). *P < 0.05, Wilcoxon matched-pairs test. *Middle*: distribution of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (open bars) on top of capsaicin (n = 7). *Right:* cumulative probabilities of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin (n = 7). ****P < 0.0001, Kolmogorov–Smirnov test.

dorsal root (L4 or L5) and recorded from large lamina I neurons in horizontal spinal cord slices, were reduced by 94 ± 7% (n = 9) by 200 nM ω-conotoxin-MVIIC (Fig. 10), consistent with a major contribution of N- and P/Q-type calcium channels. Then, similar to the experiments in Fig. 9, mEPSCs were isolated in the presence of 300 nM TTX and 200 nM ω-conotoxin-MVIIC, and with GDP-β-S (0.6 mM) in the intracellular solution to block effects mediated by GPCRs in the postsynaptic neuron. The median frequency of mEPSCs recorded in TTX (15.2 Hz) was not affected by application of 200 nM ω-conotoxin-MVIIC on top of TTX (14.8 Hz). Application of 500 nM capsaicin on top of ω-conotoxin-MVIIC increased the median frequency of mEPSCs by 367%, and subsequent application of 1 μM clonidine on top of capsaicin reduced the increased median frequency of mEPSCs to 102% (n = 7, Fig. 11). Finally, we tested whether blocking the activity of CaMKII would reduce or eliminate the effects of clonidine on the frequency of mEPSCs. For these series of experiments, spinal cord slices were pre-incubated with 10 μM KN-93 for 30–45 min. Application of 500 nM capsaicin on top of TTX and ω-conotoxin-MVIIC increased the median frequency of mEPSCs by 236%, and subsequent application of 1 μM clonidine on top of capsaicin had little additional effects (232%, Fig. 12, n = 7), confirming that the inhibitory effect of clonidine requires the activity of CaMKII as seen in isolated DRG neurons. Taken together, the data suggest that activation of presynaptic α2 adrenergic receptors reduces the frequency of capsaicin-induced mEPSCs, and that the effect is mediated by direct inhibition of presynaptic TRPV1 channels.

Discussion

The data presented here provide evidence that noradrenaline and clonidine strongly down-regulate the activity of presynaptic TRPV1 channels expressed in DRG neurons through α2 adrenergic receptor activation. Only small DRG neurons (diameter of 23.8 ± 2.5 μm and cell capacitance of 18.1 ± 3.7 pF, n = 219) sensitive to capsaicin (75% of those tested, 255/339) were included in the study, comprising a population of probable nociceptors (Cardenas *et al.* 1995; Caterina *et al.* 1997; Petruska *et al.* 2000; Ho & O'Leary, 2011).

The inhibitory effect of noradrenaline on the capsaicin-activated current was reduced by replacing GTP with GDP-β-S (a non-hydrolysable analogue of GDP) in the intracellular solution (Fig. 3), confirming that the effects of noradrenaline require activation of GPCRs as expected for adrenergic receptors (Ruffolo *et al.* 1993; Saunders & Limbird, 1999). The lack of complete abolition of the response by GDP-β-S may reflect the difficulty in completely dialysing out GTP.

Of the three isoforms of α2 adrenergic receptors cloned in humans and rat (α2A, α2B, α2C) (Bylund *et al.* 1994), DRG neurons express mainly the α2A and α2C isoforms (Nicholas *et al.* 1993; Cho *et al.* 1997; Stone *et al.* 1998; Birder & Perl, 1999), although expression of the α2B isoform has been reported (Gold *et al.* 1997). Consistent with these observations, clonidine (α2 agonist), at saturating concentrations of 0.3–1 μM, fully mimicked the inhibitory effects of noradrenaline on the capsaicin-activated current, and the effects were strongly antagonized by yohimbine (α2 antagonist), supporting the conclusion that noradrenaline down-regulates the activity of TRPV1 channels by activation of α2 adrenergic receptors. Although available pharmacological agents do not discriminate between different α2 adrenergic receptor subtypes (Giovannoni *et al.* 2009), it has been shown that degeneration of capsaicin-sensitive DRG neurons after neonatal capsaicin treatment in rats strongly decreases the expression of the α2A, but not α2C, isoform in primary afferent fibres (Stone *et al.* 1998), consistent with preferential expression of the α2A isoform in

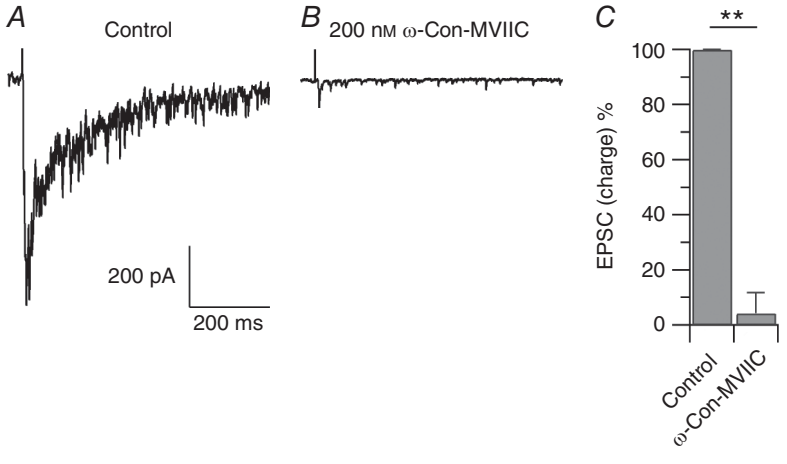

Figure 10. Contribution of N- and P/Q-type calcium channels to neurotransmitter release from primary afferent fibres to lamina I neurons
A, evoked EPSC recorded from a large lamina I neuron in a horizontal spinal cord slice. The EPSC was elicited by electrical stimulation of the L4 dorsal root attached to the spinal cord slice with 500 μA of constant current (100 μs duration). Shown is the average EPSC of three consecutive electrical stimulations at 0.016 Hz. *B*, 200 nM ω-conotoxin-MVIIC reduced the EPSC by 97%. *C*, in collected results, 200 nM ω-conotoxin-MVIIC reduced the EPSC by 94 ± 7% (n = 9), $^{**}P < 0.01$, paired t test.

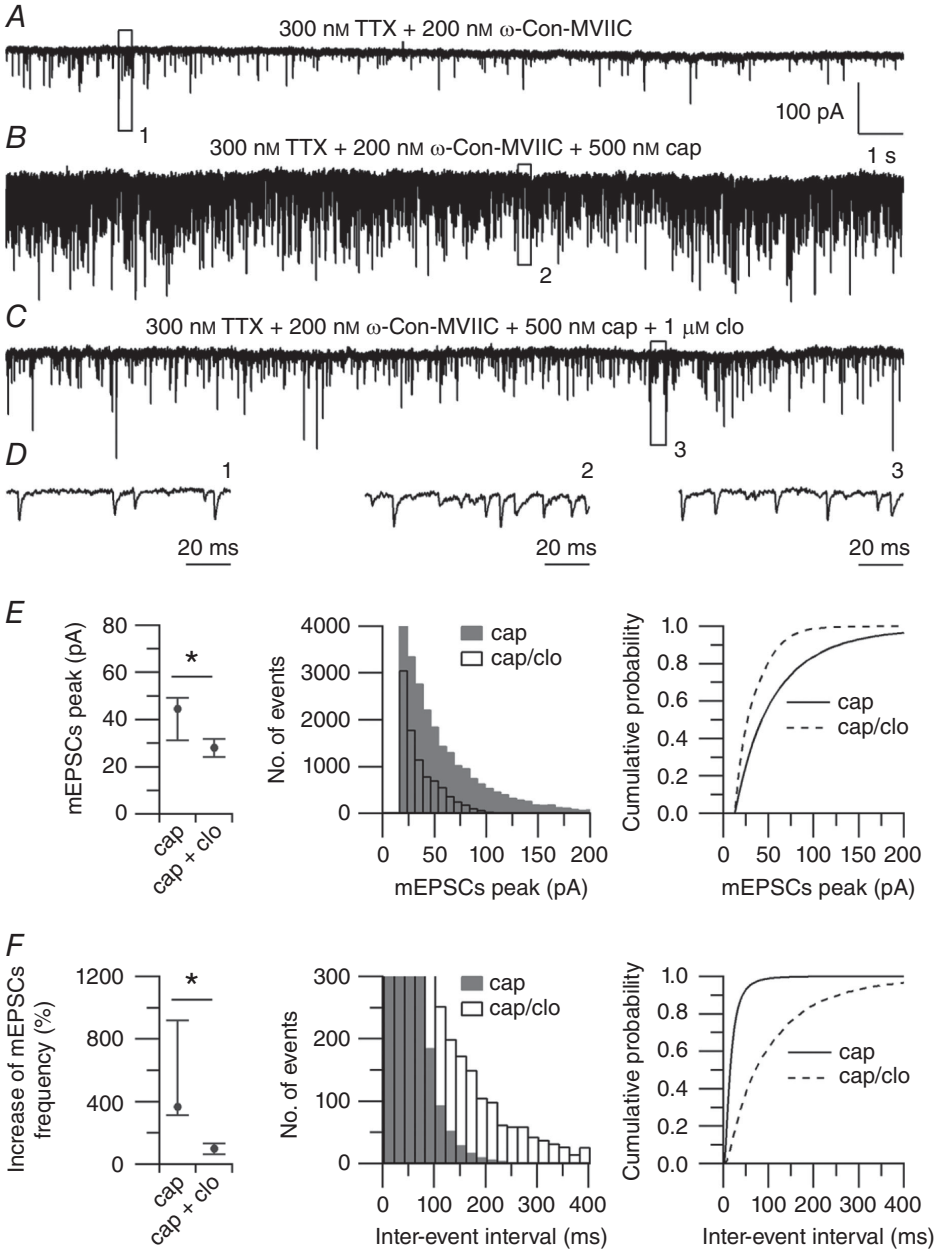

Figure 11. Effect of clonidine on capsaicin-induced mEPSCs in the presence of calcium channel blockers
A, spontaneous mEPSCs recorded from a large lamina I neuron in a horizontal spinal cord slice. mEPSCS were recorded at a holding potential (V_h) = −70 mV in the presence of 300 nM TTX + 200 nM ω-conotoxin-MVIIC, and with an intracellular solution in which Na-GTP (0.3 mM) was replaced by the non-hydrolysable analogue GDP-β-S (0.6 mM). *B*, capsaicin (500 nM) increased the median frequency of mEPSCs from 4 to 55 Hz. *C*, clonidine (1 μM), applied on top of capsaicin, reduced the median frequency of mEPSCs to 9 Hz. *D*, representative small events recorded in 300 nM TTX + 200 nM ω-Con-MVIIC (*left*, from box 1), after application of 500 nM capsaicin (*middle*, from box 2) and after application of 1 μM clonidine on top of capsaicin (*right*, from box 3). *E*, collected results showing the effects of clonidine on the peak of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile) and third quartile (75th percentile). Capsaicin (cap): median = 44.5, 25th percentile = 31.1, 75th percentile = 49.1. Capsaicin + clonidine (cap + clo): median = 28.1, 25th percentile = 24.1, 75th percentile = 31.6 (n = 7). *P < 0.05, Wilcoxon matched-pairs test. *Middle*: distribution of mEPSC peaks recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (open bars) on top of capsaicin (n = 7). *Right*: cumulative probabilities of mEPSC peaks recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin (n = 7). ****P < 0.0001, Kolmogorov–Smirnov test. *F*, collected results showing the effects of clonidine on the frequency of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile) and third quartile (75th percentile). Capsaicin (cap): median = 367%, 25th percentile = 315%,

75th percentile = 918%. Capsaicin + clonidine (cap + clo): median = 102%, 25th percentile = 65%, 75th percentile = 132% ($n = 7$). *$P < 0.05$, Wilcoxon matched-pairs test. *Middle*: distribution of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (opne bars) on top of capsaicin ($n = 7$). *Right:* cumulative probabilities of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin ($n = 7$). ****$P < 0.0001$, Kolmogorov–Smirnov test.

capsaicin-sensitive DRG neurons. Because our study was limited only to capsaicin-sensitive DRG neurons, it is likely that the effects of noradrenaline and clonidine reported here are mediated mainly by activation of α2A adrenergic receptors.

The inhibitory effects of noradrenaline and clonidine observed in acutely isolated DRG neurons, a preparation in which postsynaptic targets are removed, demonstrate that the machinery for noradrenaline inhibition of TRPV1 channels is present in DRG cell bodies. The inhibitory effects of clonidine on the capsaicin-induced mEPSCs suggest that the elements for α2 adrenergic receptor inhibition of TRPV1 currents are also operational at presynaptic terminals. This conclusion is supported by several other observations. (1) TRPV1 channels are expressed not only on the cell body and peripheral terminals of nociceptors, but also on the central terminal which make synaptic contacts with second-order neurons in the dorsal horn of the spinal cord (Holzer, 1991; Winter *et al.* 1993; Szallasi *et al.* 1995; Tominaga *et al.* 1998; Guo *et al.* 1999; Hwang *et al.* 2004). (2) Expression of α2 adrenergic receptors in the dorsal horn of the spinal cord co-localizes with substance P (Stone *et al.* 1998), consistent with expression of α2 adrenergic receptors on the central terminals of nociceptors. (3) In spinal cord slices, mEPSCs were recorded from lamina I neurons in the presence of TTX and ω-conotoxin-MVIIC, and with an intracellular solution in which GTP was replaced by the non-hydrolysable analogue GDP-β-S to block GPCRs in the postsynaptic lamina I neuron, arguing against a postsynaptic effect of clonidine. (4) The inhibitory effects of clonidine on capsaicin-induced mEPSCs reported here parallel the inhibitory effects mediated by α2A adrenergic receptors on the capsaicin-induced glutamate release in spinal cord synaptosomes (Li & Eisenach, 2001). (5) Although clonidine reduced the median peak of mEPSCs, the reduction was restricted to larger events, without any apparent reduction of the sizes of smaller events (Figs 9*D*, 11*D* and 11*E*), suggesting no effect of clonidine on quantal release of transmitter, or suppression of postsynaptic excitability. All these observations point to a presynaptic effect of noradrenaline and clonidine mediated by adrenergic receptors expressed on the central terminals of nociceptors.

Protein phosphorylation plays a major role in the modulation of TRPV1 channels. Several serine and threonine residues have been reported to be phosphorylated by PKC (Tominaga *et al.* 2001; Numazaki *et al.* 2002; Bhave *et al.* 2003; Premkumar *et al.* 2004), or by PKA (De Petrocellis *et al.* 2001; Bhave *et al.* 2002; Rathee *et al.* 2002; Vlachova *et al.* 2003; Mohapatra & Nau, 2005; Jeske *et al.* 2006), or by CaMKII (Jung *et al.* 2004; Price *et al.* 2005; Hucho *et al.* 2012). Phosphorylation by PKC sensitizes TRPV1 channels by increasing the channel open probability (Vellani *et al.* 2001), while phosphorylation by PKA may reverse the desensitization of TRPV1 channels induced by prolonged or repeated application of the agonist (Bhave *et al.* 2002). Thus, stimulation of PKC or PKA would probably produce an increase of the capsaicin-activated current. However, our data show that noradrenaline or clonidine consistently inhibited the capsaicin-activated current, arguing against a direct involvement of PKC or PKA. In contrast, the inhibitory effect of noradrenaline on the capsaicin-activated current was reduced by ~50% when the CaMKII activity was blocked, suggesting that CaMKII activity is involved in the modulation of the capsaicin-activated current by noradrenaline. We have previously shown in DRG neurons that CaMKII activity is required also for the modulation of TRPV1 channels upon activation of D1/D5 dopamine receptors (Chakraborty *et al.* 2016), raising the possibility for a convergent pathway involved in the modulation of TRPV1 channels by different catecholamines. Phosphorylation/dephosphorylation of TRPV1 channels by CaMKII/calcineurin have been suggested to modulate vanilloids binding to TRPV1 channels (Jung *et al.* 2004). Thus, an intriguing possibility is that activation of α2 adrenergic receptors may modulate the function of TRPV1 channels by affecting the binding of vanilloids to the channel. Because Ca^{2+} signalling regulates both CaMKII and calcineurin, different mechanisms have been proposed to explain the fine balance between phosphorylation and dephosphorylation, including different activation kinetics, different sensitivities to Ca^{2+} and calmodulin, or cross-talk between these two enzymes–signalling pathways (Hashimoto *et al.* 1988; Klee, 1991; Tian & Karin, 1999; Tominaga & Tominaga, 2005; Stratton *et al.* 2013; Simon *et al.* 2015). Future experiments are required to fully elucidate the interaction between α2 adrenergic receptors and the CaMKII/calcineurin pathway.

Our results show that the inhibitory effect of noradrenaline on the capsaicin-activated current is highly dependent on calcium. Removal of Ca^{2+} from the external solution strongly reduced the inhibitory effects

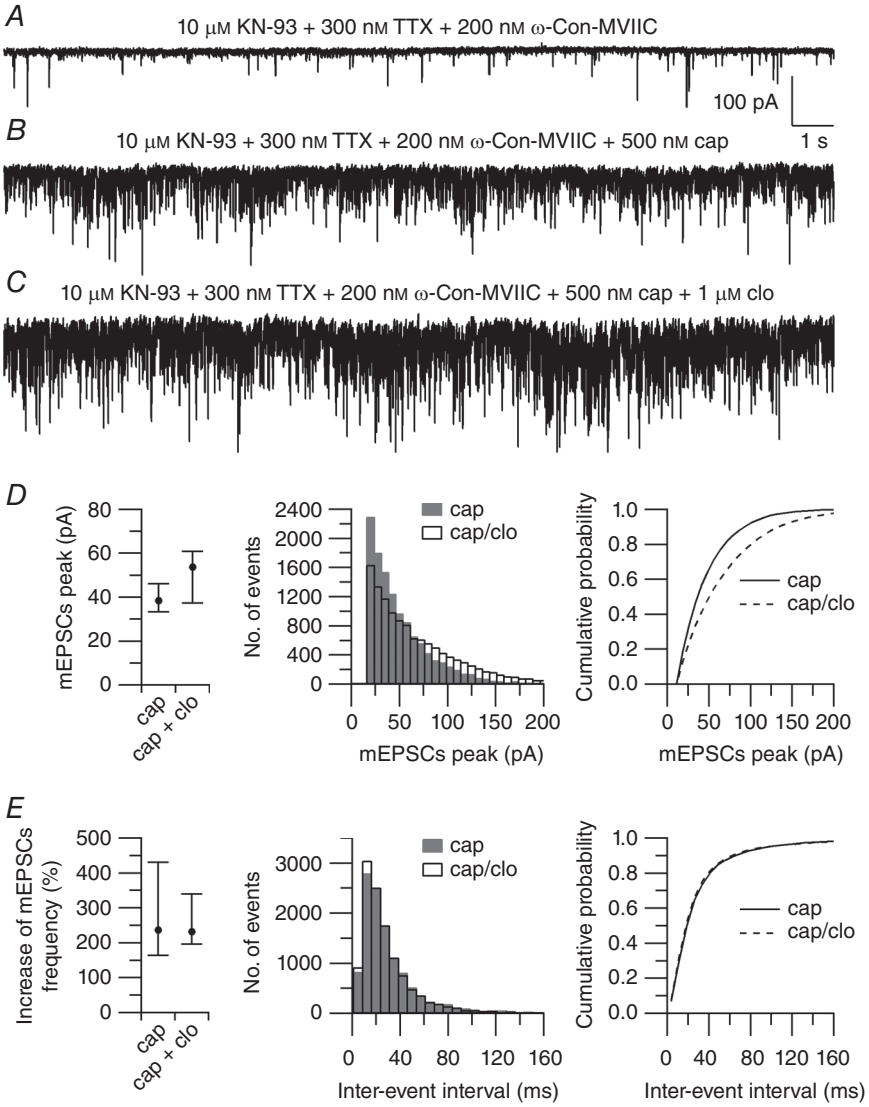

Figure 12. Effect of clonidine on capsaicin-induced mEPSCs in the presence of CaMKII blockers
A, spontaneous mEPSCs recorded from a large lamina I neuron in a horizontal spinal cord slice pre-incubated in 10 μM KN-93 for 45 min. mEPSCS were recorded at a holding potential (V_h) = −70 mV in the presence of 300 nM TTX + 200 nM ω-conotoxin-MVIIC, and with an intracellular solution in which Na-GTP (0.3 mM) was replaced by the non-hydrolysable analogue GDP-β-S (0.6 mM). *B*, capsaicin (500 nM) increased the median frequency of mEPSCs from 10 to 40 Hz. *C*, clonidine (1 μM), applied on top of capsaicin, reduced the median frequency of mEPSCs to 33 Hz. *D*, collected results showing the effects of clonidine on the peak of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile) and third quartile (75th percentile). Capsaicin (cap): median = 38.5 pA, 25th percentile = 33.4 pA, 75th percentile = 46.2 pA. Capsaicin + clonidine (cap + clo): median = 53.8 pA, 25th percentile = 37.5 pA, 75th percentile = 60.9 pA (n = 7). P = 0.078, Wilcoxon matched-pairs test. *Middle*: distribution of mEPSC peaks recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (open bars) on top of capsaicin (n = 7). *Right*: cumulative probabilities of mEPSC peaks recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin (n = 7). $^{****}P < 0.0001$, Kolmogorov–Smirnov test. *E*, collected results showing the effects of clonidine on the frequency of mEPSCs. *Left*: values are reported as median, first quartile (25th percentile) and third quartile (75th percentile). Capsaicin (cap): median = 236%, 25th percentile = 163%, 75th percentile = 430%. Capsaicin + clonidine (cap + clo): median = 232%, 25th percentile = 196%, 75th percentile = 339% (n = 7). P = 0.687, Wilcoxon matched-pairs test. *Middle*: distribution of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (shaded bars) and after application of 1 μM clonidine (open bars) on top of capsaicin (n = 7). *Right:* cumulative probabilities of inter-event intervals of mEPSCs recorded in 500 nM capsaicin (solid line) and after application of 1 μM clonidine (dashed line) on top of capsaicin (n = 7). $^{**}P < 0.01$, Kolmogorov–Smirnov test.

of noradrenaline on the capsaicin-activated current, suggesting a major contribution of Ca^{2+} influx for the rise in cytoplasmic Ca^{2+}. Under physiological conditions, activation of voltage-dependent calcium channels during action potential firing in nociceptors probably provides a major source of Ca^{2+} influx (Carbone & Lux, 1984; Scroggs & Fox, 1991, 1992; Blair & Bean, 2002; Bell *et al.* 2004; Castiglioni *et al.* 2006; Gemes *et al.* 2010), and will contribute to a rise in cytoplasmic Ca^{2+} necessary to engage downstream targets such as CaMKII. Activation of TRPV1 channels with their high permeability to Ca^{2+} ($P_{Ca}{}^{2+}/P_{Na}{}^{+} = \sim 10$) (Caterina *et al.* 1997) also results in influx of Ca^{2+}. Removal of external Ca^{2+} reduced but did not eliminate noradrenaline inhibition of TRPV1 currents, suggesting an additional contribution from Ca^{2+} released from intracellular stores, consistent with further reduction of the noradrenaline effect when stores were depleted by thapsigargin treatment (Olah *et al.* 2001; Wong *et al.* 2014).

Calcium influx is suggested to play a prominent role in the desensitization of TRPV1 channels upon repeated or sustained stimulation with the agonist (Cholewinski *et al.* 1993; Docherty *et al.* 1996; Liu & Simon, 1996; Koplas *et al.* 1997; Jung *et al.* 2004; Rosenbaum *et al.* 2004), which predicts a reduced response of nociceptors to repeated or sustained noxious stimuli. However, in our experimental conditions, in which we used a single application of capsaicin, inhibition of capsaicin-activated current by noradrenaline was consistently accompanied by rapid and complete recovery of the current upon washing out noradrenaline (Fig. 1), suggesting that the down-regulation of TRPV1 channels by noradrenaline occurs via a mechanism different from Ca^{2+}-induced desensitization.

Our experiments in spinal cord slices show that clonidine can powerfully reduce the frequency of mEPSCs induced by capsaicin in the presence of ω-conotoxin-MVIIC (Fig. 11), a condition in which N- and P/Q-type calcium channels are blocked (Fig. 10). The simplest interpretation is that under these conditions activation of presynaptic TRPV1 channels produces calcium entry into presynaptic terminals that promotes release of synaptic vesicles. This is consistent with a previous report showing a contribution of TRPV1 channels to a rise in cytoplasmic Ca^{2+} and neurotransmitter release in co-cultures of DRG neurons and dorsal horn neurons (Medvedeva *et al.* 2008). The effect of $\alpha 2$ adrenergic receptor activation to reduce the capsaicin-induced mEPSCs is then consistent with the inhibition of presynaptic TRPV1 channels like that seen in DRG cell bodies.

The inhibition of presynaptic TRPV1 channels by noradrenaline could contribute to overall inhibition of pain signaling by noradrenaline released by descending adrenergic fibres, although it remains to be determined under what circumstances presynaptic TRPV1 channels are activated. An intriguing possibility is that noradrenaline inhibition of TRPV1 signalling could also occur in peripheral terminals of primary nociceptors, but further work will be required to test this possibility.

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Additional information

Competing interests

The authors declare no competing financial interests.

Author contributions

Conception and design of the experiments: M.P, M.R., M.K. Collection, analysis and interpretation of data: S.C., V.E., M.P, M.R., M.K. Drafting the article: S.C., M.P, M.R., M.K. All authors have approved the final version of the manuscript

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Translational perspective

Neurotransmitters released from supraspinal centres can strongly modulate pain signals in the dorsal horn of the spinal cord. TRPV1 channels play a critical role in setting the excitability of nociceptors and their synaptic transmission to second-order neurons in dorsal horn of the spinal cord. Up-regulation of TRPV1 channels is involved in the development of thermal hyperalgesia associated with inflammatory pain. This work shows that noradrenaline potently down-regulates the activity of TRPV1 channels in nociceptors and inhibits the release of neurotransmitter from nociceptors to large lamina I neurons in the dorsal horn of the spinal cord. Potentiation of the descending noradrenergic system could play a prominent role in the setting of injury to reduce neurotransmitter release from nociceptors, thus inhibiting incoming noxious stimuli to the dorsal horn of the spinal cord.