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RESEARCH PAPER

Primary afferents with TRPM8 and TRPA1 profiles target distinct subpopulations of rat superficial dorsal horn neurones

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Background and purpose: The transient receptor potential (TRP) channels, transient receptor potential melastatin-1 (TRPM8) and transient receptor potential ankyrin-1 (TRPA1), are expressed in subpopulations of sensory neurones and have been proposed to mediate innocuous and noxious cold sensation respectively. The aim of this study was to compare TRPM8 and TRPA1 modulation of glutamatergic afferent transmission within the spinal dorsal horn.
Experimental approach: Whole cell patch clamp recordings were made from rat spinal cord slices *in vitro* to examine the effect of TRP agonists and temperature on glutamatergic excitatory postsynaptic currents (EPSCs).
Key results: Icilin (3 or 100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) reduced the amplitude of primary afferent evoked EPSCs in subpopulations of lamina I and II neurones. In a subpopulation of superficial neurones, innocuous cold (threshold 29°C), 3 μmol·L^{-1} icilin (EC_{50} 1.5 μmol·L^{-1}) and menthol (EC_{50} 263 μmol·L^{-1}) increased the rate of spontaneous miniature EPSCs. In the majority of lamina I and II neurones, 100 μmol·L^{-1} icilin (EC_{50} 79 μmol·L^{-1}), allyl isothiocyanate (EC_{50} 226 μmol·L^{-1}), cinnamaldehyde (EC_{50} 38 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) increased miniature EPSC rate. The response to 100 μmol·L^{-1}, but not 3 μmol·L^{-1} icilin, was abolished by ruthenium red, while neither was affected by iodoresiniferatoxin. Responsiveness to 3 μmol·L^{-1}, but not to 100 μmol·L^{-1} icilin, was highly predictive of innocuous cold responsiveness. Neurones responding to 3 μmol·L^{-1} icilin and innocuous cold were located more superficially than those responding to 100 μmol·L^{-1} icilin.
Conclusions and implications: Activation of TRPM8 and TRPA1 presynaptically modulated glutamatergic transmission onto partially overlapping but distinct populations of superficial dorsal horn neurones. Spinal TRPM8 and TRPA1 channels may therefore provide therapeutic targets in cold hyperesthesia.
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Keywords: synaptic transmission; spinal cord; cold; pain; TRP ion channel

Abbreviations: ACSF, artificial cerebrospinal fluid; CNQX, 6-cyano-2,3-dihdroxy-7-nitro-quinoxaline; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate receptor; PB, Phosphate buffer; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin-1; TRPM8, transient receptor potential melastatin-1; TRPV1, transient receptor potential vanilloid-1; TTX, tetrodotoxin

Introduction

Our understanding of thermal transduction has been facilitated by the identification of several temperature-activated ion channels belonging to the transient receptor potential (TRP) cation channel family (Dhaka *et al.*, 2006; Alexander *et al.*, 2008). Some TRP ion channels are expressed in subpopulations of dorsal root ganglion (DRG) and trigeminal ganglion neurones and are transported to the periphery where they transduce a wide range of noxious and innocuous temperatures. Two of the identified mammalian TRP ion channels have been proposed to transduce cold. Transient receptor potential melastatin-1 (TRPM8), the first to be identified, is activated by innocuous cold (or cool, <27°C), icilin, menthol and a number of other odorants and cooling compounds (McKemy *et al.*, 2002; Peier *et al.*, 2002). Transient receptor potential ankyrin-1 (TRPA1) is activated by noxious cold (<18°C), icilin (at higher concentrations than TRPM8), pungent agents such as allyl isothiocyanate (mustard oil) and cinnamaldehyde, and other irritants (Story *et al.*, 2003;

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Bandell *et al.*, 2004; Jordt *et al.*, 2004; Bautista *et al.*, 2006; McNamara *et al.*, 2007). TRPM8 and TRPA1 are also activated by a range of endogenous agents (Vanden Abeele *et al.*, 2006; Andersson *et al.*, 2008; Cruz-Orengo *et al.*, 2008). *In vitro* studies on native DRG and trigeminal ganglion neurones and *in vivo* knockout studies suggest that TRPM8 plays a major role in innocuous cold sensation; however, the role of TRPA1 in noxious cold sensation remains controversial (Reid *et al.*, 2002; Nealen *et al.*, 2003; Story *et al.*, 2003; Thut *et al.*, 2003; Babes *et al.*, 2004; Bautista *et al.*, 2006; 2007; Kwan *et al.*, 2006; Xing *et al.*, 2006; Colburn *et al.*, 2007; Dhaka *et al.*, 2007; Sawada *et al.*, 2007).

Earlier *in vivo* studies in the rat (Bester *et al.*, 2000; Zhang *et al.*, 2006), cat and monkey (Dostrovsky and Craig, 1996; Han *et al.*, 1998; Craig *et al.*, 2001) indicated that peripheral innocuous cold stimuli activated a subpopulation of lamina I and II_o (II outer) neurones, which project to supraspinal structures; however, the role of TRP ion channels in cold transmission was not examined. The transport of TRP channels to the central terminals of primary afferent fibres provides a means to pharmacologically characterize the transmission of TRP-mediated thermal information within the spinal cord using an *in vitro* preparation. Using this approach, the transient receptor potential vanilloid-1 (TRPV1) agonist capsaicin has been shown to presynaptically modulate glutamatergic primary afferent synaptic transmission onto neurones throughout laminae I and II (Yang *et al.*, 1998; 1999; Nakatsuka *et al.*, 2002; Labrakakis and MacDermott, 2003). It has also been demonstrated that innocuous cold and menthol enhance synaptic transmission between co-cultured DRG/dorsal horn neurones (Tsuzuki *et al.*, 2004), and that menthol and allyl isothiocyanate enhance spontaneous glutamatergic synaptic transmission onto lamina II neurones in spinal cord slices (Baccei *et al.*, 2003; Kosugi *et al.*, 2007). In the present study, we have used an *in vitro* approach to examine the effect of temperature reductions and TRPM8 and TRPA1 agonists on primary afferent evoked and spontaneous glutamatergic transmission onto neurones throughout laminae I–III in spinal cord slices from rats.

Methods

All animal procedures followed the guidelines of the 'NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia' and were approved by the Royal North Shore Hospital/University of Technology Sydney Animal Care and Ethics Committee. Experiments were carried out with male Sprague-Dawley rats (14–21 days old). Animals were anaesthetized with halothane (1–3% in O_2) and a laminectomy performed to expose the lumbar spinal cord. The dura was incised and the spinal column quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) of composition: (mmol·L^{-1}): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11; $NaHCO_3$, 25. The animal was then killed by decapitation. Transverse (300 μm) slices of the lumbar spinal cord (L4-6) were cut and maintained at room temperature in a submerged chamber containing ACSF equilibrated with 95% O_2 and 5% CO_2. The slices were then transferred to a chamber and superfused continuously (1.8 mL·min^{-1}) with ACSF at 34°C using an inline temperature controller (CL-100, Warner Instruments, Hampden, MA, USA). In some experiments the slice chamber temperature was modified using the inline temperature controller.

Dorsal horn neurones were visualized using infra-red Nomarski, or Dodt-tube optics on an upright microscope (Olympus BX50, Olympus, Sydney, Australia). Whole-cell voltage clamp recordings (holding potential –65 mV, liquid junction potential corrected) were made using an Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA) with patch clamp electrodes (2–5 MΩ). The internal solution contained (mmol·L^{-1}): $CsMeSO_3$ 135, ethylene glycol tetraacetic acid (EGTA) 10, HEPES 5, NaCl 10, $MgCl_2$ 1 and MgATP 2 for recordings of miniature excitatory postsynaptic currents (EPSCs), plus QX-314 3 mmol·L^{-1} for recordings of evoked EPSCs; CsCl 140, EGTA 10, HEPES 5, $CaCl_2$ 2 and MgATP 2 for recordings of inhibitory postsynaptic currents (IPSCs); and K-gluconate 95, KCl 30, NaCl 15, $MgCl_2$ 1, HEPES 10, EGTA 11, MgATP 2, NaGTP 0.3 for recordings of direct postsynaptic effects. All internal solutions were adjusted to pH 7.3 and osmolality 280–285 mosmol·L^{-1}. In some recordings biocytin (0.05%) was included in the internal solution. Series resistance (<25 MΩ) was compensated by 80% and continuously monitored during experiments.

Electrically evoked EPSCs were elicited in neurones via bipolar stimulating electrodes placed on dorsal rootlets (rate 0.03 Hz, intensity 1–15 V, 0.1–0.4 ms) in the presence of the $GABA_A$ channel blocker picrotoxin (100 μmol·L^{-1}) and the glycine receptor antagonist strychnine (3 μmol·L^{-1}). Spontaneous miniature EPSCs were recorded in the presence of tetrodotoxin (TTX, 500 nmol·L^{-1}), picrotoxin and strychnine. Spontaneous miniature IPSCs were recorded in the presence of TTX (500 nmol·L^{-1}) and the non-N-methyl-D-aspartate receptor antagonist 6-cyano-2,3-dihdroxy-7-nitroquinoxaline (CNQX, 5 μmol·L^{-1}). IPSCs and EPSCs were filtered (1, 2 kHz low-pass filter) and sampled (5, 10 kHz) in 4 and 5 s epochs, respectively, every 6 s for analysis using AxographX (Axograph Scientific, Sydney, Australia). For analysis, miniature IPSCs and EPSCs above a preset threshold (four to five standard deviations above baseline noise) were automatically detected by a sliding template algorithm and manually checked. Changes in miniature EPSC and IPSC rate are presented in absolute values, rather than percentage increases because of the variable baseline rate (range = 0.03–28.7 s^{-1}). Neurones were considered to be icilin, capsaicin or temperature responders if there was a change in miniature EPSC rate, or evoked EPSC amplitude that was three standard deviations greater than the pre-drug level for at least two consecutive epochs. Stock solutions of all drugs were diluted to working concentrations using ACSF immediately before use and applied by superfusion.

After recording, the slices containing biocytin-filled cells were placed in 4% paraformaldehyde [0.1 mol·L^{-1} phosphate buffer (PB), pH 7.4] for 60 min at room temperature. Slices were then rinsed in PB and incubated in Triton X-100 (0.3% in PB) for 2–4 days at 4°C. Slices were incubated in ExtrAvidin HRP (1:1000) overnight (4°C). To visualize biocytin-containing cells, slices were pre-incubated in a solution of 0.5% 3,3-diaminobenzidine solution containing 10% D-glucose, 4% ammonium chloride and 0.1% nickel ammo-

nium sulphate for 20 min. The reaction was catalysed by adding 1 μL of glucose oxidase per 1 mL of diaminobenzidine mixture and the reaction was allowed to proceed for 6 min before it was stopped by washing slices in PB. Slices were then mounted on gelatinized slides and dehydrated prior to coverslipping. Mounted slices were examined under light microscopy to confirm location of recorded neurones.

Data analysis

All pooled data are expressed as means ± SEM. All statistical comparisons were made using Student's paired/unpaired *t*-test, or one-way ANOVA followed by *post hoc* tests using Dunnett's adjustment for multiple comparisons.

Materials

Allyl isothiocyanate, cinnamaldehyde, picrotoxin and strychnine hydrochloride were obtained from Sigma (Sydney, Australia); icilin [AG 3-5; 3,6-dihydro-1-(2-hydroxyphenyl)-4-(3-nitrophenyl)-2(1H)-pyrimidinone] was from Cayman Chemical Co. (Ann Arbor, MI, USA); (E)-capsaicin, CNQX and LY341495 were from Tocris Cookson (Bristol, UK); QX314 and TTX were from Alomone (Jerusalem, Israel).

Results

Icilin and menthol modulate primary afferent transmission

We first examined the effect of icilin on primary afferent evoked EPSCs in lamina I and II dorsal horn neurones. In these experiments we used menthol (200 μmol·L^{-1}) and low concentrations of icilin (3 μmol·L^{-1}) to activate TRPM8 and high concentrations of icilin (100 μmol·L^{-1}) to activate both TRPM8 and TRPA1 (McKemy *et al.*, 2002; Story *et al.*, 2003). In the presence of picrotoxin (100 μmol·L^{-1}) and strychnine (3 μmol·L^{-1}), stimulation of the dorsal rootlets evoked EPSCs that had stable latencies and were abolished by TTX (500 nmol·L^{-1}, $n = 4$) and by CNQX (5 μmol·L^{-1}, $n = 5$). Superfusion of the low concentration of icilin (3 μmol·L^{-1}) produced a decrease in the amplitude of evoked EPSCs in 23% ($n = 5/22$) of neurones tested, which always reversed following washout (Figure 1A and B). The high concentration of icilin (100 μmol·L^{-1}) produced a decrease in the amplitude of evoked EPSCs in 82% ($n = 14/17$) of neurones tested. Menthol (200 μmol·L^{-1}) produced a decrease in the amplitude of evoked EPSCs in 64% ($n = 9/14$) of neurones tested. In addition, capsaicin (1 μmol·L^{-1}) produced a decrease in evoked EPSC amplitude in all neurones tested ($n = 12/12$). The inhibition of evoked EPSCs produced by icilin (100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) did not always fully reverse following washout. In the responding neurones, the inhibition of evoked EPSCs produced by icilin (100 μmol·L^{-1}) was less than that produced by icilin (3 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) (Figure 1D, $P < 0.01$).

The decrease in evoked EPSC amplitude produced by both low and high concentrations of icilin (3, 100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) was associated with an increase in the rate of spontaneous EPSCs (Figure 1A, C and E, $P = 0.007$, 0.0001, 0.02, 0.0001). In the responding neurones, the increase in spontaneous EPSC rate produced by capsaicin (1 μmol·L^{-1}) was greater than that produced by icilin (3, 100 μmol·L^{-1}) and menthol (200 μmol·L^{-1}) (Figure 1E, $P < 0.01$). In these neurones, icilin (3, 100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) did not produce a significant change in the amplitude of spontaneous EPSCs ($P = 0.4$, 0.9, 0.2, 0.09).

It was possible that the reduction in evoked synaptic transmission was due to enhanced glutamate release leading

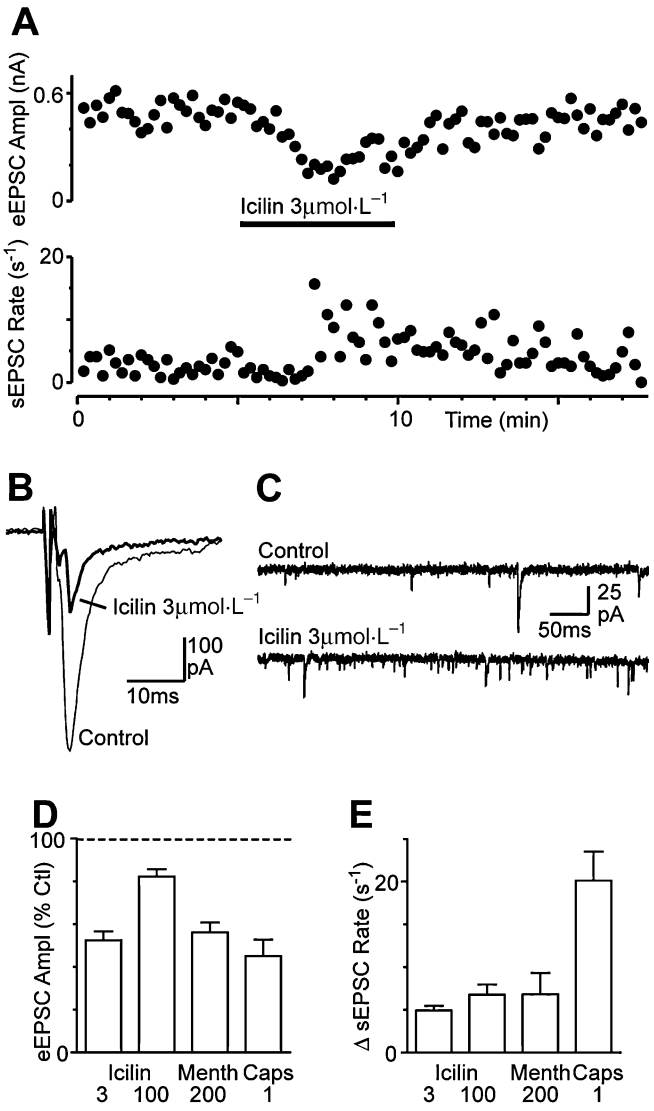

Figure 1 Icilin modulates primary afferent evoked synaptic transmission in the superficial dorsal horn. (A) Time plot of the amplitude of evoked excitatory postsynaptic currents (eEPSC) during superfusion of icilin (3 μmol·L^{-1}), plus the spontaneous EPSC (sEPSC) rate averaged over a 5 s epoch preceding each eEPSC. Raw current traces of (B) evoked EPSCs and (C) sEPSCs taken prior to (control) and during icilin (3 μmol·L^{-1}). Bar charts depicting the mean (D) eEPSC amplitude (as a percentage of pre-drug control) and (E) sEPSC rate (expressed as the absolute increase above pre-drug control) for icilin (3, 100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}). EPSCs were obtained by electrical stimulation of the dorsal rootlet in the presence of strychnine (3 μmol·L^{-1}) and picrotoxin (100 μmol·L^{-1}). (A–C) are from one neurone.

to spill-over onto inhibitory presynaptic metabotropic glutamate receptors (mGluRs), as observed in other regions of the central nervous system (see *Discussion*). In the presence of the group II and III mGluR antagonist LY341495 (3 μmol·L^{-1}), however, icilin (100 μmol·L^{-1}) produced a decrease in the amplitude of evoked EPSCs in 67% (n = 4/6) of neurones tested (79 ± 8% of pre-icilin evoked EPSC amplitude). Similarly, in the presence of LY341495 (3 μmol·L^{-1}), capsaicin (1 μmol·L^{-1}) produced a decrease in the amplitude of evoked EPSCs in all neurones tested (36 ± 6% of pre-capsaicin evoked EPSC amplitude, n = 5).

Icilin and menthol act via a presynaptic mechanism

To determine the locus of action of icilin and menthol we examined their effect on miniature EPSCs in lamina I–III dorsal horn neurones. In the presence of TTX (500 nmol·L^{-1}), picrotoxin (100 μmol·L^{-1}) and strychnine (3 μmol·L^{-1}), superfusion of the low concentration of icilin (3 μmol·L^{-1}) produced a reversible increase in the rate of miniature EPSCs in 17% (n = 14/85) of neurones tested, which was reproducible during successive applications (Figure 2A, B and F). The increase in miniature EPSC rate produced by icilin (3 μmol·L^{-1}) was associated with a leftward shift in the cumulative probability distribution of the miniature EPSC inter-event intervals (Figure 2D). In the responding neurones, icilin (3 μmol·L^{-1}) had no effect on the kinetics and amplitude of miniature EPSCs, or on the cumulative probability distributions of miniature EPSC amplitude (Figure 2C, E and G).

Superfusion of the high concentration of icilin (100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) also produced a reversible increase in the rate of miniature EPSCs in 87% (n = 52/60), 70% (n = 46/66 of neurones) and 77% (n = 72/94) of neurones tested respectively (Figure 2A, B and F). The increase in miniature EPSC rate produced by icilin (100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) was associated with a leftward shift in the cumulative probability distribution of the miniature EPSC inter-event intervals (Figure 2D). In the responding neurones, menthol (200 μmol·L^{-1}) had no effect on the kinetics and amplitude of miniature EPSCs, or on the cumulative probability distributions of miniature EPSC amplitude. Icilin (100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}), however, produced an increase in the miniature EPSC amplitude, which was associated with a rightward shift in their cumulative probability distributions (Figure 2C, E and G). The increase in miniature EPSC amplitude produced by icilin (100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) was due to summation of individual events during burst-like activity (data not shown).

The actions of low and high concentrations of icilin are consistent with TRPM8 and TRPA1 activation

The high concentration of icilin (100 μmol·L^{-1}) produced an increase in miniature EPSC rate in neurones that did (n = 4) or did not (n = 14) respond with a rate increase to a prior application of the low concentration of icilin (3 μmol·L^{-1}). We therefore determined the icilin concentration–response relationship for these two groups of neurones. In neurones responding to 3 μmol·L^{-1} icilin, the increase in miniature

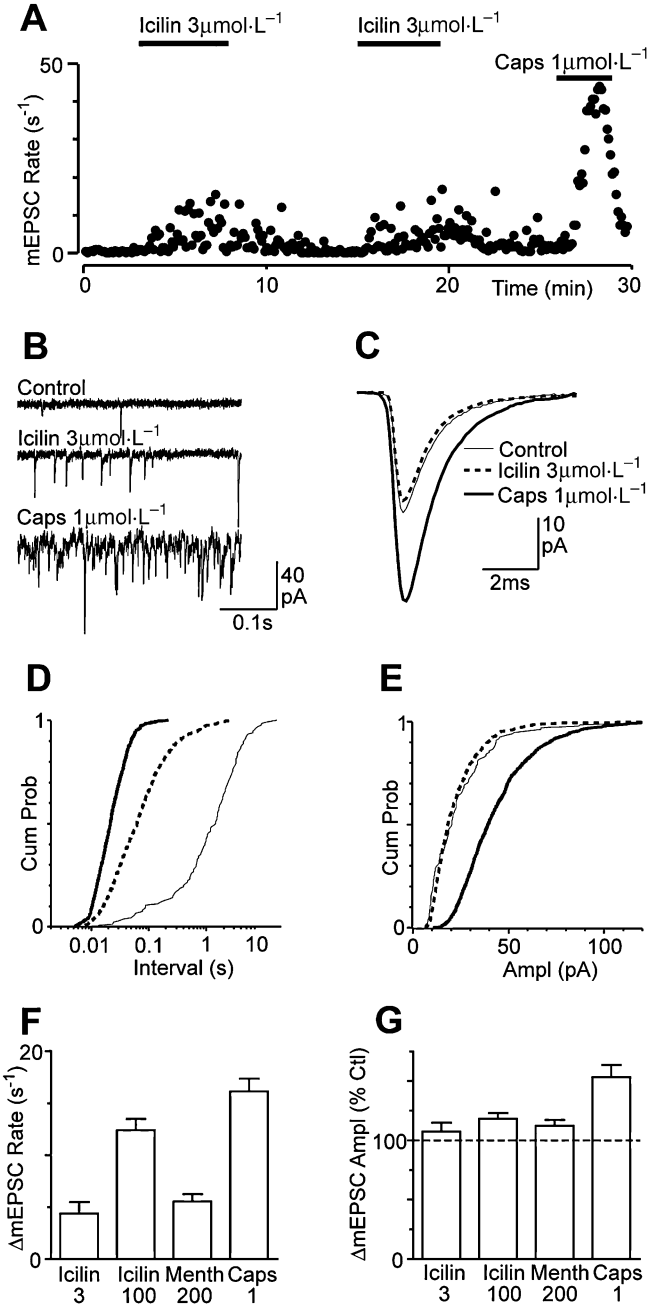

Figure 2 Icilin enhances glutamatergic transmission via a presynaptic mechanism. (A) Time plot of miniature excitatory postsynaptic current (mEPSC) rate during superfusion of icilin (3 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}). (B) Raw current traces and (C) superimposed averaged mEPSCs prior to (control) and during icilin (3 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}). Cumulative probability (cum prob) distribution plots of mEPSC (D) inter-event interval and (E) amplitude for the epochs averaged in (C). Bar charts depicting (F) mEPSC rate (expressed as the absolute increase above pre-drug control) and (G) mEPSC amplitude (as a percentage of pre-drug control) for neurones that responded to icilin (3, 100 μmol·L^{-1}), menthol (200 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}). (A–E) are taken from one neurone in the presence of TTX (500 nmol·L^{-1}), strychnine (3 μmol·L^{-1}) and picrotoxin (100 μmol·L^{-1}).

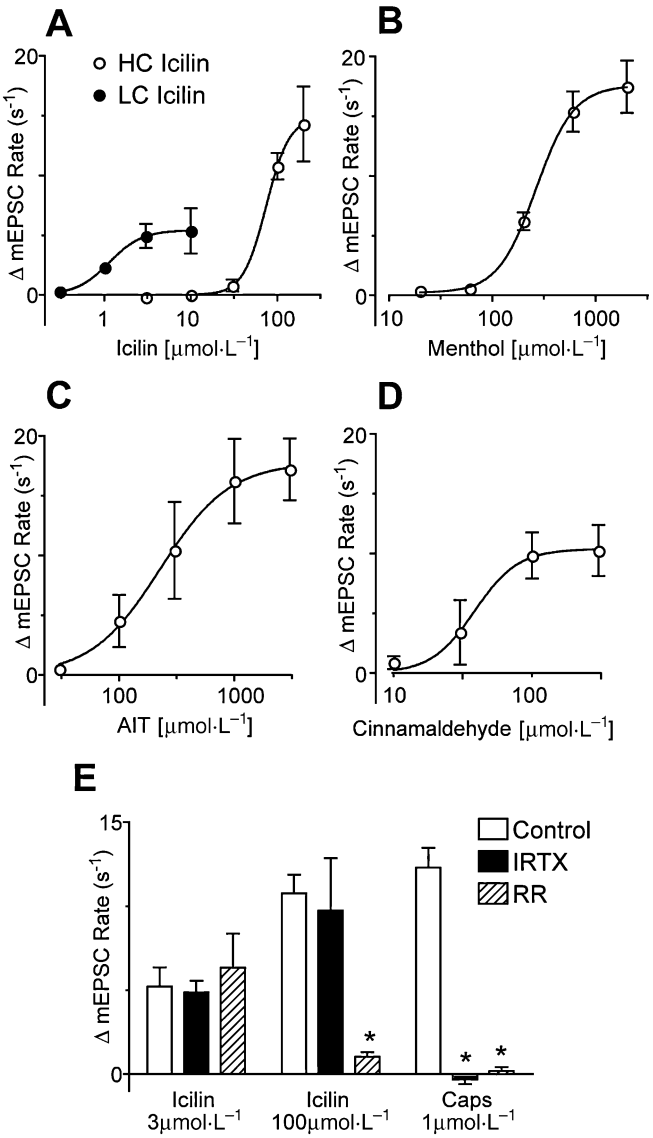

Figure 3 Icilin responsiveness predicts two presynaptic TRP ion channel types. Concentration–response curves for the increase in miniature excitatory postsynaptic current (EPSC) rate produced by (A) icilin, (B) menthol, (C) allyl isothiocyanate (AIT) and (D) cinnamaldehyde. In (A) neurones were divided into low-concentration icilin responders (LC, responded at 1–10 μmol·L^{-1}) and high-concentration icilin responders (HC, only responded >30 μmol·L^{-1}). (E) Bar chart depicting the mean increase in miniature EPSC (mEPSC) rate (expressed as the absolute increase above pre-drug level) for icilin (3, 100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) in the absence (control) and presence of iodoresiniferatoxin (IRTX, 300 nmol·L^{-1}) or ruthenium red (RR, 10 μmol·L^{-1}). In (A–D) a logistic function was fitted to each curve to determine the EC_{50}. In (A–E) change in mEPSC rate is expressed as the absolute increase above the pre-drug level. In (E) * denotes $P < 0.05$ compared with control.

EPSC rate produced by icilin was concentration-dependent, with an EC_{50} of 1.1 μmol·L^{-1} (95% confidence interval = 0.8–1.6 μmol·L^{-1}) and a Hill slope of 2.2 ± 0.1 (Figure 3A). In neurones that did not respond to 3 μmol·L^{-1} icilin, the increase in miniature EPSC rate produced by icilin was concentration-dependent, with an EC_{50} of 74 μmol·L^{-1} (95% confidence interval = 68–81 μmol·L^{-1}) and a Hill slope of 3.2 ± 0.2 (Figure 3A).

These groups of neurones responding to low and high concentrations of icilin were likely to be due to TRPM8 and TRPA1 activation respectively (see *Discussion*). We therefore determined concentration–response relationships for other TRPM8 and TRPA1 agonists. The increase in miniature EPSC rate produced by menthol was concentration-dependent, with an EC_{50} of 263 μmol·L^{-1} (95% confidence interval = 177–391 μmol·L^{-1}) and a Hill slope of 2.4 ± 0.2 (Figure 3B). The TRPA1 agonist allyl isothiocyanate (mustard oil; 1000 μmol·L^{-1}) also produced a reversible increase in the rate of miniature EPSCs in all neurones tested ($P = 0.03$), but had no significant effect on their amplitude ($P = 0.3$, $n = 6$). The increase in miniature EPSC rate produced by allyl isothiocyanate was concentration-dependent with an EC_{50} of 226 μmol·L^{-1} (95% confidence interval = 154–312 μmol·L^{-1}) and a Hill slope of 1.4 ± 0.2 (Figure 3C). The TRPA1 agonist cinnamaldehyde (100–300 μmol·L^{-1}) also produced a reversible increase in the rate of miniature EPSCs in 46% of neurones tested ($P = 0.01$, $n = 11/24$ of neurones), but had no significant effect on their amplitude ($P = 0.4$). The increase in miniature EPSC rate produced by cinnamaldehyde was concentration-dependent with an EC_{50} of 38 μmol·L^{-1} (95% confidence interval = 9–171 μmol·L^{-1}) and a Hill slope of 2.7 ± 0.8 (Figure 3D).

We next examined the effect of the TRPV1 antagonist iodoresiniferatoxin and the less selective TRP antagonist ruthenium red on the actions of low and high concentrations of icilin and capsaicin. The increase in miniature EPSC rate produced by icilin (3 μmol·L^{-1}) in the presence of ruthenium red (10 μmol·L^{-1}, $P > 0.05$, $n = 5$) and iodoresiniferatoxin (300 nmol·L^{-1}, $P > 0.05$, $n = 5$) was not significantly different from that observed in their absence (Figure 3E). The increase in miniature EPSC rate produced by icilin (100 μmol·L^{-1}) in the presence of ruthenium red (10 μmol·L^{-1}, $P < 0.05$, $n = 6$), but not iodoresiniferatoxin (300 nmol·L^{-1}, $P > 0.05$, $n = 5$), was significantly less than that observed in their absence (Figure 3E). The increase in miniature EPSC rate produced by capsaicin (1 μmol·L^{-1}) in the presence of ruthenium red (10 μmol·L^{-1}, $P < 0.05$, $n = 6$) and iodoresiniferatoxin (300 nmol·L^{-1}, $P < 0.05$, $n = 5$) was significantly less than that observed in their absence (Figure 3E).

Cooling presynaptically enhances glutamatergic synaptic transmission

The above experiments suggest that TRPM8 and TRPA1 agonists modulate primary afferent transmission via a presynaptic mechanism. We next examined whether reductions in temperature had a similar presynaptic effect in lamina I–III neurones. A reduction in bath temperature from 34°C to 25°C produced an increase in miniature EPSC rate in 23% ($n = 9/39$) neurones, with an average temperature threshold for activation of 29 ± 1°C (Figure 4A and B). In the responding neurones, there was a phasic increase in miniature EPSC rate during the period of temperature reduction, followed by a sustained increase at 25°C (Figure 4A, increase at 25°C = 11.4 ± 4.1 s^{-1}). The increase in miniature EPSC rate produced by temperature reduction to 25°C was associated with a leftward shift in the cumulative probability distribution of the miniature EPSC inter-event intervals (Figure 4D). In the responding neurones, temperature reduction to 25°C had no effect on the

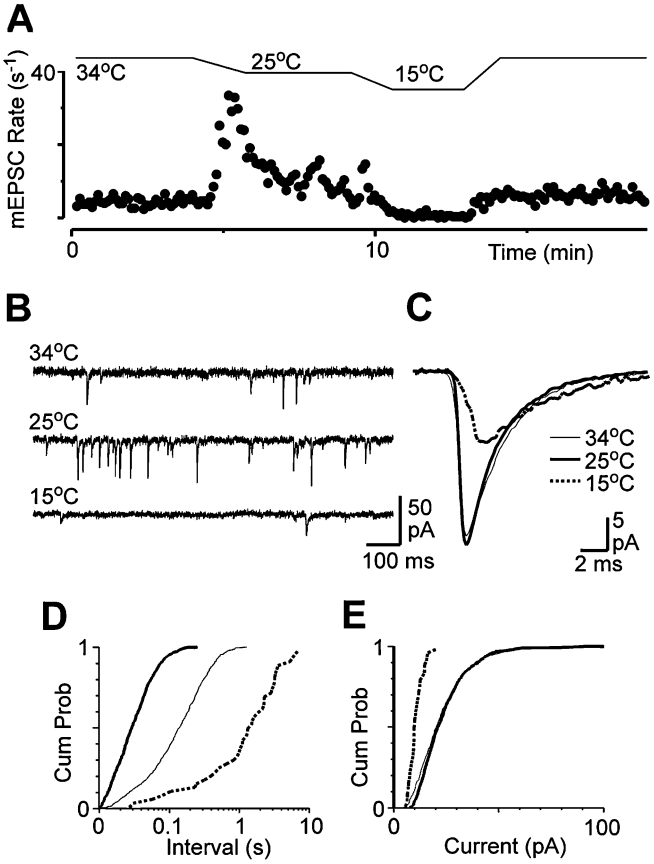

Figure 4 Temperature reductions enhance glutamatergic transmission via a presynaptic mechanism. (A) Time plot of miniature excitatory postsynaptic current (mEPSC) rate during successive temperature decreases to 25°C and 15°C. (B) Raw current traces and (C) superimposed averaged mEPSCs at the different temperatures indicated. Cumulative probability (Cum Prob) distribution plots of mEPSC (D) inter-event interval and (E) amplitude for the epochs averaged in (C). (A–E) are taken from one neurone in the presence of tetrodotoxin (500 nmol·L^{-1}), strychnine (3 μmol·L^{-1}) and picrotoxin (100 μmol·L^{-1}).

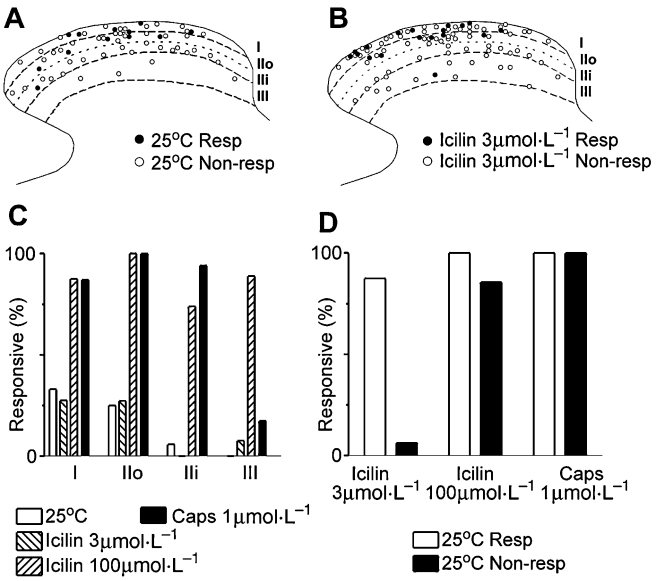

Figure 5 Cool and icilin sensitive glutamatergic inputs target a subpopulation of superficial dorsal horn neurones. Location plots of neurones that did (Resp) or did not (Non Resp) respond with an increase in miniature excitatory postsynaptic current rate to (A) a temperature reduction to 25°C, and (B) icilin (3 μmol·L^{-1}). (C) Bar chart showing the percentage of temperature reduction (25°C), icilin (3, 100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) responsive neurones in laminae I, II$_o$, II$_i$ and III. (D) Bar chart showing the percentage of 25°C responsive (open bars) and non-responsive (filled bars) neurones, which were also responsive to icilin (3, 100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}).

kinetics and amplitude of miniature EPSCs, or on the cumulative probability distributions of miniature EPSC amplitudes (Figure 4C and E). In the other neurones, a reduction in temperature to 25°C produced a reduction in miniature EPSC rate (reduction = 1.3 ± 0.4 s^{-1}, P = 0.006, n = 30).

A further reduction in bath temperature to 15°C produced a reduction in miniature EPSC rate in all neurones tested (Figure 4A and B, n = 18). This decrease in miniature EPSC rate was associated with a rightward shift in the inter-event interval cumulative probability distribution (Figure 4D). There was also a significant decrease in the amplitude, associated with a leftward shift in the amplitude cumulative probability distribution, plus a slowing of the rise time of miniature EPSCs at 15°C (Figure 4C and E).

Presynaptic fibres with a TRPM8 profile target a subpopulation of dorsal horn neurones

We next examined the distribution of neurones throughout laminae I–III that were targeted by innocuous cold-, icilin- and capsaicin-sensitive glutamatergic inputs. Temperature reduction to 25°C produced an increase in miniature EPSC rate in a subpopulation of neurones in laminae I and II$_o$, but few in lamina II$_i$ (II inner) and none in lamina III (Figure 5A and C). Similarly, low concentrations of icilin (3–10 μmol·L^{-1}) produced an increase in miniature EPSC rate in a subpopulation of neurones in laminae I and II$_o$, but none in lamina II$_i$ and few in lamina III (Figure 5B and C). By contrast, the high concentration of icilin (100 μmol·L^{-1}) produced an increase in miniature EPSC rate in relatively high proportions of neurones throughout laminae I, II$_o$ and II$_i$, and III (Figure 5C). Capsaicin (1 μmol·L^{-1}) produced an increase in miniature EPSC rate in most neurones throughout laminae I, II$_o$ and II$_i$, but few in lamina III (Figure 5C).

We next examined the correspondence between neurones that responded with an increase in miniature EPSCs rate to temperature reduction and to the TRP agonists, icilin and capsaicin. The low concentration of icilin (3 μmol·L^{-1}) produced an increase in miniature EPSC rate in most 25°C responsive neurones (n = 7/8), but in only one of the 25°C non-responsive neurones (n = 1/13) (Figure 5D). In contrast, the high concentration of icilin (100 μmol·L^{-1}) and capsaicin (1 μmol·L^{-1}) produced an increase in miniature EPSC rate in all 25°C responsive neurones (n = 3/3, 5/5) and in most 25°C non-responsive neurones (n = 6/7, 6/6) (Figure 5D).

Effect of icilin on postsynaptic membrane currents and inhibitory synaptic transmission

We next examined whether the high concentration of icilin (100 μmol·L^{-1}), which is likely to activate both TRPM8 and

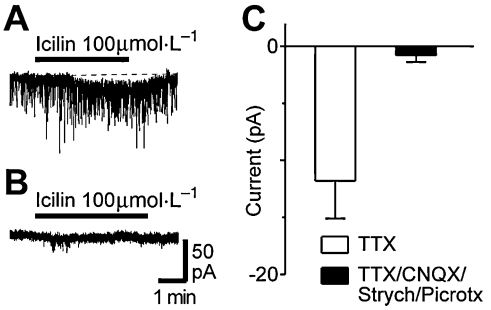

Figure 6 Postsynaptic effects of icilin in the superficial dorsal horn. Raw membrane current traces in the presence of (A) tetrodotoxin (TTX) (500 nmol·L^{-1}), or (B) TTX (500 nmol·L^{-1}), 6-cyano-2,3-dihdroxy-7-nitro-quinoxaline (CNQX) (5 μmol·L^{-1}), strychnine (Strych, 3 μmol·L^{-1}) and picrotoxin (Picrotx, 100 μmol·L^{-1}) during superfusion of icilin (100 μmol·L^{-1}). (C) Bar charts depicting the mean currents produced by icilin in the presence of TTX, or TTX, CNQX, strychnine and picrotoxin. (A) and (B) are taken from two different neurones.

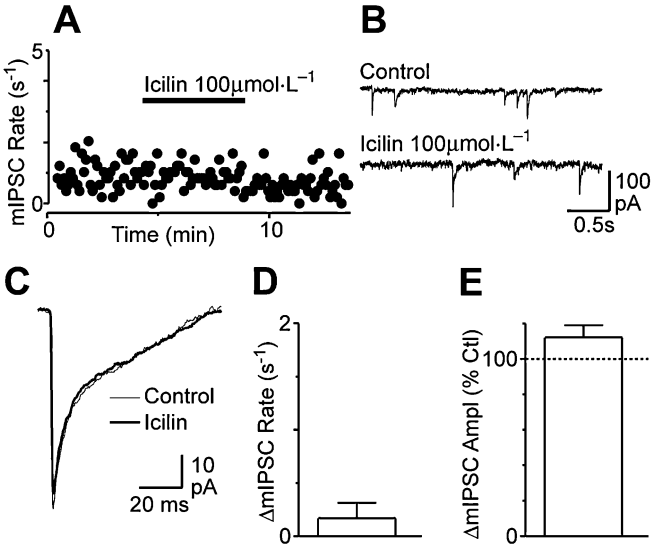

Figure 7 Effect of icilin on GABAergic/glycinergic transmission in the superficial dorsal horn. (A) Time plot of miniature inhibitory postsynaptic current (mIPSC) rate during superfusion of icilin (100 μmol·L^{-1}). (B) Raw current traces and (C) superimposed averaged mIPSCs prior to (control) and during icilin. Bar charts depicting (D) mIPSC rate (expressed as the absolute increase above pre-drug control) and (E) mIPSC amplitude (expressed as a percentage of pre-drug control) for all neurones tested. (A)–(C) are taken from one neurone in the presence of tetrodotoxin (500 nmol·L^{-1}) and 6-cyano-2,3-dihdroxy-7-nitro-quinoxaline (5 μmol·L^{-1}).

TRPA1, had a direct postsynaptic effect on dorsal horn neurones in laminae I, II_o and II_i. In the presence of TTX (500 nmol·L^{-1}), superfusion of the high concentration of icilin (100 μmol·L^{-1}) produced a significant inward current (Figure 6A and C, $P = 0.004$, $n = 19$). This current might have been due to TTX-resistant, action potential-independent neurotransmitter release. In the presence of TTX (500 nmol·L^{-1}), picrotoxin (100 μmol·L^{-1}), strychnine (3 μmol·L^{-1}) and CNQX (5 μmol·L^{-1}), the high concentration of icilin (100 μmol·L^{-1}) had no significant effect on the membrane current (Figure 6B and C, $P = 0.2$, $n = 12$).

To determine whether icilin specifically targets glutamatergic synaptic transmission, we also examined its effect on inhibitory GABAergic/glycinergic synaptic transmission in laminae I, II_o and II_i. In the presence of TTX (500 nmol·L^{-1}) and CNQX (5 μmol·L^{-1}), superfusion of the high concentration of icilin (100 μmol·L^{-1}) did not significantly affect the rate, amplitude and kinetics of miniature IPSCs (Figure 7, $P = 0.4$, 0.6, $n = 19$).

Discussion

The present study has demonstrated that, like TRPV1, activation of presynaptic TRPM8 and TRPA1 cation channels modulates glutamatergic primary afferent synaptic transmission onto superficial dorsal horn neurones. Furthermore, these findings suggest that glutamatergic inputs with TRPM8, TRPA1 and TRPV1 profiles target partially distinct but overlapping subpopulations of superficial dorsal horn neurones.

Icilin and temperature reduction presynaptically modulate primary afferent glutamatergic synaptic transmission

In the present study, the effects of icilin, menthol and temperature reduction (to 25°C) were most likely to be mediated by activation of presynaptic receptors located on primary afferent central terminals. Menthol (200 μmol·L^{-1}) and both low (3 μmol·L^{-1}) and high (100 μmol·L^{-1}) concentrations of icilin inhibited primary afferent evoked EPSCs in lamina I and II neurones. This is the first study to directly demonstrate that icilin and menthol modulate primary afferent evoked transmission in intact spinal cord slices, although it has been examined previously in co-cultured DRG/dorsal horn neurones (Tsuzuki *et al.*, 2004). The inhibition of evoked transmission was due to a direct effect on glutamatergic presynaptic terminals because icilin (100 μmol·L^{-1}) had no effect on $GABA_A$/glycine-mediated miniature IPSCs (see also Kosugi *et al.*, 2007) and had no direct postsynaptic effects. In addition, icilin, menthol, allyl isothiocyanate, cinnamaldehyde and temperature reduction (25°C) all increased TTX-resistant miniature EPSC rate, without affecting their amplitude distributions, or kinetics, as observed in earlier studies with spinal cord slices using similar concentrations of menthol, allyl isothiocyanate and cinnamaldehyde (Baccei *et al.*, 2003; Kosugi *et al.*, 2007), and for menthol and temperature reductions in co-cultured DRG/dorsal horn neurones (Tsuzuki *et al.*, 2004).

The 'paradoxical' inhibition of evoked EPSCs and increase in miniature EPSCs produced by icilin and menthol is similar to that observed for capsaicin in spinal cord slices (see also Yang *et al.*, 1999), but differs from co-cultured DRG/dorsal horn neurones where menthol produces an increase in evoked and miniature EPSCs (Tsuzuki *et al.*, 2004). The inhibition of evoked EPSCs in slices might have been due to activation of presynaptic group II and/or III mGluR autoreceptors following increased spontaneous release (Scanziani *et al.*, 1997; Vogt and Nicoll, 1999); however, icilin and capsaicin produced a decrease in evoked EPSCs in the presence of the group II/III antagonist LY341495. The paradoxical inhibition-evoked release was therefore likely to be due to

other mechanisms within afferent nerve terminals, including depolarization-induced inactivation of presynaptic voltage-dependent sodium and calcium channels, and action potential shunting (Engelman and MacDermott, 2004).

Pharmacological responses are consistent with TRMP8 and TRPA1

The present study provides pharmacological evidence for functional presynaptic TRPM8 and TRPA1 receptors. In a subpopulation of lamina I and II_o neurones, the glutamatergic miniature EPSC rate was increased by low concentrations of icilin (EC_{50} 1.5 $\mu mol \cdot L^{-1}$) and by menthol (EC_{50} 263 $\mu mol \cdot L^{-1}$). This is consistent with the cloned TRPM8 that is activated by icilin and menthol with EC_{50} values of 0.4 and 67 $\mu mol \cdot L^{-1}$ respectively (McKemy *et al.*, 2002; Peier *et al.*, 2002; Behrendt *et al.*, 2004), although it has also been reported that menthol is also a ligand at TRPA1 (Macpherson *et al.*, 2006; Karashima *et al.*, 2007). By contrast, in most lamina I and II neurones, miniature EPSC rate was increased by high concentrations of icilin (EC_{50} 79 $\mu mol \cdot L^{-1}$), but not by low concentrations of icilin (3 $\mu mol \cdot L^{-1}$). In addition, miniature EPSC rate in these neurones was increased by allyl isothiocyanate and cinnamaldehyde with EC_{50} of 206 and 38 $\mu mol \cdot L^{-1}$ respectively. This is largely consistent with the cloned TRPA1 that is only activated by icilin at concentrations above 25 $\mu mol \cdot L^{-1}$, and by allyl isothiocyanate and cinnamaldehyde with EC_{50} values of 11–22 $\mu mol \cdot L^{-1}$ and 61 $\mu mol \cdot L^{-1}$ respectively (Story *et al.*, 2003; Bandell *et al.*, 2004; Jordt *et al.*, 2004). The relatively high EC_{50} for menthol and allyl isothiocyanate in native slices is similar to that observed for other lipophilic compounds and might be due to poor drug penetration in slices, or overexpression of receptors in cell lines (Vaughan *et al.*, 2000). In addition, the increase in miniature EPSC rate produced by capsaicin and the high concentration of icilin (100 $\mu mol \cdot L^{-1}$), but not by the low concentration of icilin (3 $\mu mol \cdot L^{-1}$), was abolished by ruthenium red, which is consistent with TRPM8 and TRPA1 pharmacology (Peier *et al.*, 2002; Story *et al.*, 2003; Behrendt *et al.*, 2004; Jordt *et al.*, 2004).

In the present study, a subpopulation of lamina I and II_o neurones responded to temperature in a manner consistent with TRPM8 activation. In these neurones, glutamatergic miniature EPSC rate was increased by innocuous cold with a threshold of 29°C. While this temperature threshold is similar to that previously reported for native, cool-sensitive, sensory neurones (Reid *et al.*, 2002; Nealen *et al.*, 2003; Thut *et al.*, 2003), it differs from the threshold of 22–26°C reported for the cloned TRPM8 (McKemy *et al.*, 2002; Peier *et al.*, 2002; Story *et al.*, 2003; Bandell *et al.*,2004; Jordt *et al.*,2004). The difference in temperature thresholds between native cells and the cloned TRPM8 may be due to intracellular cascades in sensory neurones that modulate TRPM8 temperature/voltage dependence to other ion channels such as TWIK-related K+ channel-1 and epithelial sodium channel that may be involved in innocuous cold transduction (see Reid, 2005), or to the measurement of neurotransmitter release that is a downstream consequence of presynaptic TRPM8 activation. The finding that sensitivity to the low concentration of icilin (3 $\mu mol \cdot L^{-1}$) was highly predictive of innocuous cold (25°C) sensitivity suggests that both innocuous cold and low concentrations of icilin activate presynaptic TRPM8 ion channels on a subpopulation of primary afferent terminals in the spinal cord. It is, however, not possible to rule out that they act via separate presynaptic TRP-like receptors, which are located on the same or different converging primary afferent terminals.

The role of TRPA1 in noxious cold sensation remains a matter of controversy. Some studies have shown that the cloned TRPA1 is activated below a threshold of 12–24°C (Story *et al.*, 2003; Bandell *et al.*, 2004; Sawada *et al.*, 2007), while others have reported that it is temperature-insensitive (Jordt *et al.*, 2004; Nagata *et al.*, 2005; Bautista *et al.*, 2006). In addition, there are mixed reports from knockout studies as to the involvement of TRPA1 in noxious cold sensation (Bautista *et al.*, 2006; Kwan *et al.*, 2006). The present study cannot distinguish between these possibilities because temperature reduction into the noxious range (15°C) reduced glutamatergic miniature EPSC rate in all neurones. This inhibition reflects the high temperature dependence of synaptic transmission (e.g. Isaacson and Walmsley, 1995) and might have masked any potential TRPA1-mediated cold sensitivity.

Differential processing of TRPM8-, TRPV1- and TRPA1-sensitive primary afferent inputs

In the present study, it was observed that glutamatergic inputs with a TRPM8 profile (low concentrations of icilin and innocuous cold-sensitive) targeted a subpopulation of superficial lamina I and II_o neurones, which is consistent with the central termination pattern of TRPM8-containing afferents (Dhaka *et al.*, 2008). By contrast, glutamatergic inputs with TRPA1 (high concentrations of icilin, cinnamaldehyde and allyl isothiocyanate-sensitive) and TRPV1 (capsaicin-sensitive) profiles targeted most neurones throughout laminae I, II_o and II_i, although they differed in their relative terminations in lamina III. The present *in vitro* observations parallel prior *in vivo* studies in the rat (Christensen and Perl, 1970; Bester *et al.*, 2000; Zhang *et al.*, 2006), cat and monkey (Dostrovsky and Craig, 1996; Han *et al.*, 1998; Craig *et al.*, 2001) in which peripheral, innocuous cold, stimuli activate a subpopulation of superficial lamina I and II_o neurones that project to supraspinal structures, such as the parabrachial nucleus and thalamus. It is therefore possible that afferents with a TRPM8 profile might target ascending projection neurones, although this remains to be determined directly *in vitro*.

While the superficial dorsal horn projection pattern of TRPM8-expressing afferents differed from that of TRPA1/TRPV1-expressing afferents, there was a significant overlap. This is consistent with *in vivo* observations in the rat (Zhang *et al.*, 2006), but differs from the cat and monkey where innocuous cold afferent inputs are proposed to be more specific (Dostrovsky and Craig, 1996; Han *et al.*, 1998; Craig *et al.*, 2001). The differences and overlap of TRPM8- and TRPV1/TRPA1-expressing glutamatergic inputs onto dorsal horn neurones may be due to a number of factors. Several studies have shown that TRPM8 and TRPA1 are expressed in non-peptidergic and peptidergic sensory neurones respectively (Peier *et al.*, 2002; Story *et al.*, 2003; Kobayashi *et al.*, 2005; Dhaka *et al.*, 2008), while others have demonstrated partial

overlap in their expression (McKemy *et al.*, 2002; Babes *et al.*, 2004; Okazawa *et al.*, 2004; Xing *et al.*, 2006; Takashima *et al.*, 2007). The polymodal response to TRPM8 and TRPV1/TRPA1 observed in the present study might therefore be due to converging afferent inputs that express different TRP ion channel subtypes, or to co-expression of the different TRP ion channels in a common group of primary afferents.

In conclusion, while the functional role of centrally located TRP ion channels remains unclear, their presence raises the possibility of an endogenous spinal thermo-nociceptive regulatory system. It has been shown that the endocannabinoid anandamide and related arachidonic acid-amino acid conjugates activate TRPV1 (Caterina *et al.*, 1997; Huang *et al.*, 2002), and anandamide acts via TRPV1 to enhance primary afferent transmission within the dorsal horn (Morisset *et al.*, 2001; Jennings *et al.*, 2003). Similarly, an expanding range of endogenous agents have been shown to activate TRPM8 and TRPA1 (Vanden Abeele *et al.*, 2006; Andersson *et al.*, 2008; Cruz-Orengo *et al.*, 2008), although their role within the spinal cord remains to be determined. Evidence continues to emerge that these ion channels may provide a therapeutic target for cold hypesthesia associated with neuropathic pain states, both peripherally (Katsura *et al.*, 2006; Proudfoot *et al.*, 2006; Colburn *et al.*, 2007; Frederick *et al.*, 2007; Petrus *et al.*, 2007; Xing *et al.*, 2007; Wasner *et al.*, 2008) and within the spinal cord (Proudfoot *et al.*, 2006).

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Statement of conflicts of interest

None.

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