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RESEARCH PAPER

Menthol enhances phasic and tonic $GABA_A$ receptor-mediated currents in midbrain periaqueductal grey neurons

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BACKGROUND AND PURPOSE

Menthol, a naturally occurring compound in the essential oil of mint leaves, is used for its medicinal, sensory and fragrant properties. Menthol acts via transient receptor potential (TRPM8 and TRPA1) channels and as a positive allosteric modulator of recombinant $GABA_A$ receptors. Here, we examined the actions of menthol on $GABA_A$ receptor-mediated currents in intact midbrain slices.

EXPERIMENTAL APPROACH

Whole-cell voltage-clamp recordings were made from periaqueductal grey (PAG) neurons in midbrain slices from rats to determine the effects of menthol on $GABA_A$ receptor-mediated phasic IPSCs and tonic currents.

KEY RESULTS

Menthol (150–750 μM) produced a concentration-dependent prolongation of spontaneous $GABA_A$ receptor-mediated IPSCs, but not non-NMDA receptor-mediated EPSCs throughout the PAG. Menthol actions were unaffected by TRPM8 and TRPA1 antagonists, tetrodotoxin and the benzodiazepine antagonist, flumazenil. Menthol also enhanced a tonic current, which was sensitive to the $GABA_A$ receptor antagonists, picrotoxin (100 μM), bicuculline (30 μM) and Zn^{2+} (100 μM), but unaffected by gabazine (10 μM) and a $GABA_C$ receptor antagonist, 1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate (TPMPA; 50 μM). In addition, menthol potentiated currents induced by the extrasynaptic $GABA_A$ receptor agonist THIP/gaboxadol (10 μM).

CONCLUSIONS AND IMPLICATIONS

These results suggest that menthol positively modulates both synaptic and extrasynaptic populations of $GABA_A$ receptors in native PAG neurons. The development of agents that potentiate $GABA_A$-mediated tonic currents and phasic IPSCs in a manner similar to menthol could provide a basis for novel $GABA_A$-related pharmacotherapies.

Abbreviations

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride, gaboxadol; TPMPA, 1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate; TTX, tetrodotoxin

Introduction

Menthol is a naturally occurring monoterpenoid alcohol derived from the essential oil of mint plants. Menthol has been widely used as a food additive, local anaesthetic, topical analgesic, antipruritic, antimicrobial and gastric sedative. Topical application of menthol to the skin produces cooling sensations, which can range in quality from pleasant to painful, via activation of transient receptor potential (TRP) channels, TRPM8 and TRPA1 (channel nomenclature follows Alexander *et al.*, 2103a), located on the peripheral terminals of cold-sensitive nociceptive afferents (Wasner *et al.*, 2004; Hatem *et al.*, 2006; Vriens *et al.*, 2008).

In addition to peripheral actions, a number of studies have demonstrated that menthol has actions within the CNS. In animal behavioural tests, systemic or direct central administration of menthol produces a variety of effects, including ambulation promoting (Umezu *et al.*, 2001), sedative/general anaesthetic (Watt *et al.*, 2008), anticonvulsant (Zhang *et al.*, 2008), analgesic (Proudfoot *et al.*, 2006; Su *et al.*, 2011; Pan *et al.*, 2012) and nootropic (Bhadania *et al.*, 2012) actions. In the sensory system, menthol is thought to act at TRPM8 channels located predominantly on primary afferent neurons, whereas expression levels for this channel are low or absent in spinal cord and brain tissues (Peier *et al.*, 2002). Other potential cellular targets for menthol within the CNS include voltage-gated sodium (Haeseler *et al.*, 2002; Gaudioso *et al.*, 2012; Pan *et al.*, 2012) and calcium channels (Swandulla *et al.*, 1987; Baylie *et al.*, 2010; Pan *et al.*, 2012; Cheang *et al.*, 2013), 5-HT_3 receptors (Ashoor *et al.*, 2013a), nicotinic ACh receptors (Hans *et al.*, 2012; Ashoor *et al.*, 2013b), glycine receptors (Hall *et al.*, 2004) and $GABA_A$ receptors (Hall *et al.*, 2004; Watt *et al.*, 2008). In the case of $GABA_A$ receptors, menthol acts as a potent positive allosteric modulator of recombinant human $GABA_A$ receptors, possibly via binding sites shared with the i.v. anaesthetic agent propofol (Hall *et al.*, 2004; Watt *et al.*, 2008). Consistent with this, recent studies have shown that menthol enhances $GABA_A$ receptor-mediated currents in spinal cord dorsal horn (Pan *et al.*, 2012) and hippocampal CA1 pyramidal neurons *in vitro* (Zhang *et al.*, 2008). In addition, menthol was found to suppress respiratory rhythm generation in a brainstem–spinal cord preparation via a $GABA_A$ receptor-dependent mechanism (Tani *et al.*, 2010).

$GABA_A$ receptors are pentameric anion channels composed of various combinations of α1–6, β1–3, γ1–3, δ, ε, π, θ and ρ1–3 subunits (Alexander *et al.*, 2013b). As a general rule, $GABA_A$ receptors containing α1–3 and γ2 subunits (α1–3βxγ2) are enriched in synapses and mediate phasic synaptic inhibition, whereas extrasynaptic $GABA_A$ receptors mediate tonic inhibition and most commonly contain α4–6 subunits together with either a γ2 or δ subunit (α4/6βxδ and α5βxγ2) (Farrant and Nusser, 2005). In the present study, we examined the effects of menthol on phasic and tonic $GABA_A$ receptor-mediated currents in the midbrain periaqueductal grey (PAG), a region that plays a pivotal role in organizing an organism's behavioural responses to threat, stress and pain (Keay and Bandler, 2001). We found that menthol prolonged synaptic $GABA_A$ receptor-mediated currents in a concentration-dependent manner, and enhanced a $GABA_A$ receptor-mediated tonic current in neurons throughout PAG. These effects were likely to be mediated via interactions with distinct populations of synaptic and δ subunit-lacking extrasynaptic $GABA_A$ receptors. The findings implicate modulation of GABAergic activity in the CNS as a possible cellular substrate for behavioural changes observed following central administration of menthol.

Methods

Brain slice preparation

All animal care and experimental procedures complied with guidelines set out by the National Health and Medical Research Council 'Australian code of practice for the care and use of animals for scientific purposes' and were approved by the Institutional Animal Care and Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 62 animals were used in the experiments described here.

Experiments were carried out on male and female Sprague-Dawley rats (2–4 weeks old) obtained from Animal Resources Centre (Canning Vale, Australia). Animals were deeply anaesthetized with isoflurane and then decapitated. Transverse midbrain slices (300 μm) containing the PAG were cut using a vibratome (VT1200S, Leica Microsystems, Nussloch, Germany) in ice-cold artificial CSF (ACSF), as described previously (Drew *et al.*, 2008). PAG slices were maintained at 34°C in a submerged chamber containing ACSF equilibrated with 95% O_2 and 5% CO_2. The slices were then individually transferred to a chamber and superfused continuously (2.0–2.5 mL·min^{-1}) with ACSF (34°C) of composition (in mM): 126 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose and 25 $NaHCO_3$.

Electrophysiology

PAG neurons were visualized using infrared Dodt-tube contrast gradient optics on an upright microscope (BX51; Olympus, Sydney, Australia). Whole-cell voltage-clamp recordings (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA) of synaptic currents (holding potential, −70 mV) were made from ventrolateral, lateral and dorsolateral PAG neurons using a CsCl-based internal solution containing (in mM): 140 CsCl, 0.2 EGTA, 10 HEPES, 1 $MgCl_2$, 2 MgATP and 0.3 NaGTP (pH 7.3; osmolarity, 280–285 mOsm·L^{-1}). Series resistance (<25 MΩ) was compensated by 80% and continuously monitored during experiments. Liquid junction potentials of −4 mV were corrected. All recordings of spontaneous IPSCs and tonic $GABA_A$ receptor-mediated currents were obtained in the presence of the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (5 μM) and the glycine receptor antagonist strychnine (3 μM), whereas miniature IPSCs were isolated by further addition of the sodium channel blocker tetrodotoxin (TTX) (0.3 μM). All recordings of spontaneous EPSCs were obtained in the presence of the $GABA_A$ receptor antagonist picrotoxin (100 μM) and strychnine (3 μM). IPSCs and EPSCs were inward currents with the internal/externals solutions used in these experiments.

Data analysis

IPSCs and EPSCs were filtered (2, 5 kHz low-pass filter) and sampled (10, 20 kHz) for online and later offline analysis

(Axograph X; AxoGraph Scientific, Sydney, Australia). Spontaneous IPSCs and EPSCs above a preset threshold (2.5–4.5 SDs above baseline noise) were automatically detected by a sliding template algorithm, then manually checked offline. Multi-peak PSCs were excluded from analysis of PSC kinetics, whereas all PSCs, including multi-peak events, were included in the analysis of PSC frequency. The IPSC and EPSC decay phases were best fit by two exponentials, and results are presented as weighted decay time, $\tau_w = [A_{fast} \times \tau_{fast} + A_{slow} \times \tau_{slow}]/[A_{fast} + A_{slow}]$, where A and τ are the amplitudes and time constants for the fast and slow components of IPSC decay phases. Synaptic charge transfer, Q, was calculated as the area under averaged PSC traces. The membrane holding current ($I_{holding}$) and noise/variance (I_{var}) were measured in segments of traces where PSCs were absent.

All numerical data are expressed as mean ± SEM. Statistical comparisons of individual drug effects were made using paired Student's *t*-test and comparisons between treatment groups with an unpaired Student's *t*-test or one-way ANOVA using Dunnett's correction for *post hoc* comparisons. Differences were considered significant if $P < 0.05$.

Materials

Bicuculline, CNQX, HC-030031, picrotoxin, strychnine hydrochloride, (–)-menthol, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride (THIP, gaboxadol), $ZnCl_2$ and (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid hydrate (TPMPA) were obtained from Sigma (Sydney, Australia); capsazepine and gabazine (SR95531) from Tocris Bioscience (Bristol, UK); flumazenil and TTX from Ascent Scientific (Bristol, UK); and icilin from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of drugs were made in distilled water, except menthol (in ethanol vehicle, ethanol:ACSF ≤ 1:2000), flumazenil, capsazepine, HC-030031 and icilin (in dimethyl sulfoxide vehicle, dimethyl sulfoxide:ACSF ≤ 1:3000), then diluted to working concentrations in ACSF immediately before use and applied by superfusion.

Results

Menthol prolongs the duration of spontaneous IPSCs, but not EPSCs

Superfusion of menthol (450 μM) prolonged the duration of spontaneous IPSCs in all PAG neurons tested (Figures 1A,B and 5A; $n = 17$). Specifically, menthol produced an increase in the IPSC 10–90% rise time, weighted decay time (τ_w) and synaptic charge transfer (Q) (Figure 1E; $P < 0.001$–0.05; Table 1). The increase in τ_w produced by menthol resulted from a similar increase in both τ_{fast} (323 ± 52% of baseline) and τ_{slow} (276 ± 89% of baseline). Menthol had no significant effect on IPSC amplitude ($P = 0.9$) and produced a variable but significant increase in IPSC frequency ($P = 0.04$) (Figure 1E; Table 1). The effect of menthol on these spontaneous IPSC parameters was similar in the ventrolateral/lateral ($n = 7$) and dorsolateral subregions ($n = 10$) of the PAG ($P > 0.05$). IPSCs recorded in the presence of menthol were abolished by the competitive $GABA_A$ receptor antagonist gabazine (10 μM, $n = 3$; Figure 1A), confirming that they were mediated by $GABA_A$ receptors. Furthermore, superfusion of ethanol vehicle (dilution 1:2000 absolute ethanol in ACSF) had no effect on spontaneous IPSCs or $I_{holding}$ ($P > 0.05$, $n = 4$, data not shown).

The slowing of spontaneous IPSC decay times by menthol was concentration dependent, with significant increases detected at concentrations ≥150 μM (Figure 1F; $n = 3$–17). In contrast, IPSC rise times did not appear to be affected by menthol in a concentration-dependent manner, with a significant increase being observed only at 450 μM (Figure 1F). Furthermore, there was no correlation between the effect of menthol (450 μM) on IPSC rise and decay times in individual neurons (Pearson's correlation coefficient, $r^2 = 0.02$, $P > 0.05$, $n = 17$), suggesting that the change in rise times may have been a non-specific effect of menthol. At higher concentrations (≥750 μM), menthol dramatically increased baseline noise, which obscured many IPSCs and precluded the determination of the EC_{50} of menthol's effect on IPSC decay time. The menthol (450 μM)-induced changes in IPSC rise and

Table 1

Effect of menthol on spontaneous IPSCs and EPSCs

	Ampl (pA)	Freq (s^{-1})	Rise time (ms)	Decay time (ms)	Charge transfer (fC)
sIPSC					
Pre	42 (5)	2.7 (1.0)	0.58 (0.04)	6.9 (0.6)	336 (25)
+Menthol	38 (3)	5.0 (2.0)*	0.83 (0.06)***	28.0 (3.9)***	960 (85)***
mIPSC					
Pre	42 (3)	1.5 (0.4)	0.59 (0.01)	7.4 (0.4)	318 (17)
+Menthol	51 (5)	2.1 (0.5)	0.79 (0.05)*	29.8 (5.8)*	1264 (151)**
sEPSC					
Pre	22 (2)	3.9 (1.3)	0.52 (0.10)	2.0 (0.2)	60 (4)
+Menthol	19 (2)	5.6 (1.9)	0.59 (0.08)*	2.2 (0.2)	56 (3)

Data shown as mean (SEM) before and during application of menthol (450 μM) for spontaneous IPSCs (sIPSCs), miniature IPSCs (mIPSCs) and spontaneous EPSCs (sEPSCs). * $P < 0.05$, **0.01, ***0.001 for Pre versus +Menthol (sIPSC, $n = 17$; mIPSC, $n = 5$; sEPSC, $n = 7$).

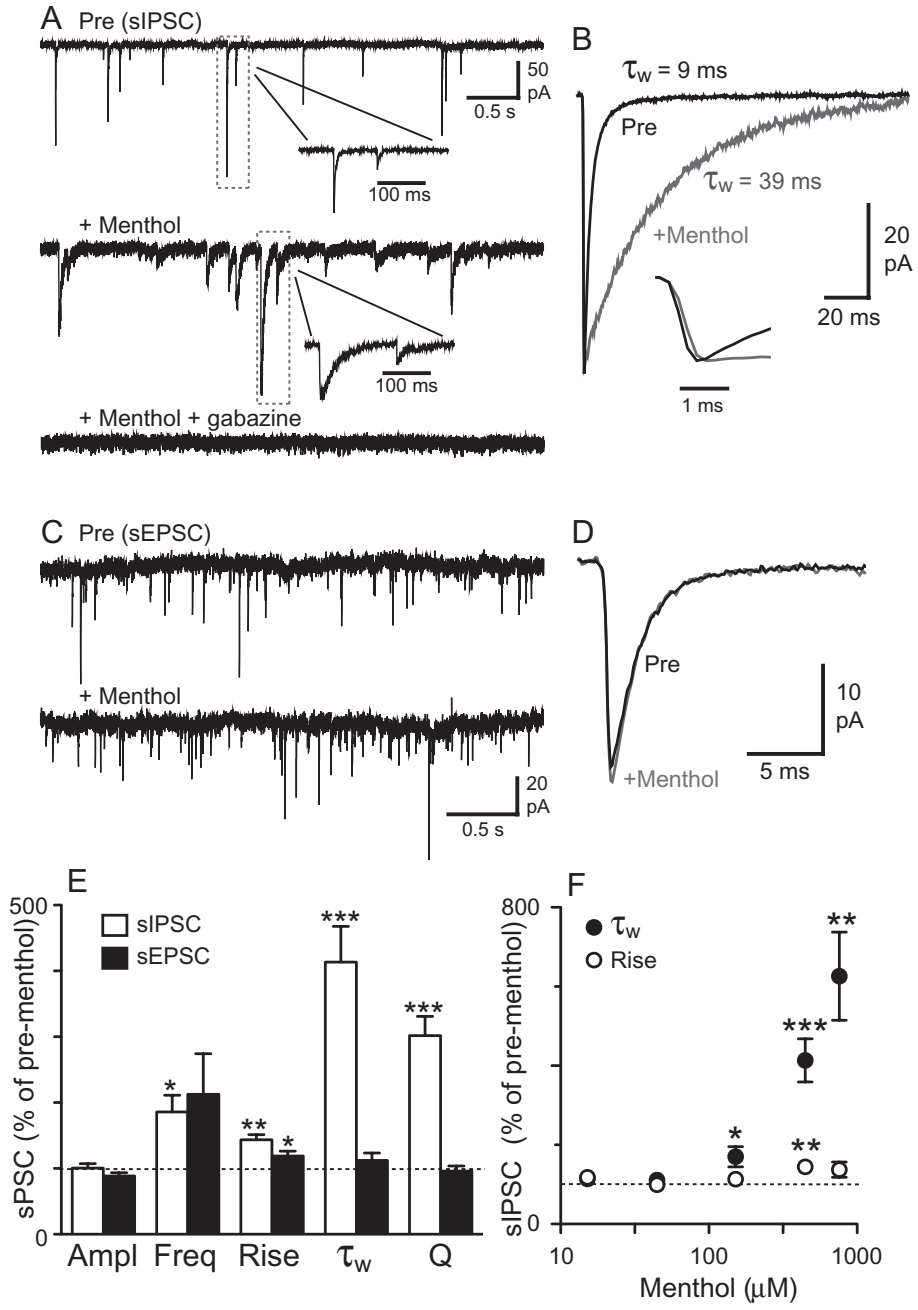

Figure 1

Menthol prolongs the duration of spontaneous $GABA_A$ IPSCs, but not non-NMDA EPSCs in PAG neurons. (A) Representative traces of spontaneous IPSCs before and during superfusion of menthol (450 μM) and following addition of gabazine (10 μM). (B) Averaged traces of IPSCs from the same neuron as shown in (A), recorded before (pre) and during menthol. Inset: expanded normalized IPSCs, demonstrating a slowing of the rise time during menthol. (C) Representative traces of spontaneous EPSCs before and during superfusion of menthol (450 μM). (D) Averaged traces of EPSCs from the same neuron as shown in (B), recorded before (pre) and during menthol. (E) Summary bar chart showing the effects of menthol (450 μM) on spontaneous IPSC and EPSC amplitude (Ampl), frequency (Freq), 10–90% rise time (Rise), weighted decay time (τ_w) and synaptic charge transfer (Q). (F) Concentration–response relationship for the effect of menthol on spontaneous IPSC-weighted decay time and rise time (at concentrations 15, 45, 150, 450 and 750 μM, n = 3, 5, 6, 17, 4). Each point represents the mean ± SEM of 3–17 neurons. *P < 0.05, **P < 0.01 and ***P < 0.001 versus baseline pre-menthol level.

decay times and charge transfer persisted in the presence of TTX (0.3 μM, Table 1, n = 5), indicating that these effects of menthol were action potential independent and likely to be mediated by a direct effect at the GABAergic synapse.

In contrast to IPSCs, superfusion of menthol (450 μM) had no significant effect on spontaneous EPSC frequency, amplitude, decay time or charge transfer (Table 1, Figure 1C–E; P > 0.05, n = 7). As observed for IPSCs, however,

menthol produced a significant increase in the rise time of spontaneous EPSCs (Figure 1E; $P = 0.04$). The spontaneous EPSCs were abolished by addition of CNQX (5 μM, $n = 6$, data not shown).

Menthol actions occur independently of TRP channel activation

Menthol interacts with several members of the TRP channel family including, primarily, TRPM8 and TRPA1. To determine whether these channels may have contributed to the actions of menthol, we examined the effects of several TRPM8/A1 agonists and antagonists. Superfusion of the TRPM8 agonist icilin (3 μM) had no significant effect on spontaneous IPSC amplitude, frequency, rise time, decay time or charge transfer (Figure 2A–C; $P > 0.05$, $n = 10$). In slices pretreated with the non-selective TRPM8/TRPV1 antagonist capsazepine (20 μM) or the TRPA1 antagonist HC-030031 (50 μM), menthol (450 μM) increased the weighted decay time of IPSCs (Figure 2D; $P < 0.01$ in each case, $n = 5$ and 5 respectively). The effect of menthol in the presence of capsazepine and HC-030031 was not significantly different to that observed under control conditions ($P > 0.05$). These results indicate that TRPM8 and TRPA1 channels do not mediate the effects of menthol on spontaneous IPSCs.

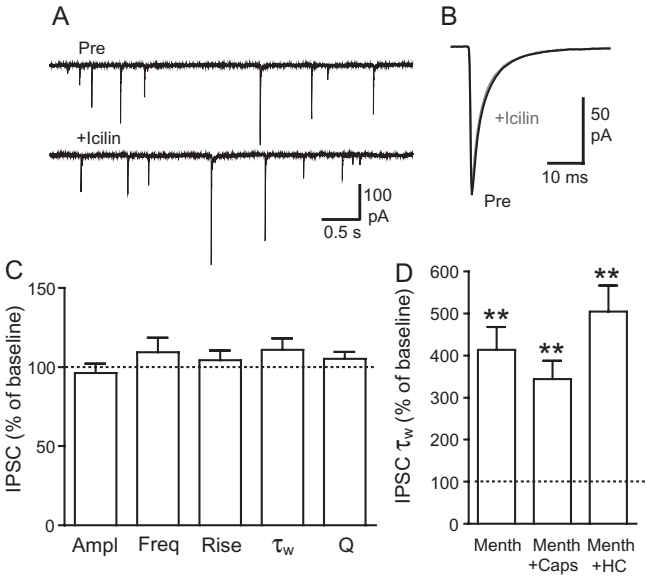

Figure 2

Menthol effects on IPSCs are not dependent on TRPM8 or TRPA1 channel activation. (A) Representative traces showing spontaneous IPSCs before and during superfusion of icilin (3 μM). (B) Averaged traces of IPSCs from the same neuron as shown in (A), recorded before (control) and during icilin. (C) Summary bar chart of icilin effects on IPSC amplitude (Ampl), frequency (Freq), 10–90% rise time (Rise), weighted decay time (τ_w) and synaptic charge transfer (Q). (D) Bar chart showing the effects of menthol (450 μM) on IPSC-weighted decay times in the absence and presence of capsazepine (Caps, 20 μM) and HC-030031 (HC, 50 μM). $^{**}P < 0.01$ versus baseline control.

Menthol actions are not mediated via the $GABA_A$ receptor benzodiazepine binding site

Previous studies have demonstrated menthol to be a positive allosteric modulator of recombinant $GABA_A$ receptors via a binding site distinct to that of benzodiazepines (Hall *et al.*, 2004; Watt *et al.*, 2008). We examined whether this was also the case in native PAG neurons. In slices pretreated with the benzodiazepine antagonist flumazenil (10 μM), menthol produced changes in spontaneous IPSC rise time, decay time and charge transfer that were not significantly different to those observed with menthol alone ($P > 0.05$, $n = 6$) (Figure 3). This indicates that menthol modulation of IPSC kinetics in PAG neurons also occurs via a mechanism distinct to that of benzodiazepines.

Characterization of $GABA_A$ receptor-mediated tonic currents

We next investigated whether tonic $GABA_A$ receptor-mediated currents occur within PAG, as previously described in other brain regions, by examining the effect of a range of $GABA_{A/C}$ receptor antagonists (Farrant and Nusser, 2005). Superfusion of the $GABA_A$ receptor channel blocker picrotoxin (100 μM, $n = 7$) abolished spontaneous IPSCs and produced an outward shift in $I_{holding}$ (Figure 4A and E; $P = 0.005$). This picrotoxin-induced decrease in $I_{holding}$ was accompanied by a reduction in I_{var} (Figure 4A and F; $P = 0.04$), consistent with the suppression of a tonic $GABA_A$ receptor-mediated

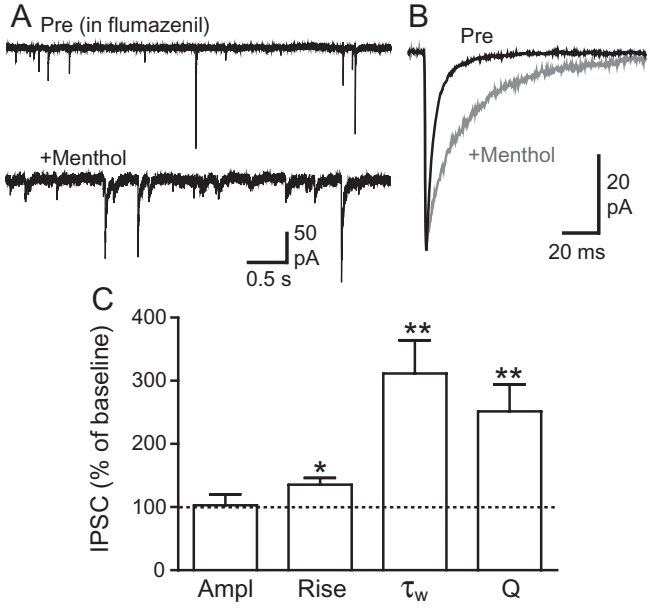

Figure 3

Menthol effects on IPSCs are not mediated via the benzodiazepine binding site on $GABA_A$ receptors. (A) Representative traces showing spontaneous IPSCs before (pre) and during menthol (450 μM) in the presence of flumazenil (10 μM). (B) Averaged traces of IPSCs from the same neuron as shown in (A), recorded before (pre) and during menthol. (C) Summary bar chart showing the effects of menthol on IPSCs in the presence of flumazenil. $^{*}P < 0.05$, $^{**}P < 0.01$ versus baseline control.

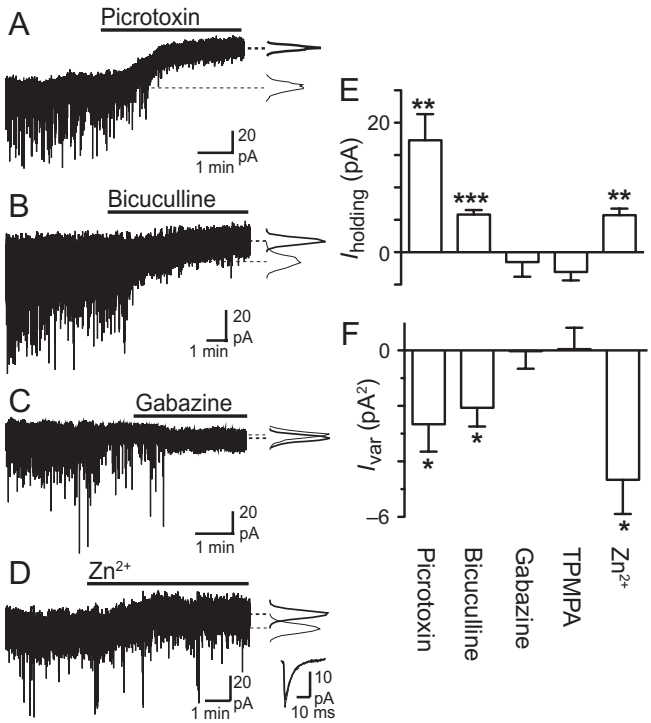

Figure 4

A tonic $GABA_A$ receptor-mediated current is present in PAG neurons. Representative current traces from different PAG neurons during superfusion of (A) picrotoxin (100 μM), (B) bicuculline (30 μM), (C) gabazine (10 μM) and (D) Zn^{2+} (100 μM). Insets in (A–D) are probability density distributions of holding current, and in (D) averaged spontaneous IPSCs before and during Zn^{2+} (thin and thick lines respectively). (E, F) Summary bar charts showing the effects of picrotoxin (100 μM), bicuculline (30 μM), gabazine (10 μM), TPMPA (50 μM) and Zn^{2+} (100 μM) on (E) holding current ($I_{holding}$) and (F) holding current variance (I_{var}). *P < 0.05, **P < 0.01 and ***P < 0.001 when comparing values before and during addition of each agent.

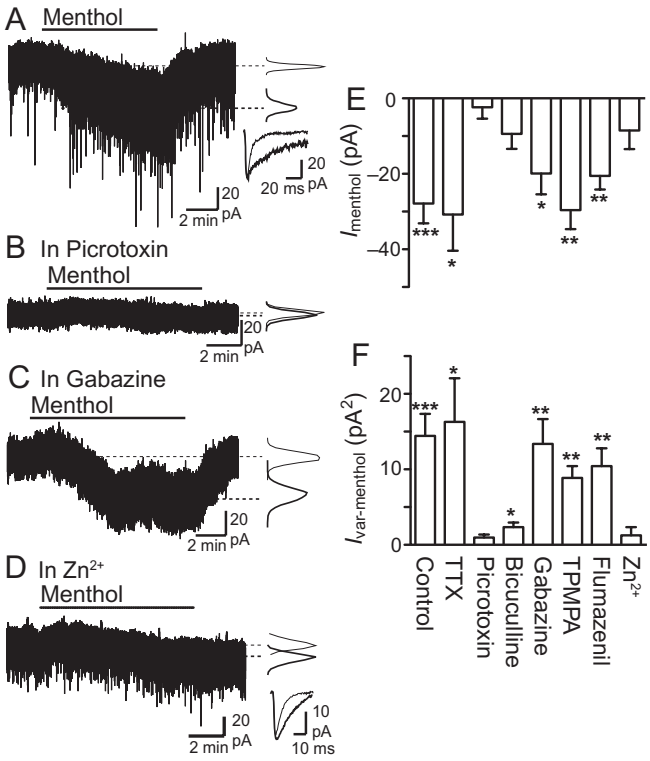

Figure 5

Menthol enhances the tonic $GABA_A$ receptor-mediated current. Representative current traces from different PAG neurons during superfusion of menthol (450 μM) in (A) control untreated slices, and slices pre-incubated in (B) picrotoxin (100 μM), (C) gabazine (10 μM) and (D) Zn^{2+} (100 μM). Insets in (A–D) are probability density distributions of holding current, and in (A, D) averaged spontaneous IPSCs before and during menthol (thin and thick lines respectively). (E, F) Summary bar charts showing changes in (E) holding current ($I_{menthol}$) and (F) holding current variance ($I_{var\text{-}menthol}$), produced by menthol in untreated slices (control) and slices pre-incubated in TTX (0.3 μM), picrotoxin (100 μM), bicuculline (30 μM), gabazine (10 μM), TPMPA (50 μM), flumazenil (10 μM) and Zn^{2+} (100 μM). *P < 0.05, **P < 0.01 and ***P < 0.001 when comparing values before and during addition of menthol.

conductance. The competitive $GABA_A$ receptor antagonist bicuculline (30 μM, n = 6) also abolished spontaneous IPSCs and produced a small but significant outward shift in $I_{holding}$ and a decrease in I_{var} (Figure 4B, E and F; P = 0.004 and 0.03). In contrast, gabazine (10 μM, n = 8) abolished spontaneous IPSCs, but had no effect on either $I_{holding}$ or I_{var} (Figure 4C, E and F; P = 0.5, 0.9). The $GABA_C$ receptor antagonist TPMPA (50 μM, n = 6) had no significant effect on spontaneous IPSCs (data not shown), $I_{holding}$ or I_{var} (Figure 4E and F; P = 0.07 and 0.9).

This pharmacological profile is consistent with menthol acting at extrasynaptic $GABA_A$ receptors. To further investigate this possibility, we examined the effect of Zn^{2+} on basal tonic currents in the PAG. In the micromolar range, Zn^{2+} has little effect on synaptic γ2 subunit-containing $GABA_A$ receptors, but greatly reduces currents mediated by $GABA_A$ receptors that lack γ2 subunits, including αβ and δ-$GABA_A$ receptors (Saxena and Macdonald, 1994; 1996; Krishek *et al.*, 1998). Superfusion of Zn^{2+} (100 μM, n = 5) produced an outward shift in $I_{holding}$ and a decrease in I_{var} (Figure 4D, E and F; P = 0.005 and 0.02), similar to that observed with bicuculline. In these cells, Zn^{2+} also produced a small but significant decrease in the amplitude of spontaneous IPSCs (P = 0.001, amplitude = 34 ± 7 and 31 ± 6 pA before and during Zn^{2+}, respectively), but had no effect on their kinetics (Figure 4D; rise time = 0.71 ± 0.07 and 0.72 ± 0.12, P = 0.9, τ_w = 5.5 ± 0.4 and 5.2 ± 0.5 ms, P = 0.5, before and during Zn^{2+} respectively). These results confirm the presence of an extrasynaptic $GABA_A$ receptor-mediated tonic current in our PAG slice preparation.

Menthol enhances tonic currents

Next, we examined whether modulation of $GABA_A$ receptors by menthol could enhance this basal tonic current. Superfusion of menthol (450 μM) for 8–10 min caused a slowly developing and reversible inward shift in $I_{holding}$ (P < 0.001) in 14 out of 16 PAG neurons tested (Figure 5A and E). This inward shift in $I_{holding}$ was accompanied by a marked increase in I_{var} (P < 0.001) (Figure 5A and F). There was no correlation between the magnitude of the change in $I_{holding}$ during menthol appli-

cation and the change in IPSC decay time for each cell (Pearson's correlation coefficient, $r^2 = -0.16$, $P > 0.05$, $n = 13$). The menthol-induced increase in $I_{holding}$ and I_{var} persisted in the additional presence of TTX (0.3 μM, $P = 0.03$ and 0.04, respectively, $n = 5$) (Figure 5E and F). Menthol also produced an inward shift in $I_{holding}$ of 37 ± 6 pA ($P < 0.01$) and 26 ± 5 pA ($P < 0.05$) in the presence of capsazepine (20 μM, $n = 5$) and HC-030031 (50 μM, $n = 4$), respectively, suggesting that TRP channels had no role in the menthol-induced tonic current.

To further investigate the potential molecular targets of menthol, we examined the effect of a range of $GABA_{A/C}$ receptor ligands on the menthol-induced tonic current. In the presence of picrotoxin (100 μM, $n = 6$), menthol did not produce a significant change in either $I_{holding}$ ($P = 0.5$) or I_{var} ($P = 0.06$) (Figure 5B, E and F). In the presence of bicuculline (30 μM, $n = 6$), menthol did not significantly alter $I_{holding}$ ($P = 0.06$), but produced a small increase in I_{var} ($P = 0.02$) (Figure 5E and F). In contrast, gabazine (10 μM, $n = 5$), TPMPA (50 μM, $n = 5$) and flumazenil (10 μM, $n = 6$) had no effect on menthol-induced increases in either $I_{holding}$ ($P < 0.05$) or I_{var} ($P < 0.05$) (Figure 5C, E and F). Finally, menthol did not significantly alter $I_{holding}$ ($P = 0.1$) or I_{var} ($P = 0.3$) in the presence of Zn^{2+} (100 μM, $n = 6$) (Figure 5D, E and F). Additionally, although menthol increased the decay time of spontaneous IPSCs in the presence of Zn^{2+} (Figure 5D; $P = 0.01$, $n = 6$, τ_w = 5.2 ± 0.5 and 10.6 ± 1.4 ms before and during Zn^{2+}, respectively), this effect was significantly less than observed under control conditions ($P = 0.03$, τ_w = 201 ± 26% and 414 ± 54% of the pre-menthol value in the presence and absence of Zn^{2+} respectively).

Collectively, these results suggest that menthol might be acting at Zn^{2+}-sensitive peri/extrasynaptic δ-$GABA_A$ receptors to potentiate basal tonic currents. In the PAG, extrasynaptic α4βδ $GABA_A$ receptors have been proposed as a likely candidate for generating tonic currents, as α4 and δ subunits are present on neurons throughout this brain region (Lovick *et al.*, 2005) and are known to contribute to tonic currents elsewhere in the CNS (Farrant and Nusser, 2005). To test this possibility, we investigated the effect of menthol in the presence of the $GABA_A$ receptor agonist THIP (gaboxadol). Superfusion of THIP (1 μM, $n = 6$), at a concentration that is selective for δ-$GABA_A$ receptors (Herd *et al.*, 2009; Meera *et al.*, 2011), produced small but significant increases in $I_{holding}$ ($P = 0.009$) and I_{var} ($P = 0.04$) (Figure 6B and C). At a higher concentration, THIP (10 μM, $n = 6$) markedly increased both $I_{holding}$ ($P = 0.01$) and I_{var} ($P = 0.03$) (Figure 6A–C). These effects were significantly larger than observed using 1 μM THIP ($P = 0.01$ and 0.02 for $I_{holding}$ and I_{var} respectively). Subsequent addition of menthol (450 μM, $n = 6$) produced increases in $I_{holding}$ and I_{var} at both 1 and 10 μM THIP concentrations (Figure 6A, D and E; $P < 0.0001$–.05). However, menthol-induced currents were potentiated above control levels only in the presence of the higher concentration of THIP ($P < 0.05$ for both $I_{holding}$ and I_{var}) (Figure 6D and E), implying that distinct THIP-sensitive receptor subtypes may be differentially targeted by menthol. It should also be noted that, in several cells (see Figure 6A), the frequency of spontaneous IPSCs appeared to be greatly reduced by 10 μM THIP, which is consistent with the inhibitory actions of THIP on network activity and GABA release described in a previous study (Gao and Smith, 2010). This effect could not be adequately analysed, however, because the increase in noise precluded reliable identification of individual IPSCs.

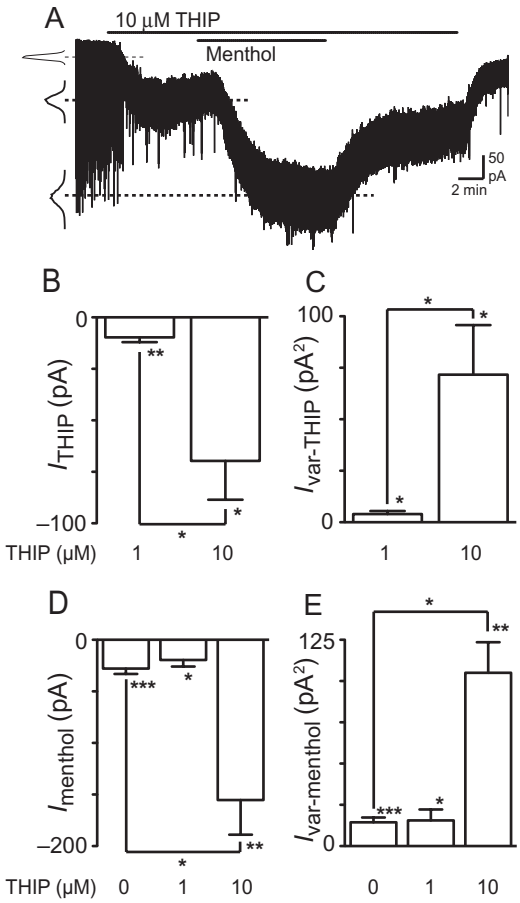

Figure 6

THIP produces a tonic current that is enhanced by menthol. (A) Representative current trace from a PAG neuron during superfusion of THIP (10 μM), then addition of menthol (450 μM). Insets are probability density distributions of holding current before and during addition of THIP, then menthol. (B, C) Summary bar charts showing changes in (B) holding current (I_{THIP}) and (C) holding current variance ($I_{var\text{-}THIP}$) produced by 1 and 10 μM THIP. (D, E) Summary bar charts showing changes in (D) holding current ($I_{menthol}$) and (E) holding current variance ($I_{var\text{-}menthol}$) produced by menthol (450 μM) in the absence (0 μM) and presence of 1 and 10 μM THIP. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ when comparing values before and during addition of each agent and comparing between treatment groups.

Discussion

The present study has demonstrated that menthol potentiates both phasic and tonic $GABA_A$ receptor-mediated currents in neurons located within all subregions of the PAG *in vitro*. The effect of menthol on the tonic current appears to be mediated by extrasynaptic $GABA_A$ receptors that lack that a δ subunit. This menthol-induced enhancement of GABAergic inhibition within the PAG has the potential to modulate the analgesic and anxiolytic functions of this brain region.

Menthol prolonged the decay time of spontaneous IPSCs in PAG neurons, resulting in an approximately threefold increase in synaptic charge transfer at the concentration used in this study. This differs to a previous study in which menthol enhanced a tonic current but had no effect on IPSC kinetics in hippocampal CA1 pyramidal neurons (Zhang *et al.*, 2008). The effect of menthol on IPSC decay time was concentration dependent, with significant changes observed at concentrations of 150 μM and above, which is in accord with concentration–response profiles previously reported for menthol modulation of $GABA_A$ receptor activity (Hall *et al.*, 2004; Watt *et al.*, 2008; Zhang *et al.*, 2008; Pan *et al.*, 2012). The increase in IPSC decay time produced by menthol was not accompanied by changes in IPSC amplitude, consistent with its proposed role as an allosteric modulator of $GABA_A$ receptors (Hall *et al.*, 2004; Watt *et al.*, 2008). Menthol effects were unaltered by TRPM8 and TRPV1/TRPA1 channel antagonists and not mimicked by the selective TRPM8 agonist icilin, ruling out possible TRP channel involvement. The menthol enhancement of IPSC decay times and tonic current were also unaffected by flumazenil, indicating that menthol was not acting via the benzodiazepine binding site on $GABA_A$ receptors. This is in agreement with a previous study in which modulation of recombinant α1β2γ2 ('synaptic-type') $GABA_A$ receptor activity by menthol, but not benzodiazepines, was abolished by point mutations in the β2 subunit (Watt *et al.*, 2008). Collectively, these results suggest that menthol acts as positive modulator of synaptic $GABA_A$ receptors in PAG neurons, possibly via interaction with β2 subunits.

Despite having little effect on IPSCs *per se*, we found that Zn^{2+} greatly reduced the effect of menthol on IPSC decay time. Although there is currently little information available regarding the subcellular localization of $GABA_A$ receptor subtypes in the PAG, at other synapses α4, α6 and/or δ subunit-containing $GABA_A$ receptors are concentrated at perisynaptic sites, where they are thought to contribute to IPSC decay kinetics during GABA spillover following synaptic release (Rossi and Hamann, 1998; Wei *et al.*, 2003; Bright *et al.*, 2011; Herd *et al.*, 2013). Indeed, the δ-$GABA_A$ receptor modulator AA29504 was recently shown to prolong miniature IPSC decay time in mouse dentate gyrus granule cells, an effect that was abolished by Zn^{2+} (Vardya *et al.*, 2012). The authors proposed that AA29504 might selectively increase the GABA affinity of perisynaptic δ-$GABA_A$ receptors, thereby promoting their activation during synaptic transmission. It is possible that menthol produces a similar effect on Zn^{2+}-sensitive perisynaptic $GABA_A$ receptors in the PAG. Alternatively, Zn^{2+}, an allosteric modulator of $GABA_A$ receptor gating (Barberis *et al.*, 2000), could potentially interfere with menthol binding and/or actions at synaptic $GABA_A$ receptors. Further research will be required to address these possibilities. In addition to IPSC kinetics, menthol produced a variable increase in the frequency of spontaneous IPSCs. Interestingly, in tractus solitarius neurons, propofol increases spontaneous IPSC frequency by potentiating $GABA_A$ receptor-mediated depolarization of presynaptic GABAergic terminals (Jin *et al.*, 2009). It is not known, however, if such a mechanism also occurs in PAG. Regardless of underlying mechanisms, these results indicate that menthol is likely to produce complex neuronal network effects within the PAG.

In the present study, we observed a tonic GABAergic current under basal conditions in PAG slices, which was enhanced by menthol. The tonic current was likely to be mediated by $GABA_A$ receptors because it was observed in the presence of strychnine and CNQX, was sensitive to picrotoxin and bicuculline, but was unaffected by the $GABA_C$ receptor antagonist TPMPA. The observed enhancement of tonic current by menthol is similar to that reported in hippocampal CA1 pyramidal neurons, where menthol increased a bicuculline-sensitive tonic current (Zhang *et al.*, 2008). Several lines of evidence indicate that distinct populations of $GABA_A$ receptors were responsible for the tonic current and phasic IPSCs reported here. First, there was no correlation between the effect of menthol on tonic current and IPSC decay time in individual neurons, implying that the two phenomena occurred independently. Second, these currents could be differentiated pharmacologically based on their sensitivity to various $GABA_A$ receptor antagonists. Thus, gabazine abolished phasic IPSCs but had no effect on the tonic basal and menthol-induced currents. This pharmacological profile is similar to tonic $GABA_A$ receptor-mediated currents observed in the hippocampus (Bai *et al.*, 2001; Yeung *et al.*, 2003; McCartney *et al.*, 2007). Conversely, Zn^{2+} greatly reduced tonic basal and menthol-induced currents, while producing only a modest (~10%) reduction in the amplitude of IPSCs. This is consistent with the lower Zn^{2+} sensitivity of γ2 subunit-containing synaptic $GABA_A$ receptors (Draguhn *et al.*, 1990; Hosie *et al.*, 2003).

Although application of 1 μM THIP produced only a small (~10 pA) tonic current in PAG neurons and had no effect on menthol-induced tonic currents, it produced a substantial inward current and marked potentiation of menthol-induced tonic currents at a concentration of 10 μM. Together, these observations suggest that δ-$GABA_A$ receptors are likely to contribute only partly to the tonic current observed in PAG slices under our experimental conditions, and that these receptors are unlikely to be a target for menthol because δ-subunits confer sub-micromolar sensitivity to THIP (Herd *et al.*, 2009; Meera *et al.*, 2011). These observations also argue against involvement of γ2 subunit-containing $GABA_A$ receptors because αβγ2 receptor combinations display relatively weak sensitivity to THIP, with EC_{50}s typically above 100 μM (Ebert *et al.*, 1994; Meera *et al.*, 2011). Thus, menthol is likely to be acting on a population of a THIP- and Zn^{2+}-sensitive, γ2/δ subunit-lacking extrasynaptic $GABA_A$ receptors in PAG neurons. In native cerebellar neurons, THIP produces a tonic current in δ-subunit knockout mice with a similar mid-range concentration dependence (EC_{50} ~20 μM) as γ2/δ-lacking α4/6β3 $GABA_A$ receptors (Meera *et al.*, 2011). Indeed, it has been suggested that αβ $GABA_A$ receptor combinations are abundant in brain tissue (Bencsits *et al.*, 1999), are sensitive to Zn^{2+} (Draguhn *et al.*, 1990; Smart *et al.*, 1991) and have been implicated in the generation of tonic currents in hippocampal pyramidal neurons (Mortensen and Smart, 2006).

Tonic $GABA_A$ receptor-mediated currents are generally thought to require the continued presence of low levels of extracellular GABA (Semyanov *et al.*, 2004). One such source of extrasynaptic GABA could arise from action potential-dependent spillover of GABA following synaptic release (Hamann *et al.*, 2002; Bright *et al.*, 2007; Gao and Smith, 2010). The menthol-induced tonic current was unaffected by

TTX, however, suggesting that GABA spillover did not contribute significantly to this current under our experimental conditions. Alternatively, tonic currents could arise from the constitutive activity of extrasynaptic $GABA_A$ receptors in the absence of ambient GABA (Birnir *et al.*, 2000; McCartney *et al.*, 2007; Wlodarczyk *et al.*, 2013). Indeed, in the present study, the insensitivity of the basal tonic current to gabazine and the small effect produced by bicuculline (relative to that of picrotoxin) is very similar to the pharmacological profile reported in the earlier studies. Thus, the results could be explained by the neutral and inverse agonist properties of these two competitive ligands, respectively, when binding to constitutively active $GABA_A$ receptors in the absence of GABA (Ueno *et al.*, 1997; McCartney *et al.*, 2007; Wlodarczyk *et al.*, 2013). In contrast, picrotoxin, an open channel blocker, would be expected to inhibit $GABA_A$ receptor activity regardless of whether it was mediated by GABA-dependent or independent gating. Additional research will be required to elucidate the source of tonic $GABA_A$ receptor-mediated currents in the PAG slice preparation.

There is accumulating evidence that centrally administered menthol produces a variety of behavioural effects in animal models and that some of these are mediated by $GABA_A$ receptor-mediated mechanisms (Watt *et al.*, 2008; Zhang *et al.*, 2008; Tani *et al.*, 2010). Interestingly, $GABA_A$ receptors are a major target for many sedatives and general anaesthetics (Millan, 2003; Hemmings *et al.*, 2005). In particular, potentiation of tonic $GABA_A$ receptor-mediated inhibition has been proposed to play an important role in the suppression of neuronal excitability by general anaesthetics (Bieda and MacIver, 2004). The development of agents that target $GABA_A$-mediated tonic currents and phasic IPSCs in a manner similar to menthol could provide a basis for novel $GABA_A$-related pharmacotherapies.

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Conflict of interest

The authors declare no conflicts of interest.

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