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Modulation of human $GABA_A$ and glycine receptor currents by menthol and related monoterpenoids

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Abstract

Effects of common monoterpenoid alcohols and ketones were investigated on recombinant human γ-aminobutyric acid A ($GABA_A$; $\alpha_1\beta_2\gamma_{2s}$) and glycine ($\alpha_1$ homomers) receptors expressed in *Xenopus* oocytes. GABA currents were enhanced by coapplications of 10–300 μM: (+)-menthol>(−)-menthol>(−)-borneol≫(−)-menthone=camphor enantiomers>carvone enantiomers, with menthol acting stereoselectively. By contrast, thujone diastereomers inhibited $GABA_A$ receptor currents while glycine currents were only markedly potentiated by menthol. Positive modulation by (+)-menthol was explored given its pronounced effects (e.g., at 100 μM, GABA and glycine EC_{20} responses increased by 496±113% and 135±56%, respectively). (+)-Menthol, 100 μM, reduced EC_{50} values for GABA and glycine from 82.8±9.9 to 25.0±1.8 μM, and from 98.7±8.6 to 75.7±9.4 μM respectively, with negligible effects on maximal currents. This study reveals a novel neuroactive role for menthol as a stereoselective modulator of inhibitory ligand-gated channels.

Keywords: $GABA_A$ receptor; Glycine receptor; Monoterpenoid; Menthol

1. Introduction

Monoterpenoids, like menthol, camphor, and thujone, are naturally occurring oxygenated C_{10} compounds (ketones and alcohols) derived from plant extracts. These fragrant compounds are widely used as insect repellents, antimicrobials, and antifungal remedies (Tirillini et al., 1996), and are additives in pharmaceutical preparations, cosmetics, food, cigarettes, and alcoholic drinks (Eccles, 2000; Olsen, 2000; Burk, 2003). Monoterpenoids can act as local anesthetic (Galeotti et al., 2001), topical analgesic (Galeotti et al., 2002), antipruritic and gastric sedative agents, and can exert profound effects on animal behaviour when administered intravenously (Umezu et al., 2001). These agents are reported to have neuroactive properties by affecting neuronal intracellular signaling or by modulation of neurotransmitter-gated currents. For instance, the alcohol menthol (Fig. 1), an activator of the cold- and menthol-sensitive receptor-1 (CMR1) or transient receptor potential (TRPM8) channel, was recently shown to induce Ca^{2+} release from intracellular stores, resulting in enhanced neurotransmission at sensory synapses (Tsuzuki et al., 2004). Camphor (a ketone; Fig. 1) and borneol (the corresponding alcohol) are potent non-competitive inhibitors of nicotinic acetylcholine receptors that belong to the ionotropic ligand-gated ion channel (LGIC) superfamily (Park et al., 2001, 2003). α-Thujone, the principal active and convulsant ingredient in the opalescent drink absinthe, inhibits currents of another LGIC, the γ-aminobutyric acid A ($GABA_A$) receptor (Hold et al., 2000). By contrast, thymol, a phenol derived from thyme oil, demonstrated positive allosteric modulation of $GABA_A$ receptor currents (Priestley et al., 2003).

$GABA_A$ and glycine receptors are the principal ionotropic receptors for fast inhibitory neurotransmission in the mammalian central nervous system (CNS). Their pentame-

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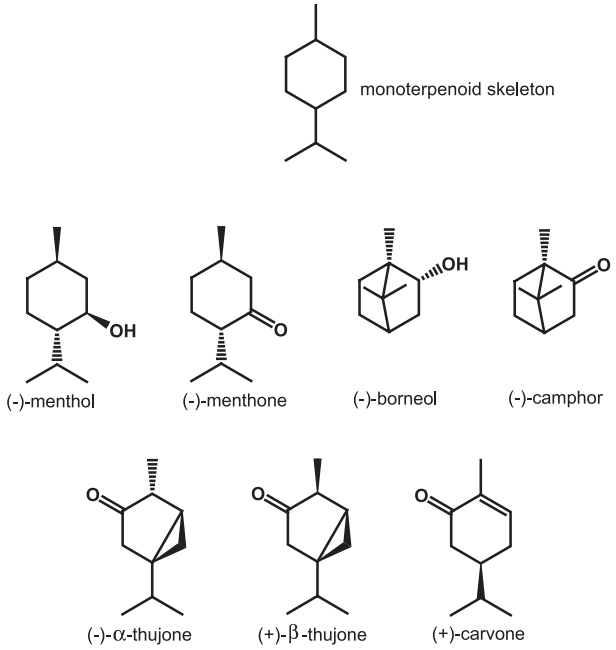

Fig. 1. Structures of a selection of the monoterpenoid enantiomers used in the study. The top structure depicts the monoterpenoid skeleton, common to this group of compounds. (−)-Menthol and (−)-borneol are alcohols, whereas the other compounds are all ketones.

ric structure is composed of different subunits, forming membrane-spanning Cl^--selective ion channel complexes activated by the binding of GABA or glycine, respectively (Barnard et al., 1998; Cascio, 2004). In mammalian neurones, the predominant $GABA_A$ receptor combination appears to be $\alpha_1\beta_2\gamma_2$ (Tretter et al., 1997), while glycine receptors are typically composed of $\alpha_1\beta_1$ subunits (Langosch et al., 1988). Both receptors are recognized as important targets for modulation by sedative, anxiolytic, general anesthetic (Franks and Lieb, 1994; Krasowski and Harrison, 1999), and convulsant agents (Olsen, 1981).

In the following study, using the *Xenopus* oocyte expression system, we investigated the modulation of both recombinant human $GABA_A$ ($\alpha_1\beta_2\gamma_{2s}$) and glycine ($\alpha_1$ homomers) receptor currents by stereoisomers of a number of common monoterpenoid ketones (menthone, camphor, thujone, and carvone) and alcohols (menthol and borneol). Interestingly, these compounds had pronounced effects on the LGICs, both by inhibiting (e.g., α-thujone) and potentiating (e.g., menthol) responses to submaximal concentrations of agonists. Structure–activity relationships for receptor modulation by the monoterpenoids are discussed.

2. Materials and methods

2.1. Xenopus oocyte expression

cDNAs encoding for the α_1, β_2, and γ_{2s} subunits of human $GABA_A$ receptors and the α_1 subunit of the human glycine receptor were kindly provided by Dr. Paul J. Whiting (Merck, Sharp, and Dohme Research Laboratories, UK) and Dr. John Mihic (University of Texas, Austin, TX, USA), respectively. The $GABA_A$ receptor subunit cDNAs were prepared in pcDNA3.1$^+$ vector as described previously (Hall et al., 2004). The α_1 glycine subunit cDNA was incorporated in a modified pBK-CMV vector (see Mihic et al., 1997). Both were stored at −20 °C prior to oocyte injection.

$GABA_A$ receptors were expressed as a wild-type $\alpha_1\beta_2\gamma_{2s}$ combination, whereas glycine receptors were expressed as homomers of α_1 subunits. Briefly, *Xenopus laevis* (Xenopus Express, Plant City, FL, USA) were anesthesized with 1.25% tricaine/1.5% sodium bicarbonate and oocytes were harvested through laparotomy. Eggs were treated with 1 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN, USA) in (mM) 82 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.6) for 80 min at room temperature on a shaking platform. Oocytes were transferred to a solution (ND-96) containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 5 μg/ml tetracycline, pH 7.6 (antibiotics from Invitrogen, Rockville, MD, USA). Eggs were defolliculated manually by repetitive rolling on plastic Petri dishes. Plasmids were introduced by nuclear injection using a Nanoject II (Drummond Scientific, Broomall, PA, USA). For all eggs, the injection volume was 32 nl, with concentrations of the GABA receptor α_1 and β_2 subunit cDNAs at 12 ng/μl and the γ_{2s} subunit at 6 ng/μl, and of the glycine receptor α_1 subunits at 5 ng/μl. Injected oocytes were maintained in ND-96 with antibiotics at 16 °C. Animal maintenance and oocyte harvest procedures were approved by the Smith College's Institutional Animal Care and Use Committee (IACUC).

2.2. Electrophysiology

Between 1 and 3 days after cDNA injection, injected oocytes were screened for either GABA- or glycine-evoked currents in a 100-μl oocyte chamber (Warner Instruments, Hamden, CT, USA). All experiments were performed at room temperature (20–23 °C) unless stated otherwise. Eggs were placed in a small depression, animal pole face up, and continually superfused at 5 ml/min with ND-96 (less antibiotics). Recordings were made using standard two-electrode voltage-clamp technique with an OC-75C clamp (Warner Instruments). Glass micropipettes (World Precision Instruments, Sarasota, FL, USA) were fabricated using a two-stage pull (Narishige, Tokyo, Japan) and filled with 3 M KCl, giving resistances of 1–3 MΩ. For all experiments, impaled oocytes were voltage-clamped at −50 mV. All drugs were dissolved in ND-96 immediately prior to use, and solutions were applied via gravity feed (5 ml/min) using an automated switching device (ALA Scientific Instruments, Westbury, NY, USA). Currents were digitized at 200 Hz, recorded, and analyzed using pClamp 6.0 software (Axon

Instruments, Union City, CA, USA). Solution switches to GABA or glycine (in the absence or presence of the monoterpenoids) were applied until currents were determined to have achieved peak amplitude. There was at least 2-min exposure to control recording solution between drug switches to allow adequate washout and recovery from receptor desensitization.

2.3. GABA, glycine, and monoterpenoid concentrations

Currents evoked by increasing concentrations of GABA (1 μM–1 mM) or glycine (3 μM–3 mM) were measured, and concentration–response data were plotted. GABA currents were completely blocked by bicuculline and picrotoxin (data not shown). Incorporation of the γ_{2s} subunit was confirmed by insensitivity of the evoked currents to block by Zn^{2+}, and by a rightward shift in the GABA dose–response plots relative to recordings from wild-type $\alpha_1\beta_2$ receptors (see Hall et al., 2004). α_1 Homomer glycine currents were completely blocked by strychnine and picrotoxin (data not shown).

Dilutions of the monoterpenoids (0.3–300 μM) α-thujone (Fluka), α/β-thujone (Fluka), (+)-menthol and (−)-menthol, (+)-camphor and (−)-camphor, (−)-borneol, (+)-carvone and (−)-carvone, and (−)-menthone (Fig. 1) were prepared by adding quantities of 1 M stock solutions of the monoterpenoid in dimethyl sulphoxide (DMSO) to the recording solution. Testing higher doses of monoterpenoids was considered unreliable given the insolubility of some of these agents at high concentrations (≥1 mM). All reservoirs for control and drug applications contained equivalent amounts of DMSO (0.1%), a concentration that was determined to have no effect on either GABA or glycine responses (data not shown). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.4. Data analysis

Currents were measured using pClamp 6.0 software (Axon Instruments). Concentration–response data for agonist-evoked currents were fitted with the Hill equation (below) using Origin software (OriginLab, Northampton, MA, USA):

$$I = \frac{I_{max}[\text{Agonist}]^{n_H}}{[\text{Agonist}]^{n_H} + (EC_{50})^{n_H}}$$

where I is the amplitude of the agonist-evoked current at a given concentration, I_{max} is the peak current at saturating [Agonist], EC_{50} is the concentration of agonist that elicits a half-maximal response, and n_H is the Hill coefficient. All collated data are expressed as mean±standard error of the mean (S.E.M.), and were calculated from at least n=6 individual oocytes for every data point. Data sets were tested for statistically significant differences using Student's t test with $P<0.05$.

3. Results

Oocytes were routinely screened for receptor expression by applications of either 30 μM GABA or 50 μM glycine that evoked ~EC_{20} currents (effective concentration that evoked 20% of maximal current) for both receptor types (see Fig. 5). Coapplications of 100 μM monoterpenoids produced a variety of effects on control GABA and glycine responses (Figs. 2–4), ranging from positive modulation (e.g., menthol enantiomers and (−)-borneol), to minimal effect (e.g., camphor and carvone enantiomers, and (−)-menthone) to inhibition (e.g., thujone diastereomers on $GABA_A$ receptors). For both receptor types, all monoterpenoid effects were fully reversible upon washout (see Figs. 2 and 4). Notably, the rate of activation of submaximal currents often appeared reduced in the presence of the monoterpenoids, regardless of the level of current amplitude modulation. Preexposure to these agents did not affect the extent of current modulation upon subsequent coapplication (data not shown). Also none of the monoterpenoids tested (up to 300 μM) evoked any

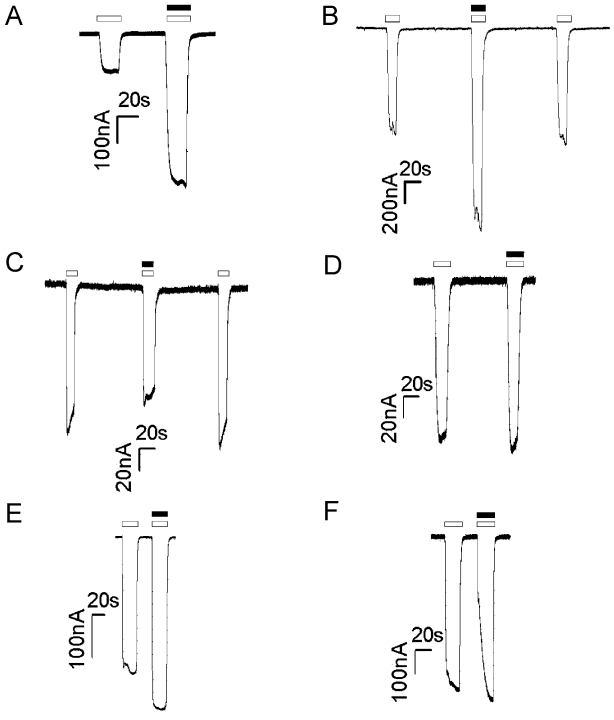

Fig. 2. Current recordings illustrating modulation by monoterpenoids of $GABA_A$ receptor activity. Oocytes were held at −50 mV, and 30 μM GABA was applied for the duration of the open boxes above each trace. Coapplication of 100 μM monoterpenoid is indicated by the filled boxes with (A) (−)-menthol, (B) (−)-borneol, (C) α/β-thujone, (D) (−)-carvone, (E) (+)-camphor, and (F) (−)-menthone. Effects were fully reversible upon washout for all monoterpenoids tested, as shown in (B) and (C).

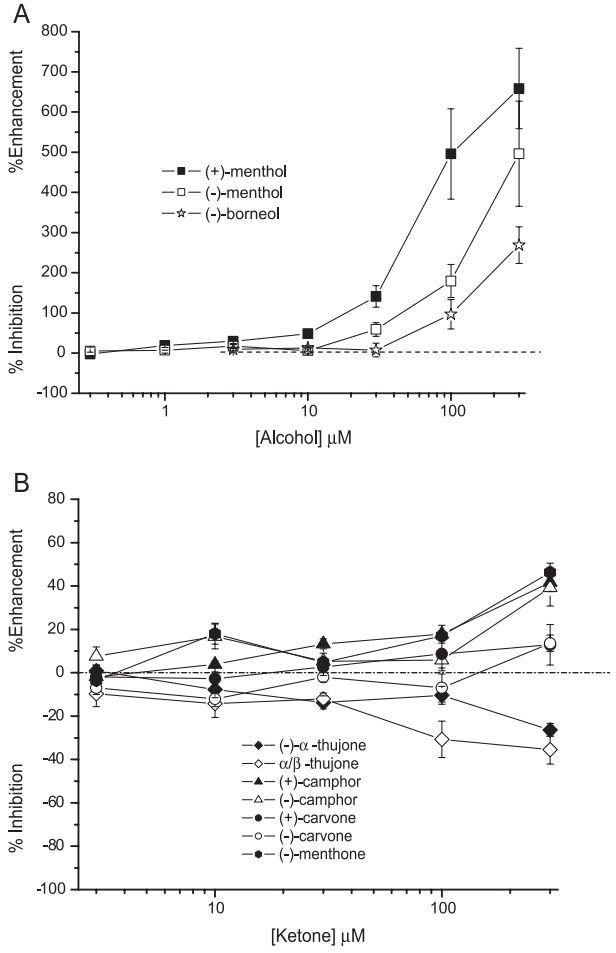

Fig. 3. The monoterpenoid alcohols are more potent positive modulators of $GABA_A$ receptors than the corresponding ketones. Relative modulations of EC_{20} GABA (30 μM) responses were compared by coapplying increasing concentrations of alcohols (menthol enantiomers and (−)-borneol) and ketones (stereoisomers of thujone, camphor, carvone, and (−)-menthone). (A) Dose-dependency for effects of the alcohols on $GABA_A$ receptors. (+)-Menthol and (−)-menthol demonstrated potent positive modulation of GABA currents with stereoselectively at 10–100 μM ($P<0.05$). (B) Dose-dependency for effects of ketones on $GABA_A$ receptors. The ketones (camphors, carvones, and (−)-menthone) were considerably less potent modulators of GABA currents (~10-fold less than the corresponding alcohols) with no evidence for stereoselectivity of their action. Exceptions were the thujone diastereomers ((−)-α-thujone and α/β-thujone) that consistently inhibited GABA currents when coapplied at 30–300 μM.

directly activated current in expressing or uninjected oocytes (data not shown).

3.1. $GABA_A$ receptor modulation

For $GABA_A$ receptors, coapplications of 100 μM monoterpenoid alcohols consistently resulted in enhanced EC_{20} GABA responses (Fig. 2A and B). By comparison, the effects of the ketones were minimal, ranging from small potentiations (camphor, menthone, and carvone; Fig. 2D–F) to current inhibition (thujones; Fig. 2C). The dose-dependency for modulation of $GABA_A$ receptor activity by the monoterpenoid enantiomers (0.3–300 μM) was further investigated (Fig. 3). The rank order for positive modulation of EC_{20} currents was: (+)-menthol>(−)-menthol>(−)-borneol≫(−)-menthone=(+)-camphor=(−)-camphor >(−)-carvone=(+)-carvone. Enhancements by(+)-menthol were pronounced with 496 ±113% (n=6) potentiation at 100 μM (Fig. 3A). Moreover, potentiations by 10–100 μM (+)-menthol were typically more pronounced than for equivalent concentrations of (−)-menthol, indicating stereoselectivity for the action of the menthol enantiomers on $GABA_A$ receptors.

Positive modulations by the camphor enantiomers, (−)-menthone and particularly the carvone enantiomers, were minimal compared to their corresponding alcohols. For instance, at 300 μM, camphors and (−)-menthone produced only 40–50% current enhancement (Fig. 3B). By contrast, $GABA_A$ receptors were dose-dependently inhibited by the thujone diastereomers (Figs. 2C and 3B) with IC_{50}>300 μM (concentration for half-maximal inhibition).

3.2. Glycine receptor modulation

Glycine receptor responses were also positively modulated by coapplications of the menthol enantiomers (Fig. 4). The rank order for current enhancement of EC_{20} glycine currents by the alcohols was: (−)-menthol=(+)-menthol≫(−)-borneol. In common with $GABA_A$ receptor modulations, the menthol stereoisomers again produced the most pronounced effects with ca. 150–200% potentiation when coapplied at 100 μM (Fig. 4A and D). However, there was no evidence for stereoselectivity of action of the menthol enantiomers and minimal current modulation by (−)-borneol (Fig. 4B and C). Furthermore, glycine receptors were largely insensitive to coapplications of all the ketones at 100 μM (e.g., Fig. 4C). Addition of 300 μM (−)-menthone did potentiate the glycine responses by 37.6±10.3% (n=6), while at this concentration, α/β-thujone also resulted in nominal enhancement of the EC_{20} glycine responses (45.0±12.2%, n=6). Note that this is in contrast to the inhibitions observed for the thujone diastereomers acting on $GABA_A$ receptors.

3.3. Relative sensitivities of $GABA_A$ and glycine receptors to modulation by menthol

Given the pronounced positive modulation of both currents by (+)-menthol, we further investigated the effects of this enantiomer on GABA and glycine concentration–response curves (Fig. 5). With addition of 100 μM (+)-menthol, the EC_{50} for GABA was reduced from 82.8±9.9 to 25.0±1.8 μM ($P<0.05$), while the Hill slope (n_H) and maximum GABA response did not differ significantly ($P<0.05$). For glycine, the EC_{50} in the presence of the monoterpenoid was marginally, although significantly, reduced from 98.7±8.6 to 75.7±9.4 μM ($P<0.05$, n=6), while there was no significant difference in Hill slope or

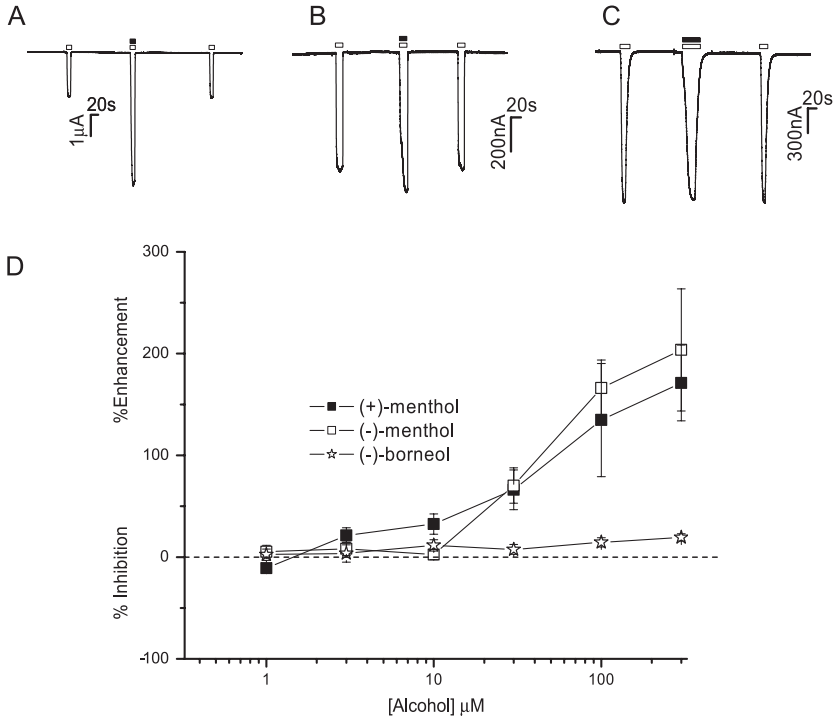

Fig. 4. Positive modulation of glycine receptor currents by the monoterpenoid alcohols. Oocytes were held at −50 mV and 50 μM glycine was applied for the duration of the open boxes above each trace. Coapplication of 100 μM monoterpenoid is indicated by the filled boxes with (A) (−)-menthol, (B) (−)-borneol, and (C) α/β-thujone. Effects were fully reversible upon washout for all monoterpenoids tested. (D) Dose-dependency for effects of the alcohols on glycine (50 μM) currents. While (−)-borneol had minimal effect, at 30–300 μM, (+)-menthol and (−)-menthol resulted in enhancements of EC_{20} glycine currents (with no evidence of stereoselectivity).

maximum glycine current ($P<0.05$). Thus, (+)-menthol was a more potent positive modulator of human $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors than α_1 homomer glycine receptors.

4. Discussion

$GABA_A$ and glycine receptors are the main ionotropic receptors responsible for fast inhibitory neurotransmission throughout the mammalian nervous system. Positive modulation of these LGICs has a profound influence on neuronal activity, often resulting in sedation and anesthesia. Our major findings are: (1) the monoterpenoid alcohol, menthol, is a potent and stereoselective positive allosteric modulator of $GABA_A$ receptors; (2) menthol enantiomers enhance glycine receptor responses; (3) ketone monoterpenoids (camphor, menthone, and carvone) have minimal effect on either LGIC; and (4) thujone diastereomers are inhibitors of $GABA_A$ receptors.

The effects of the monoterpenoids were dependent on their structure and associated functional groups (–OH or =O), and the type of receptor studied. For instance, in agreement with previous studies (Hold et al., 2000), the diastereomers of the bicyclohexanone thujone consistently inhibited $GABA_A$ receptors (see Figs. 2 and 3), although recombinant receptors appeared less sensitive (IC_{50}>300 μM) than those recorded from native membranes (IC_{50}=21 μM). By contrast, recombinant glycine receptor currents were slightly increased (45.0±12.2%, n=6) by equivalent concentrations of the thujone diastereomers—an important observation given that $GABA_A$ and glycine receptors often share similar pharmacology. Other monoterpenoid ketones tested (enantiomers of camphor, carvone, and menthone) produced minimal modulation of both receptor responses with little evidence for stereoselectivity (see Figs. 2–4). For instance, 300 μM (+)-camphor had negligible effects on glycine receptors (3.5±5.8%, n=6, data not shown) and enhanced GABA currents by only 41.7±4.2% (n=6; Fig. 3B). By contrast, equivalent concentrations completely inhibited nicotinic acetylcholine receptors, also members of the LGIC superfamily (Park et al., 2001).

The most pronounced effects on the inhibitory LGICs were observed with coapplications of the alcohols, (+)-menthol and (−)-menthol and (−)-borneol (the corresponding alcohol of camphor). In accord with these results, Aoshima and Tenpaku (1997) showed that $GABA_A$ receptor currents were modulated by food additives including menthol, while recent reports described borneol and thymol (a phenolic constituent of thyme oil) as potent modulators of nicotinic acetylcholine and $GABA_A$ receptors, respectively (Park et al., 2003; Priestley et al., 2003). In our study, (+)-menthol induced leftward shifts in both the GABA and glycine concentration–response curves, and corresponding reductions in their respective EC_{50} values (Fig. 5). Thus, menthol acts as a positive allosteric modulator either by affecting agonist binding or, alternatively, the consequent

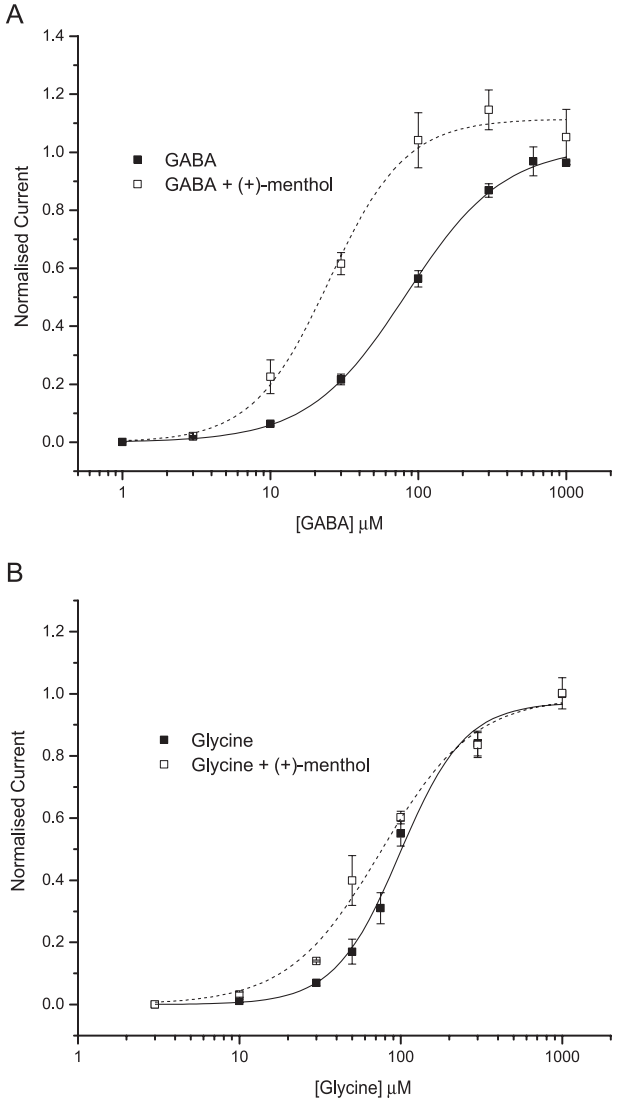

Fig. 5. GABA receptors are more sensitive than glycine receptors to positive allosteric modulation by (+)-menthol. For both receptors, currents were normalised against the maximum current evoked by any concentration of agonist without coapplication of (+)-menthol. Data ($n \geq 6$, mean±S.E.M.) were fitted with the Hill equation (see Materials and Methods) in the absence (solid lines) and presence of 100 μM (+)-menthol (dotted lines). (A) Leftward shift of the GABA concentration–response curve in the presence of the monoterpenoid. For control currents, EC_{50} for $\alpha_1\beta_2\gamma_{2s}$ GABA receptors was 82.8±9.9 μM with a Hill coefficient (n_H) of 1.3±0.2. In the presence of (+)-menthol, the EC_{50} was shifted to 25.0±1.8 μM with a Hill coefficient of 1.7±0.2. (B) Minimal leftward shift of the glycine concentration–response curve in the presence of the monoterpenoid. For control currents, EC_{50} for α_1 homomer glycine receptors was 98.7±8.6 μM with a Hill coefficient (n_H) of 2.1±0.4. In the presence of (+)-menthol (100 μM), the EC_{50} was shifted to 75.7±9.4 μM with a Hill coefficient of 1.5±0.2.

gating of inhibitory LGICs. Moreover, we observed stereoselectivity of the action of the menthol enantiomers on $GABA_A$ receptors. Stereoselectivity for positive modulation of LGIC currents is not unprecedented, particularly by enantiomers of general anesthetics (e.g., Hall et al., 1994; Tomlin et al., 1998). However, in contrast to anesthetic pharmacology (see Pearce, 2001), neither menthol enantiomers nor any other of the monoterpenoids tested gave any direct activation of GABA or glycine currents.

Menthol is recognised as an activator of the CMR1, also known as the transient receptor potential channel, TRPM8 (McKemy et al., 2002), and evokes a cation current in cell lines expressing TRPM8 (Peier et al., 2002). Indeed presynaptic TRPM8 receptor activation by menthol facilitates transmission at sensory synapses via mobilisation of intracellular Ca^{2+} (Tsuzuki et al., 2004). An endogenous TRP-like receptor (*xTRP*) has been cloned from *X. laevis* and is expressed in the oocytes (Bobanovic et al., 1999). However, xTRP is highly homologous to human TRPC-1, which is activated by G-protein-coupled receptors and receptor tyrosine kinases rather than by cooling compounds like menthol (Brereton et al., 2000; Clapham, 2003). Moreover, although a substantial endogenous Ca^{2+}-dependent chloride conductance is present in all oocytes, neither menthol nor other monoterpenoids directly activated any current in uninjected or expressing oocytes. We decided to investigate the effects of cooling oocytes to 4 °C on EC_{20} $GABA_A$ receptor responses (data not shown). Although the rate of activation of the GABA currents was reduced at this temperature, there was no effect on current amplitude compared to recordings at room temperature (n=5). Thus, our study suggests a novel role for menthol in modulating transmission at predominantly postsynaptic receptor sites by direct binding to $GABA_A$ and glycine receptors.

In assessing the extent of positive modulation by the monoterpenoid alcohols compared to ketones, it is evident that the hydroxyl group is a critical component for the reported effects, suggesting a polar interaction at the monoterpenoid receptor binding site. Interestingly, menthol has some structural similarities to the intravenous general anesthetic, propofol (Fig. 6). Although propofol is a

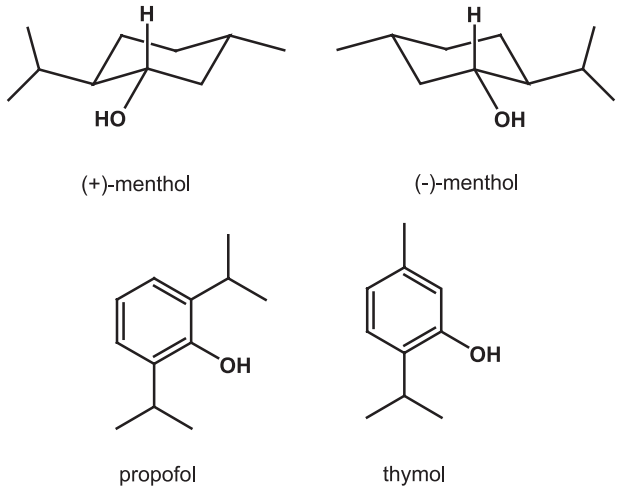

Fig. 6. Structures of menthol enantiomers versus the intravenous anesthetic, propofol, and the phenolic monoterpenoid, thymol. Although all the compounds share similar positioning of one isopropyl group adjacent to their functional –OH groups, (+)-menthol (a cyclohexanol) has a chair structure, while propofol and thymol are both phenols and therefore planar.

phenol (pK_a=11.0, planar ring structure) and menthol is a neutral cyclohexanol (chair structure), they share equivalent positioning of an isopropyl adjacent to their respective hydroxyl groups. The *ortho* positioning of an aliphatic chain was shown to be an important requirement for the activity of propofol analogs for modulation (Krasowski et al., 2001, 2002) and for direct activation of $GABA_A$ receptor currents (Mohammadi et al., 2001). In our experiments, although menthol was a potent positive modulator of GABA currents, neither enantiomer activated a chloride current in the absence of agonist. By comparing the structure of menthol to borneol (Fig. 1), the reduced modulatory activity of the latter may result from the fact that the isopropyl methyl groups, which are fixed in space in a rigid bicyclic configuration, are located farther away from the hydroxyl group. In contrast, the isopropyl group in menthol, as well as in propofol and thymol, is capable of free rotation (Fig. 6). Thus, in certain rotomeric conformations, the isopropyl methyls can assume close proximity to the hydroxyl group. Furthermore, the difference in activity observed for the enantiomeric menthols suggests that chiral recognition may also be taking place at the binding site.

We cannot draw conclusions about exact sites on the receptors for menthol binding. $GABA_A$ receptors appear equally sensitive to modulation by (+)-menthol and thymol (e.g., at 100 μM, ca. 150–200% enhancements of GABA EC_{20} currents for both monoterpenoids; see Priestley et al., 2003), while propofol is evidently a more potent modulator (Krasowski et al., 2001). Interestingly, thymol does not compete with other GABA ligands, including propofol, suggesting a novel binding site for this monoterpenoid (Priestley et al., 2003). We will continue to investigate the precise nature of the menthol–$GABA_A$ receptor interaction as an avenue of future research.

Menthol is a well-known additive in foods (Aoshima and Tenpaku, 1997), cigarettes, and pharmaceutical products (Eccles, 2000); is an antipruretic and gastric sedative; and has pronounced effects on animal behaviour. For instance, when administered to rodents by injection, menthol promotes ambulation (Umezu et al., 2001), while the (−)-enantiomer is endowed with analgesic properties mediated through activation of kappa opioid receptors (Galeotti et al., 2002). When applied topically, menthol is an effective local anesthetic (Galeotti et al., 2001), possibly via a voltage-dependent block of neuronal sodium channels (Haeseler et al., 2002). Borneol has also been used for centuries in Asian cultures for its medicinal properties, including treatment of sore throat, swollen eyes, convulsions, coma, and coronary heart disease. In the present study, we have demonstrated that menthol and borneol act as potent positive allosteric modulators at $GABA_A$ and glycine receptors. In conclusion, the enhancement of inhibitory neurotransmission by common monoterpenoids may be an important component of the neuroactive properties of monoterpenoid alcohols and further explain their widespread medicinal use.

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References

Aoshima, H., Tenpaku, Y., 1997. Modulation of GABA receptors expressed in *Xenopus* oocytes by 13-L-hydroxylinoleic acid and food additives. Biosci. Biotechnol. Biochem. 61, 2051–2057.

Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N., Langer, S.Z., 1998. Subtypes of γ-aminobutyric acid A receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev. 50, 291–313.

Bobanovic, L.K., Laine, M., Petersen, C.C.H., Bennett, D.L., Berridge, M.J., Lipp, P., Ripley, S.J., Bootman, M.D., 1999. Molecular cloning and immunolocalization of a novel vertebrate *trp* homologue from *Xenopus*. Biochem. J. 340, 593–599.

Brereton, H.M., Harland, M.L., Auld, A.M., Barritt, G.J., 2000. Evidence that the TRP-1 protein is unlikely to account for store-operated Ca^{2+} inflow in *Xenopus laevis* oocytes. Mol. Cell. Biochem. 214, 63–74.

Burk, L.A., 2003. Absinthe—chemistry and culture. In: Patey, D.L. (Ed.), Of Human Bondage, Historical Perspectives on Addiction. Smith College Studies in History LII, pp. 95–132.

Cascio, M., 2004. Structure and function of the glycine receptor and related nicotinicoid receptors. J. Biol. Chem. 279, 19383–19386.

Clapham, D.E., 2003. TRP channels as cellular sensors. Nature 426, 517–524.

Eccles, R., 2000. Role of cold receptors and menthol in thirst, the drive to breathe and arousal. Appetite 34, 29–35.

Franks, N.P., Lieb, W.R., 1994. Molecular and cellular mechanisms of general anesthesia. Nature 367, 607–614.

Galeotti, N., Ghelardini, C., Di Cesare Mannelli, L., Mazzanti, G., Baghirolli, L., Bartolini, A., 2001. Local anesthetic activity of (+)- and (−)-menthol. Planta Med. 67, 174–176.

Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A., Ghelardini, C., 2002. Menthol: a natural analgesic compound. Neurosci. Lett. 322, 145–148.

Haeseler, G., Maue, D., Grosskreutz, J., Butler, J., Nentwig, B., Piepenbrock, R., Dengler, R., Leuwer, M., 2002. Voltage-dependent block of neuronal and skeletal muscle sodium channels by thymol and menthol. Eur. J. Anaesthesiol. 19, 571–579.

Hall, A.C., Lieb, W.R., Franks, N.P., 1994. Stereoselective and non-stereoselective actions of isoflurane on the $GABA_A$ receptor. Br. J. Pharmacol. 112, 906–910.

Hall, A.C., Rowan, K.C., Stevens, R.J.N., Kelley, J.C., Harrison, N.L., 2004. The effects of isoflurane on desensitized wild-type and α_1(S270H) γ-aminobutyric acid type A receptors. Anesth. Analg. 98, 1297–1304.

Hold, K.M., Sirisoma, N.S., Ikeda, T., Narahashi, T., Casida, J.E., 2000. α-Thujone (the active component of absinthe): γ-aminobutyric acid type A receptor modulation and metabolic detoxification. Proc. Natl. Acad. Sci. 97, 3826–3831.

Krasowski, M.D., Harrison, N.L., 1999. General anesthetic actions on ligand-gated ion channels. Cell. Mol. Life Sci. 55, 1278–1303.

Krasowski, M.D., Jenkins, A., Flood, P., Kung, A.Y., Hopfinger, A.J., Harrison, N.L., 2001. General anesthetic potencies of a series of

propofol analogs correlate with potency for potentiation of γ-aminobutyric acid (GABA) current at the $GABA_A$ receptor but not with lipid solubility. J. Pharmacol. Exp. Ther. 297, 1–14.

Krasowski, M.D., Hong, X., Hopfinger, A.J., Harrison, N.L., 2002. 4D-QSAR analysis of a set of propofol analogues: mapping binding sites for an anesthetic phenol on the $GABA_A$ receptor. J. Med. Chem. 25, 3210–3221.

Langosch, D., Thomas, L., Betz, H., 1988. Conserved quaternary structure of ligand-gated ion channels: the postsynaptic glycine receptor is a pentamer. Proc. Natl. Acad. Sci. 85, 7394–7398.

McKemy, D.D., Neuhasser, W.M., Julius, D., 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416, 52–58.

Mihic, S.J., Ye, Q., Wick, M.J., Kotchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R.A., Harrison, N.L., 1997. Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. Nature 389, 385–389.

Mohammadi, B., Haeseler, G., Leuwer, M., Dengler, R., Krampfl, K., Bufler, J., 2001. Structural requirements of phenol derivatives for direct activation of chloride currents via $GABA_A$ receptors. Eur. J. Pharmacol. 421, 85–91.

Olsen, R.W., 1981. The GABA postsynaptic membrane receptor–ionophore complex. Site of action of convulsant and anticonvulsant drugs. Mol. Cell. Biochem. 39, 261–279.

Olsen, R.W., 2000. Absinthe and γ-aminobutyric acid receptors. Proc. Natl. Acad. Sci. 97, 4417–4418.

Park, T.-J., Seo, H.-K., Kang, B.-J., Kim, K.-T., 2001. Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors. Biochem. Pharmacol. 61, 787–793.

Park, T.-J., Park, Y.-S., Lee, T.-G., Ha, H., Kim, K.-T., 2003. Inhibition of acetylcholine-mediated effects by borneol. Biochem. Pharmacol. 65, 83–90.

Pearce, R.A., 2001. Effects of volatile anesthetics on $GABA_A$ receptors: electrophysiologic studies. In: Moody, E., Skolnick, P. (Eds.), Molecular Bases of Anesthesia. CRC Press, Baton Rouge, FL, USA, pp. 245–272.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S., Patapoutian, A., 2002. A TRP channel that senses cold stimuli and menthol. Cell 108, 705–715.

Priestley, C.M., Williamson, E.M., Wafford, K.A., Sattelle, D.B., 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. Br. J. Pharmacol. 140, 1363–1372.

Tirillini, B., Velasquez, E.R., Pellegrino, R., 1996. Chemical composition and antimicrobial activity of essential oil of *Piper angustifolium*. Planta Med. 62, 372–373.

Tomlin, S.L., Jenkins, A., Lieb, W.R., Franks, N.P., 1998. Stereoselective effects of etomidate optical isomers on gamma-aminobutyric acid A receptors and animals. Anesthesiology 88, 708–717.

Tretter, V., Ehya, N., Fuchs, K., Sieghart, W., 1997. Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J. Neurosci. 17, 2728–2737.

Tsuzuki, K., Xing, H., Ling, J., Gu, J.G., 2004. Menthol-induced Ca^{2+} release from presynaptic Ca^{2+} stores potentiates sensory synaptic transmission. J. Neurosci. 24, 762–771.

Umezu, T., Sakata, A., Ito, H., 2001. Ambulation-promoting effect of peppermint oil and identification of its active constituents. Pharmacol. Biochem. Behav. 69, 383–390.