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(+)- And (−)-borneol: efficacious positive modulators of GABA action at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

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Abstract

(+)-Borneol is a bicyclic monoterpene used for analgesia and anaesthesia in traditional Chinese and Japanese medicine and is found in the essential oils of medicinal herbs, such as valerian. (+)-Borneol was found to have a highly efficacious positive modulating action at $GABA_A$ receptors, as did its enantiomer (−)-borneol. The effects of these bicyclic monoterpenes alone and with GABA were evaluated at recombinant human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp electrophysiology. (+)-Borneol (EC_{50} 248 μM) and (−)-borneol (EC_{50} 237 μM) enhanced the action of low concentrations of GABA by more than 1000%. These enhancing effects were highly dependent on the relative concentrations of the borneol enantiomer and GABA, and were insensitive to flumazenil indicating that (+)- and (−)-borneol were not acting at classical benzodiazepine sites. The maximal responses to GABA were enhanced 19% by (+)-borneol and reduced 21% by (−)-borneol. The borneol analogues isoborneol, (−)-bornyl acetate and camphor, produced less marked effects. At high concentrations (>1.5 mM) (+)- and (−)-borneol directly activated $GABA_A$ receptors producing 89% and 84%, respectively, of the maximal GABA response indicative of a weak partial agonist action. Although of lower potency, the highly efficacious positive modulatory actions of (+)- and (−)-borneol on GABA responses were at least equivalent to that of the anaesthetic etomidate and much greater than that of diazepam or 5α-pregnan-3α-ol-20-one. The relatively rigid cage structure of these bicyclic monoterpenes and their high efficacy may aid in a greater understanding of molecular aspects of positive modulation of the activation of $GABA_A$ receptors.

Keywords: Borneol; $GABA_A$ receptor; Monoterpene; *Valeriana officinalis*; *Xenopus* oocyte

1. Introduction

Monoterpenes are the primary components of plant essential oils and the effects of many medicinal herbs have been attributed to them [1–6]. (+)-Borneol (Fig. 1) is a bicyclic monoterpene present in the essential oils of numerous medicinal plants, including valerian (*Valeriana officinalis*), chamomile (*Matricaria chamomilla*) and lavender (*Lavandula officinalis*). Extracts of these plants are used traditionally to relieve anxiety, restlessness and insomnia [7–10]. Valerian extracts and essential oil demonstrate significant sedative activity in animal and human studies, providing equivalent sedation to conventional sedative and hypnotic agents [11], while also increasing sleep depth [12]. (+)-Borneol and its structural analogue camphor (Fig. 1) are used for analgesia and anaesthesia in traditional Chinese and Japanese medicine [13].

While many possible mechanisms for the actions of sedative herbal medicines have been proposed, these herbs have been primarily linked with functions associated with the neurotransmitter GABA [14–18]. GABA is the predominant inhibitory transmitter in the mammalian central nervous system, present in 40% of all mammalian CNS neurons. $GABA_A$ and $GABA_C$ receptors are ligand-gated chloride channels that mediate an inhibitory effect by increasing the chloride influx into neurons, inducing

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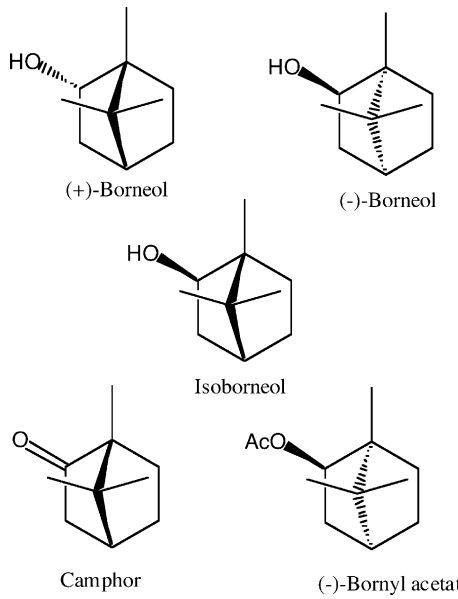

Fig. 1. Bicyclic monoterpenes present in *Valeriana officinalis*.

membrane hyperpolarisation and neuronal inhibition [19]. $GABA_A$ receptors comprise of five protein subunits chosen from α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π and θ, with $\alpha_1\beta_2\gamma_2$ being the most common arrangement in the mammalian brain [20]. Stimulation of $GABA_A$ receptors by GABA (and added positive modulators, such as benzodiazepines and barbiturates) produces anxiolysis, sedation, anaesthesia and myorelaxation [21], akin to the effects of these sedative herbs. Valerian extracts have been previously shown to prevent GABA re-uptake [18], bind at the GABA and benzodiazepine sites of the $GABA_A$ receptor [22] and facilitate GABA transport to the brain [23]. Additionally, lavender essential oil mildly potentiates the effects of GABA at $GABA_A$ receptors [24]. Thus, traditional sedative herbs like valerian and lavender most likely contain GABAergic compounds. Determining their structure and activity could result in new drug leads for the treatment of anxiety and insomnia.

(+)-Borneol produces mild sedation in mice when inhaled; yet, its isomer isoborneol increases locomotion [25] which may indicate divergent actions at $GABA_A$ receptors, despite their structural similarity. Previous research indicates some monoterpenes, including citronellol and α-pinene, produce positive modulation of GABA at $\alpha_1\beta_1$ $GABA_A$ receptors [24], suggesting (+)-borneol and analogues (Fig. 1) may also act via $GABA_A$ receptors to contribute to the traditionally acknowledged sedative and anxiolytic effects of valerian.

The present study compares the effects of bicyclic monoterpenes found in essential oils, particularly (+)- and (−)-borneol, on GABA-induced chloride currents at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp electrophysiology.

2. Materials and methods

2.1. Materials

Human α_1, β_2 and γ_{2L} cDNAs subcloned in pcDM8 (Stratagene, La Jolla, CA, USA) were kindly provided by Dr. Paul Whiting, Merck Sharp and Dohme Research Laboratories, Harlow, Essex, UK). $GABA_A$ receptor mRNA ($\alpha_1\beta_2\gamma_{2L}$) were the kind gifts of Ms. Shelley Huang and Ms. Belinda Hall (The University of Sydney, Sydney, Australia). GABA, (+)-borneol, (−)-borneol, isoborneol, (−)-bornyl acetate, zinc sulfate, and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Camphor was obtained from BDH Laboratory Supplies (Poole, England, UK). Diazepam and flumazenil were gifts from Hoffman-La Roche (Nutley, NJ, USA).

2.2. Expression of GABA receptors in X. laevis oocytes

Female *X. laevis* were anaesthetized with 0.17% ethyl 3-aminobenzoate in saline and a lobe of the ovaries was surgically removed, in a procedure approved by the Animal Ethics Committee of the University of Sydney. The lobe was rinsed with OR-2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 5 mM HEPES, pH 7.4) and incubated in collagenase A solution (Boehringer, Mannheim, Germany—2 mg/mL in OR-2) for up to 2 h. Defolliculated stages V–VI oocytes were collected and rinsed with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). cRNAs were injected into the cytoplasm of individual oocytes using a 15–20 μm diameter tip micropipette (micropipette puller, Sutter Instruments, USA) made from Drummond $3^{1/2}$ in. glass tube, using a Nanoject injector (Drummond Scientific Company, Broomali, PA, USA) at approximately 10 ng/50 nL. A 1:1:2.5 mixture was used for α_1, $\beta_2$and γ_{2L}. Oocytes were stored at 16 °C with constant rotation on an orbital shaker for 2–7 days in ND96 buffer with 2.5 mM sodium pyruvate, 0.5 mM theophylline and 50 μg/mL gentamycin, and replaced daily.

2.3. Electrophysiological recording

Receptor activity was measured using two-electrode voltage-clamp electrophysiology 2–5 days after injection. Two glass microelectrodes (Harvard Apparatus, Edenbridge, Kent, UK) made with a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) filled with 3 M KCl solution were inserted into the cell, with voltage clamped at −60 mV using a Geneclamp 500 Amplifier (Axon Instruments, Foster City, CA, USA). During recording, cells were constantly superfused with ND96 buffer.

Current traces were recorded using MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia) and Chart v3.5.2 for Apple Macintosh. Cells were screened for

receptor expression by superfusion of the cell with a maximal dose of GABA (1000 μM) dissolved in ND96 buffer. GABA stock solutions (1 M) were prepared with milli-Q water. Stock solutions of other compounds, also 1 M, were dissolved in DMSO. Compounds were diluted on the day of experimentation from the stock solutions to the desired concentration using ND96, to give a total volume of 25 mL, with a maximum of 200 μL DMSO (0.8%), at which concentration DMSO had no effects on healthy cells.

Compounds were tested for both direct activity at the receptors and for modulation of GABA activity, expressed as a function of the effective concentration to produce a certain response range to GABA ($EC_{1–4}$, $EC_{5–14}$, $EC_{15–24}$, $EC_{25–39}$, $EC_{40–59}$, $EC_{60–99}$ and EC_{100}. When testing for direct activity, the screening dose of 1000 μM GABA was first applied, and then a series of escalating concentrations of the monoterpenes were added to the cell bath (0.01–3000 μM) until a consistent maximum response or maximum dissolvable concentrations were reached. When testing for modulation, the screening dose of 1000 μM was applied first, followed by the modulatory GABA dose, and then a series of escalating concentrations of the monoterpenes with the chosen modulatory GABA dose were added to the cell bath (0.01–3000 μM) until a consistent maximum response or maximum dissolvable concentrations were reached. In both experiments, a washout period of 3–5 min between each application was used to avoid receptor desensitization. Both the maximal GABA doses and when used, the modulatory GABA doses, were reapplied periodically during testing to ensure adequate washout periods had been used.

We varied the sequence of application for different borneol concentrations (ascending or descending) and GABA test concentrations, in order to ensure adequate washout had occurred. We have found that dose sequence had no effect on current responses.

2.4. Data analysis

Data obtained from Chart v3.5.2 recordings were interpreted as a change in the current during the application of a dose. The peak amplitude of current in response to each concentration of compound was recorded and standardized using the calculation: $\%I_{max} = (I/I_{max}) \times 100$, where I is the peak amplitude at a given dose of compound and I_{max} is the maximal current generated by the compound or GABA, where appropriate. Responses were also calculated as a percentage of the modulating GABA dose (ECx) to determine maximum efficacy, where 100% indicates no potentiating effect. All values were analysed using the Sigmoidal Fit (variable slope) Equation from GraphPad Prism 3.0 (GraphPad Software, San Francisco, CA, USA) as follows: $Y = \text{maximum} + ((\text{maximum} - \text{minimum})/1 + 10^{(\log\ EC_{50} - X) \times \text{Hill slope})})$ where EC_{50} is the concentration of the compound producing 50% of I_{max} All results were analyzed for linear regression (departure of linearity with runs tests and 95% confidence interval (CI) of regression line). Unless otherwise noted, EC_{50} values are reported as mean ± 95% confidence interval, where the confidence interval gives the range of values which is 95% certain to include the EC_{50}. Hill slope values are reported as mean ± S.E.M. Significant differences were determined using two-way ANOVAs, generating F- and P-values, which have been quoted where appropriate.

3. Results

3.1. Expression of functional $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

GABA-sensitive channels were produced via injection of α_1, β_2, γ_{2L} ($GABA_A$) cRNAs in *X. laevis* oocytes. At a holding potential of −60 mV, the injected oocytes responded to bath applied GABA with an inward current response. Current traces are shown in Fig. 2. Incorporation of the γ_{2L} subunit was confirmed via insensitivity to Zn^{2+} ions. GABA-evoked currents were concentration-dependent, exhibiting comparable pharmacological profiles to those previously described, with GABA EC_{50} (32.9 μM; 95% CI: 29.2–37.0; $n = 8$) and Hill slope (n_H) (1.23 ± 0.08) similar to results reported of these receptors [26,27]. A complete summary of results can be seen in Table 1.

3.2. Potentiation of GABA activity at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors by (+)-borneol and analogues

3.2.1. Modulation of the $EC_{1–4}$ GABA response

(+)- And (−)-borneol produced dose-dependent positive modulation of the Cl^- conductance generated by extremely low dose ($EC_{1–4}$) GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Figs. 3 and 4). While isoborneol, (−)-bornyl acetate and camphor produced some enhancement and antagonism at selected doses, no meaningful dose–response curves could

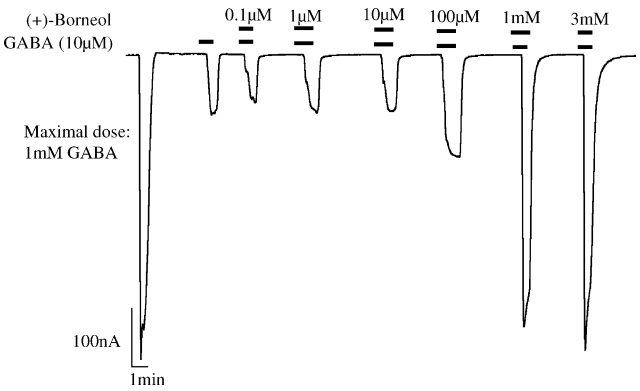

Fig. 2. Current traces produced by (+)-borneol in the presence and absence of 10 μM GABA (solid bar) on human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, compared to the maximal GABA response.

Table 1
GABA-modulatory action of (+)-borneol and related monoterpenes in *X. laevis* oocytes expressing recombinant $GABA_A$ receptors; EC_{50} values are mean ± 95% confidence interval; Hill slope, % of modulatory and maximal GABA response values are mean ± standard error for the indicated number (*n*) of oocytes

Modulatory [GABA]	*n*	EC_{50} (μM)	Hill slope (n_H)	% of modulatory GABA response	% of maximal GABA response
(+)-Borneol					
$EC_{1–4}$ GABA	4	609.5 (439.5–845.2)	2.79 ± n/a	1095 ± 94	92 ± 5
$EC_{5–14}$ GABA	4	247.7 (176.1–348.2)	1.66 ± 0.40	1251 ± 88	107 ± 7
$EC_{15–24}$ GABA	10	451.0 (234.2–868.3)	1.04 ± 0.24	848 ± 107	152 ± 12
$EC_{25–39}$ GABA	3	349.6 (276.5–395.9)	1.01 ± 0.20	325 ± 20	104 ± 8
$EC_{40–59}$ GABA	5	234.5 (74.8–735.3)	0.96 ± 0.38	203 ± 18	96 ± 6
$EC_{60–99}$ GABA	3	0.2 (−)	10.63 ± n/a	101 ± 5	88 ± 4
EC_{100} GABA	3	287.5 (−)	2.31 ± 3.72	119 ± 4	119 ± 4
(−)-Borneol					
$EC_{1–4}$ GABA	5	183.6 (146.8–229.6)	2.79 ± 0.71	884 ± 47	103 ± 10
$EC_{5–14}$ GABA	3	281.7 (205.1–386.8)	1.75 ± 0.40	1106 ± 73	148 ± 8
$EC_{15–24}$ GABA	3	236.9 (202.6–276.9)	2.41 ± 0.43	903 ± 50	154 ± 6
$EC_{25–39}$ GABA	3	342.7 (285.7–398.2)	1.41 ± 0.43	306 ± 17	99 ± 8
$EC_{40–59}$ GABA	5	43.3 (−)	1.21 ± n/a	102 ± 10	37 ± 8
$EC_{60–99}$ GABA	3	93.7 (−)	10.49 ± n/a	137 ± 5	93 ± 3
EC_{100} GABA	5	1233 (−)	6.44 ± n/a	79 ± 4	79 ± 4
Isoborneol					
$EC_{1–4}$ GABA	4	–	–	–	–
$EC_{5–14}$ GABA	3	360.1 (217.5–596.3)	1.48 ± 0.49	986 ± 88	90 ± 7
$EC_{15–24}$ GABA	3	190.5 (141.0–257.3)	1.77 ± 0.40	419 ± 26	92 ± 3
$EC_{25–39}$ GABA	3	435.2 (351.7–501.6)	1.48 ± 0.37	323 ± 22	79 ± 6
$EC_{40–59}$ GABA	5	6246 (−)	−1.79 ± n/a	88 ± 5	63 ± 8
$EC_{60–99}$ GABA	3	–	–	–	–
EC_{100} GABA	3	1517 (−)	–1.45 ± 3.76	56 ± 4	56 ± 4
(−)–Bornyl acetate					
$EC_{1–4}$ GABA	4	–	–	–	–
$EC_{5–14}$ GABA	3	333.5 (199.2–558.3)	1.41 ± 0.46	571 ± 123	67 ± 6
$EC_{15–24}$ GABA	4	140.4 (84.2–234.2)	0.90 ± 0.19	238 ± 20	57 ± 4
$EC_{25–39}$ GABA	3	111.2 (35.1–.352.8)	0.80 ± 0.32	323 ± 40	95 ± 11
$EC_{40–59}$ GABA	3	241.8 (−)	0.99 ± n/a	78 ± 32	69 ± 20
$EC_{60–99}$ GABA	3	35.6 (4.7–267.9)	0.84 ± 0.50	153 ± 13	107 ± 29
EC_{100} GABA	3	–	–	–	–
Camphor					
$EC_{1–4}$ GABA	5	–	–	–	–
$EC_{5–14}$ GABA	3	836.2 (211.7–3302)	2.08 ± 2.46	377 ± 156	42 ± 6
$EC_{15–24}$ GABA	3	469.1 (196.1–1122)	2.87 ± 2.57	187 ± 15	36 ± 2
$EC_{25–39}$ GABA	3	497.5 (−)	2.56 ± n/a	180 ± 14	38 ± 8
$EC_{40–59}$ GABA	3	3×10^8 (−)	−0.17 ± 1.642	75 ± 85	40 ± 40
$EC_{60–99}$ GABA	3	–	–	–	–
EC_{100} GABA	3	–	–	–	–

be constructed. The response to (+)-borneol with GABA did not reach a maximum within its solubility range, and hence response minimum and Hill slope were constrained to the values obtained for (−)-borneol. With these constraints, (+)-borneol produced a maximum enhancement of 1095% of the $EC_{1–4}$ GABA response (Figs. 3 and 4), generating significantly greater enhancement than the response produced by (−)-borneol (884%, Fig. 4).

3.2.2. Modulation of the $EC_{5–14}$ GABA response

All tested monoterpenes produced dose-dependent positive modulation of the Cl^- conductance generated by low ($EC_{5–14}$) GABA concentrations at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. (+)-Borneol produced the greatest proportion of potentiation at this GABA dose range. Trace recordings at this GABA modulatory dose can be seen in Fig. 2. (+)-Borneol enhanced the $EC_{5–14}$ GABA response by 1251%, significantly lower than that seen with (−)-borneol, which potentiated the $EC_{5–14}$ GABA response by 1106% (Fig. 5). Isoborneol, (−)-bornyl acetate and camphor produced 986%, 571% and 377% enhancement of the $EC_{5–14}$ GABA response, respectively, a reduced response compared to those of the borneol enantiomers, and with an elevated EC_{50} (Table 1).

3.2.3. Modulation of the $EC_{15–24}$ GABA response

All tested monoterpenes produced dose-dependent positive modulation of the Cl^- conductance generated by a moderately low ($EC_{5–14}$) GABA dose at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. As with $EC_{5–14}$ GABA, (−)-borneol produced

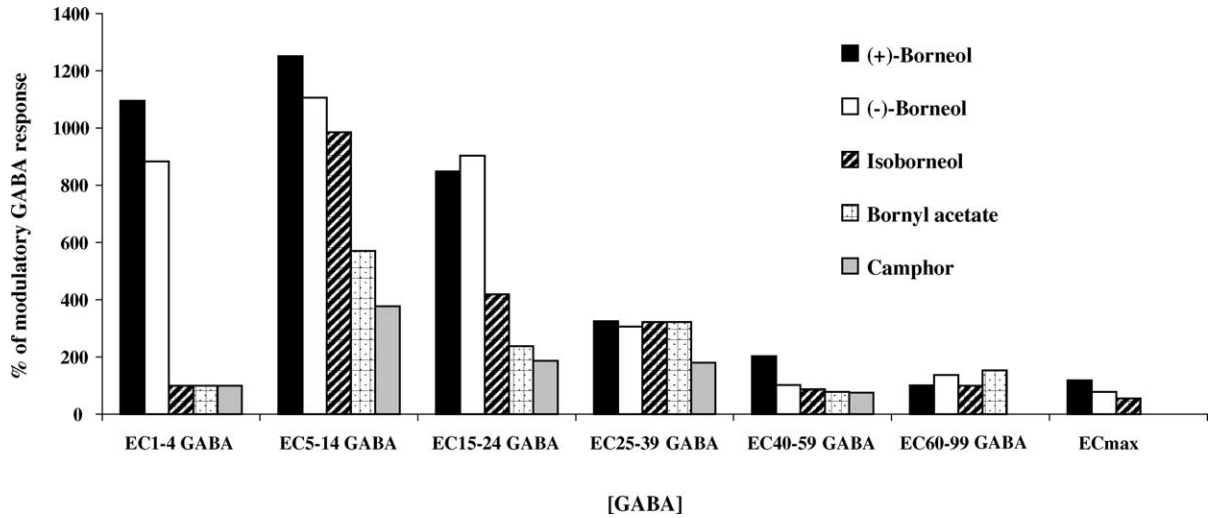

Fig. 3. Bar graph showing the potentiation produced by all monoterpenes tested at all GABA concentrations tested. (+)- And (−)-borneol produce extreme potentiation at low GABA concentrations, and produce the greatest potentiation throughout the GABA concentration range, except with $EC_{60–99}$ GABA, where (−)-bornyl acetate produces the greatest effect.

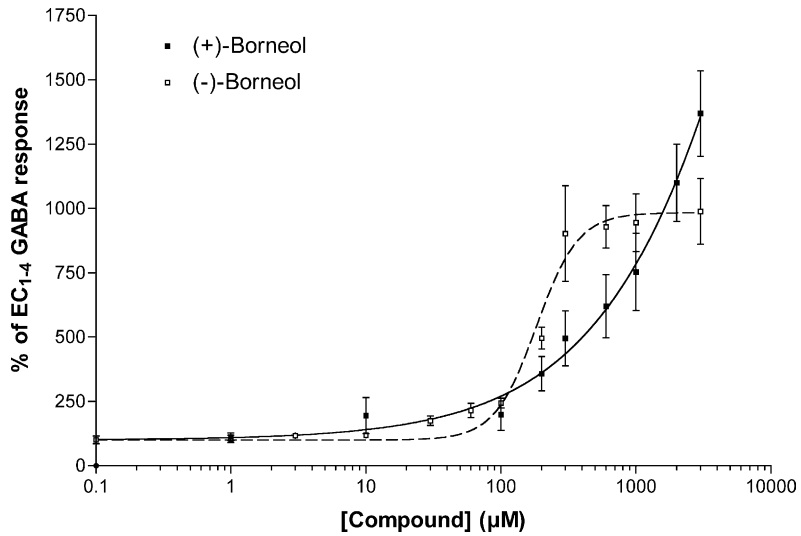

Fig. 4. (+)-Borneol (■) produced almost three times the potentiation of extremely low dose GABA ($EC_{1–4}$) at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, when compared (−)-borneol (□), where 100% is the response generated by the maximal GABA dose ($n = 5$ oocytes).

the greatest enhancement, generating 903% of the $EC_{15–24}$ GABA response (Fig. 6), compared to 848% produced by (+)-borneol. Both compounds exceeding the maximal response to GABA, producing 154% and 152% of this GABA response. However, the plateau maximal response for (+)-borneol was not reached within the limits of solubility, indicating this compound may produce a greater response than (−)-borneol, given improved solubility. Isoborneol produced less than half (419%) of the $EC_{15–24}$ GABA response, with (−)-bornyl acetate (238%) and camphor (187%) generating smaller responses again. Results for the modulation of moderately low GABA concentrations indicate a similar order of efficacy to the $EC_{5–14}$ GABA data, favouring (+)- and (−)-borneol (see Fig. 3 and Table 1).

3.2.4. Modulation of intermediate ($EC_{25–39}$, $EC_{40–59}$, $EC_{60–99}$) GABA responses

(+)- And (−)-borneol produced a much smaller degree of potentiation with moderate to high GABA concentrations, when compared to the results above, as shown in Fig. 3. (+)- And (−)-borneol, isoborneol and (−)-bornyl acetate all produced up to 300% increase in the $EC_{25–39}$ GABA response. Only (+)-borneol produced a discernible potentiation with $EC_{40–59}$ GABA, potentiating the GABA response by 200%. (−)-Bornyl acetate was the only compound to produce a significant dose-dependent relationship with $EC_{60–99}$ GABA. Camphor produced no significant dose–response relationship at any GABA dose in these ranges.

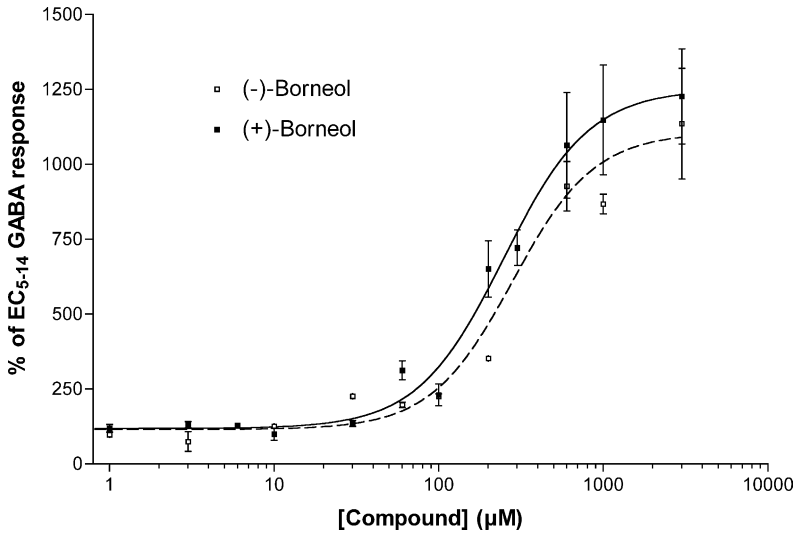

Fig. 5. (−)-Borneol (□) produced the greatest potentiation in the presence of $EC_{5–14}$ GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, when compared to (+)-borneol (■) and other monoterpenes tested ($n = 3–4$ oocytes).

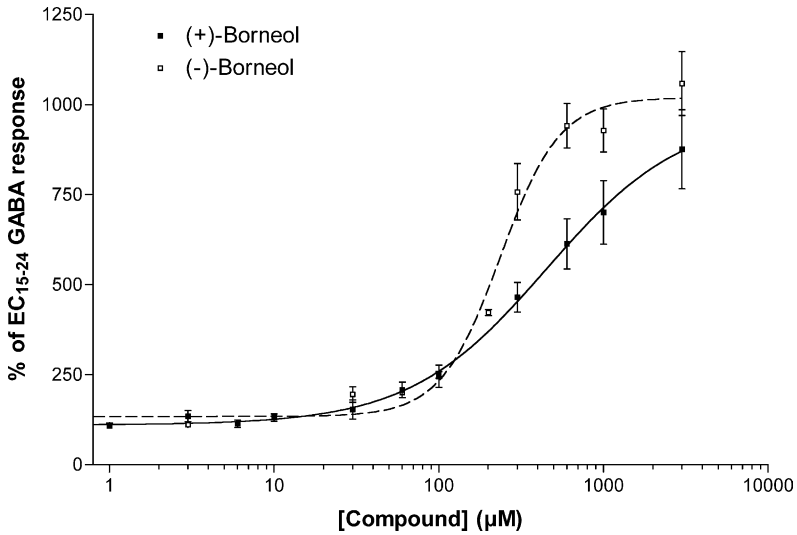

Fig. 6. (−)-Borneol (□) produced the greatest potentiation in the presence of $EC_{15–24}$ GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, when compared to (+)-borneol (■) and other monoterpenes tested ($n = 3–9$ oocytes).

3.2.5. Modulation of the EC_{100} (maximal) GABA response

(+)-, (−)-Borneol and isoborneol produced dose-dependent modulation of the Cl^- conductance generated by the maximal GABA concentration (EC_{100}) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. While (−)-bornyl acetate and camphor produced some enhancement and antagonism at selected doses, no meaningful dose–response curves could be constructed. Broad dose–response parameters have been constructed for the remaining compounds tested. (+)-Borneol was the only compound to produce enhancement of this maximal GABA response, generating 19% potentiation (Fig. 7), whereas both (−)-borneol and isoborneol produced a significant reduction in the maximal GABA response (21% and 44%, respectively). Compounds tested at this GABA concentration produced a less distinct dose–response relationship, as evidenced by the larger or incalculable EC_{50} 95% confidence interval. However, it is clear that the borneol enantiomers produce divergent actions in combination with a maximal GABA concentration.

3.3. Modulation of the GABA dose–response curve by (+)-borneol

(+)-Borneol at 500 μM shifted the GABA dose–response curve to the left ($n = 6$, $F = 4.416$, $P = 0.038$), decreasing the mean EC_{50} at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors by $34 \pm 8\%$ to 25.03 μM (95% CI: 14.83–35.37, Fig. 8), where GABA $EC_{50} = 47.32$ (95% CI: 27.53–62.79). (+)-

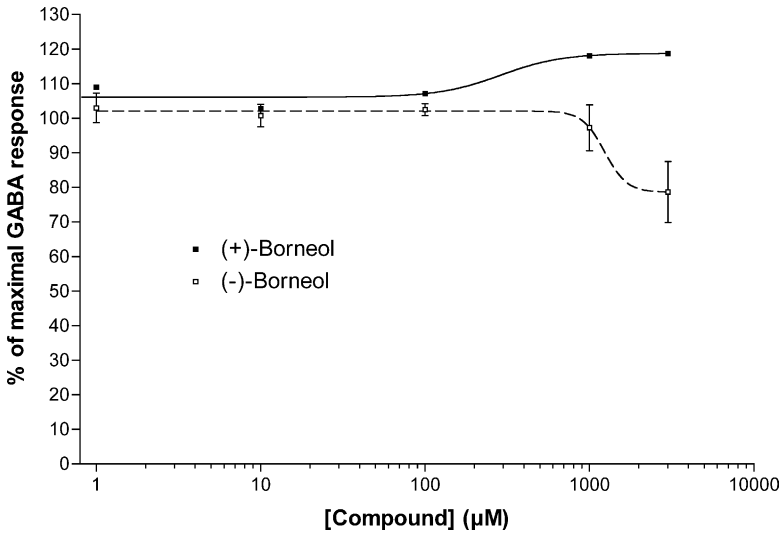

Fig. 7. (+)-Borneol (■) potentiated the effect of EC_{100} (maximal) GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, while (−)-borneol (□) reduced this response ($n = 3$–5 oocytes). Where error bars are not visible, they are smaller than the data points.

Borneol did reduce the maximal response to GABA ($(I_{max} - 100\%)$ by $7 \pm 5\%$ ($93.43 \pm 5.99\%$), but this result was not significant. The addition of 400 μM (+)-borneol had no significant effect on the GABA dose–response curve ($n = 6$, $F = 2.272$, $P = 0.135$).

3.4. The effect of antagonists on (+)-borneol

Flumazenil (0.1–100 μM) did not block the enhancement produced by 0.1–3000 μM (+)-borneol at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, whereas under the same conditions 1 μM flumazenil completely blocked the response to 0.6 μM diazepam. Flumazenil (1 μM) did not have any significant effect on the dose–response curve for (+)-borneol in the presence of 10 μM GABA ($n = 6$, $F = 0.1958$, $P = 0.6596$) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

Bicuculline produced some inhibition of the (+)-borneol-induced current, but only when high concentrations of (+)-borneol were added. Bicuculline (10 μM) produced up to 30% inhibition and 100 μM bicuculline produced 76% inhibition of the response to 1000 μM (+)-borneol alone. Picrotoxinin (30 μM) produced up to 60% inhibition of the response to 1000 μM (+)-borneol. The same degree of response inhibition was seen with all of the tested compounds for both antagonists.

3.5. Modulation of the $GABA_A$ receptor response by (+)-borneol and analogues

(+)-Borneol generated the greatest direct action of the compounds tested at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, producing up to 89% of the maximal GABA response in a

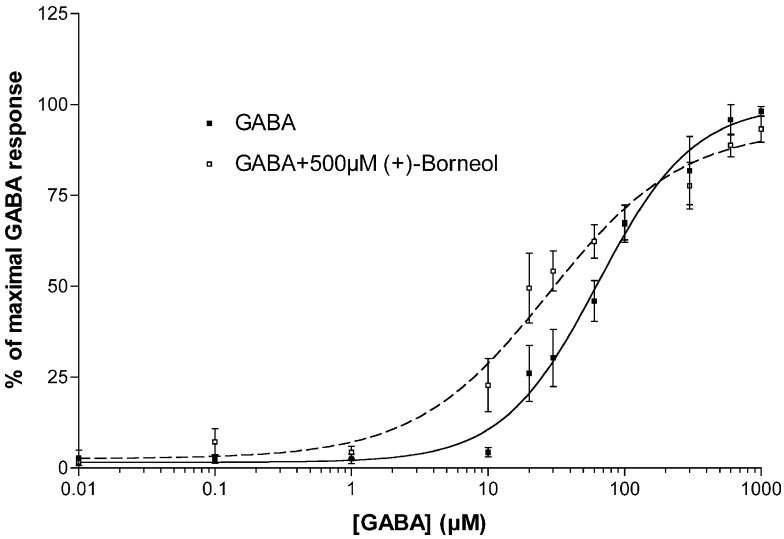

Fig. 8. (+)-Borneol (500 μM, ■) shifts the GABA dose–response curve (□) to the left at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, decreasing the mean EC_{50} by $34 \pm 8\%$. Data are mean ± S.E.M. ($n = 6$ oocytes).

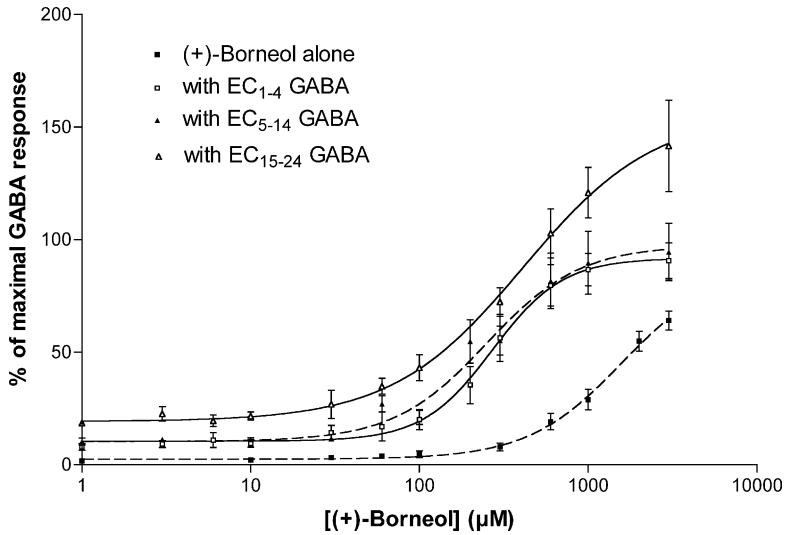

Fig. 9. (+)-Borneol (■) produced direct activity in the absence of GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors of up to 65% of the maximal GABA response, compared to more than 100% with $EC_{1–4}$ (□), $EC_{5–14}$ (▲) and $EC_{15–24}$ (△) GABA concentrations.

concentration-dependent and reversible manner (Fig. 9), with a threshold concentration of 300 μM. (−)-Borneol produced a slightly lower response, up to 84% and isoborneol produced 51% of the maximal GABA response. (−)-Bornyl acetate also produced a direct action, with a much lower 12% of maximal GABA response. The plateau phase of the dose–response curves was unachievable for all compounds except for (−)-bornyl acetate, as the limits of solubility of all other compounds were reached. Estimations of EC_{50} concentrations were generated for (+)-borneol ($EC_{50} \sim 1573$ μM; 95% CI: 963.6–2569; n_H: 1.79 ± 0.35), (−)-borneol ($EC_{50} \sim 2179$ μM; 95% CI: 467.7–10149; n_H: 1.34 ± 0.46), isoborneol ($EC_{50} \sim 1139$ μM; 95% CI: 827–1569; n_H 2.58 ± 0.80) and (−)-bornyl acetate ($EC_{50} \sim 458.8$ μM (95% CI: 263.1–891.4); n_H: 2.17 ± 1.34), all of which produced non-linear regression with good fit. While camphor produced a very small direct action (1.6%), no significant non-linear relationship was found, and hence no meaningful dose–response curve could be constructed. The concentrations required for direct modulation were notably higher than those required for GABA-enhancement, although the general order of compound potency was maintained, with (+)-borneol and (−)-borneol producing the greatest effect. None of the tested compounds, including (+)-borneol, produced any effect at uninjected or water-injected oocytes, with or without GABA.

4. Discussion

Many studies have shown that traditional herbal extracts have GABAergic effects, such as sedation and anxiolysis. Furthermore, a range of reports have shown that some monoterpenes present within the essential oils of such herbal medicines have modulatory activity with GABA at several $GABA_A$ receptor subtypes, but these monoterpenes had not been tested at $\alpha_1\beta_2\gamma_{2L}$ receptors, thought to comprise 40% of GABA receptors in the mammalian CNS [20]. The current study demonstrates that a number of bicyclic monoterpenes, particularly (+)- and (−)-borneol are highly efficacious as positive modulators the effects of low GABA concentrations at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

(+)- And (−)-borneol produced the greatest enhancement of GABA throughout the GABA concentration range, although the enantiomers had different patterns of efficacy. The difference in response between the enantiomers was greatly increased when exceptionally low ($EC_{1–4}$) or high (EC_{100}) GABA concentrations were used, where (+)-borneol produced greater enhancement compared to (−)-borneol at extremely low ($EC_{1–4}$) and extremely high (EC_{100}) GABA concentrations. (−)-Borneol produced greater potentiation at moderately low GABA concentrations, although the difference between the activity of the two enantiomers was less marked at these GABA dose ranges. As shown in Fig. 1, (+)- and (−)-borneol possess hydroxyl substituents, which are oriented in the opposite direction to the geminal dimethyl bridge. Isoborneol, possessing the same functional groups although its hydroxyl group is oriented in the same direction as its bridge, consistently produced an intermediate effect between the borneol enantiomers and with (−)-bornyl acetate and camphor. Both (−)-bornyl acetate and camphor possess the same structure as borneol, but with an acetyl and ketone group instead of borneol's hydroxyl substituent, respectively. Camphor consistently produced little to no modulatory effect or alone, with (−)-bornyl acetate producing a slightly greater effect in both cases. The order of efficacy within tested compounds was well maintained throughout all GABA concentrations used, indicating that the presence and orientation of the

(+)- and (−)-borneol hydroxyl group heightens the response to these compounds.

The appearance of inhibition with maximal GABA (EC_{100}) in the response to (−)-borneol indicates that it acts as a partial agonist as does isoborneol. (+)-, (−)-Borneol and isoborneol at high concentrations produced greater than 50% of the maximal GABA response in the absence of GABA, while (−)-bornyl acetate produced a very mild GABA-mimetic effect, and camphor produced no effect at $\alpha_1\beta_2\gamma_{2L}$ receptors in the absence of GABA. These results maintain the order observed with GABA-modulation. Previous tests of monoterpenes at $GABA_A$ receptors had not reported GABA-mimetic responses. While the results suggest that (+)-borneol produces a significant direct effect, Fig. 9 shows that an additive effect of the responses to (+)-borneol and GABA separately could not account for the responses seen when (+)-borneol was combined with low GABA concentrations, which produced vastly greater proportions of the maximal GABA response, indicating a modulatory effect. For example, the maximum direct activation seen with (+)-borneol was around 65% of the maximal GABA response. In the case of $EC_{5–14}$ GABA, a simple additive effect would yield 69–79% of the maximal GABA response. In fact what was observed was up to 100% of the maximal GABA response, i.e., the observed effects are not simply additive but represent positive modulation of the GABA response by borneol. Other GABA modulators have significant direct agonist activity at $GABA_A$ receptors, including the anaesthetics propofol (producing 48% of the maximal GABA response), etomidate (19%) and pentobarbitone (16%) [28].

The positive modulatory efficacy of (+)- and (−)-borneol is also comparable to that of other modulators at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Where (+)-borneol potentiated the $EC_{1–4}$ and $EC_{5–14}$ GABA responses by 1095% and 1251% potentiation, respectively, diazepam potentiation by 500% and 200% [29]. (+)-Borneol also produced 107% of the maximal GABA response at $\alpha_1\beta_2\gamma_{2L}$ receptors, statistically equivalent to the effects of pentobarbitone, etomidate and propofol, which all produce ∼127% of the maximal GABA response with EC_{10} GABA. The effects of (+)-borneol were also greater than those of neurosteroid 5α-pregnan-3α-ol-20-one, which produces 75% [28].

While the compounds tested here are less potent than known GABA modulators, they compare favorably in terms of potency and efficacy to previous electrophysiological studies examining monoterpenes at other $GABA_A$ receptors. At $\alpha_1\beta_1$ receptors, cineol, citral and eugenol all produce 2–3-fold enhancement of low dose GABA [30], where citral and eugenol are structurally markedly different from borneol, and cineol possesses the bicyclic backbone but with an oxygen ether, and not the hydroxyl substituent. Thymol possesses a hydroxyl substituent, but not the geminal dimethyl bridge and produces four-fold enhancement of low dose GABA at $\alpha_1\beta_1\gamma_{2S}$, $\alpha_6\beta_3\gamma_{2S}$ and $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$ receptors [31]. Similarly, the lower activity of camphor and (−)-bornyl acetate compared to (+)- and (−)-borneol may be further evidenced in support of the hydroxyl group's importance. The relatively rigid cage structure of these bicyclic monoterpenes may aid in a greater understanding of molecular aspects of positive modulation of the activation of $GABA_A$ receptors.

At least 11 distinct binding sites have been proposed to exist on the $GABA_A$ receptor [19]. So far, modulatory sites for GABA, benzodiazepines, barbiturates, steroids, picrotoxin, ethanol and certain enzymes have been explored as likely targets for the action of sedative herbs [21,32]. The direct actions of (+)-borneol were inhibited to some extent by competitive $GABA_A$ receptor antagonist bicuculline (76%) and mixed competitive/non-competitive antagonist picrotoxinin (60%). These results suggest that while (+)-borneol may act at their respective binding sites, additional sites may be involved in order for (+)-borneol to produce its direct and modulatory effects. As (+)-borneol was found to be insensitive to the benzodiazepine antagonist flumazenil, it is unlikely that it binds to the high-affinity benzodiazepine site. It may however bind at the low-affinity benzodiazepine site, as high concentrations of diazepam produce up to 800% potentiation of low GABA doses and this effect is flumazenil insensitive [29]. Similar results were seen with (+)-borneol, indicating it and related monoterpenes may bind at this benzodiazepine site. Another monoterpene, thymol, is also flumazenil-insensitive and potentiates currents induced by pentobarbital and propofol, indicating thymol does share the sites of action of these common $GABA_A$ receptor modulators [31]. (+)-Borneol and analogues, with similar structural and functional qualities, may possess a similar binding profile. In addition to these possibilities, an alcohol potentiation site has been proposed on the α_1/β_1 subunits, consisting of a hydrophilic region for hydroxyl group binding in a hydrophobic large region for binding of up to 10 carbon atoms [30], equivalent to the size of (+)-borneol and its analogues. This is also a possible candidate site for the action of monoterpene alcohols, like (+)- and (−)-borneol. None of the compounds tested here directly activated uninjected oocytes, indicating that these monoterpenes do not act at endogenous *X. laevis* oocyte receptors, including Ca^{2+}-activated chloride channels and muscarinic receptors. Radioligand binding studies may assist in determining (+)- and (−)-borneol's site of action.

Based on the activity found in this study, the concentrations of compounds required to modulate GABA receptors in animals or humans would be reasonably large. However, as large quantities of valerian root are used to make a standard extract, a typical valerian dose (2–3 g of dried herb in 200 mL water) contains up to 320 μM borneol, a dose greater than the EC_{50} concentrations found for (+)- and (−)-borneol mediated GABA-modulation in this paper. However, this concentration of (+)- or (−)-borneol is unlikely to reach the brain. While some in vitro studies

attribute the action of medicinal plants like valerian to their GABA content [16,23], GABA does not pass the blood brain barrier [22] and some studies indicate that GABA may be destroyed before it reaches CNS targets in vivo [33]. However, based on its structure, (−)-bornyl acetate may penetrate the blood brain barrier much more readily than (−)-borneol. It may be a pro-drug, being de-esterified to (−)-borneol in the brain in much the same way as heroin (diacetylmorphine) is de-esterified to morphine. (−)-Bornyl acetate (Fig. 1) comprises the largest percentage of the monoterpene component of valerian essential oil (up to 36%) [34]. Furthermore, it is the major constituent of *Centella asiatica*, the extract of which also has sedative and anti-depressant effects in humans, which have been attributed to its monoterpene content [35]. In mice, a racemic mixture of bornyl acetate produces a mild sedative effect when inhaled and is more effective than a racemic mixture of (+)- and (−)-borneol [25]. Isoborneol produces a significant increase in mouse locomotion, contrary to the predictions, which would be made from the results in this paper. The reason for this discrepancy is unclear, and further research is required to reconcile this discrepancy. It may be that isoborneol acts at additional receptors and receptor subunits, for example, camphor is a non-competitive nicotinic receptor antagonist [36].

In summary, the present study demonstrates that (+)- and (−)-borneol produce notable enhancement of the actions of GABA at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, as well as a moderate direct action at these same receptors. The activity of (+)- and (−)-borneol in comparison to their enantiomers indicates the presence and orientation of the hydroxyl group may be important for monoterpene activity at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The modulatory effects of (+)-borneol at low GABA concentrations were at least equivalent to that of anaesthetic etomidate and much greater than that of diazepam and 5α-pregnan-3α-ol-20-one. The efficacy of each enantiomer varied markedly with the GABA concentration present. These findings provide further evidence as to the GABAergic mechanisms of components within the essential oils of sedative medicinal plants such as *V. officinalis*, particularly bicyclic monoterpenes. While naturally-occurring flavonoids are known to have diverse actions on $GABA_A$ receptors [36], the actions of terpenoids such as (+)-borneol may also contribute to the effects of herbal extracts on $GABA_A$ receptors.

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References

[1] Broitman SA, Wilkinson IV J, Cerda S and Branch SK. Effects of monoterpenes and mevinolin on murine colon tumor CT-26 in vitro and its hepatic ‘‘metastases" in vivo. In: Research AIfC, editors. Dietary phytochemicals in cancer prevention and treatment. London: Plenum Press, 1995. pp. 111-30401

[2] Gherlardini C, Galeotti N, Mazzanti G. Local anaesthetic activity of monoterpenes and phenylpropanes of essential oils. Planta Med 2001; 67:564–6.

[3] Harney JW, Barofsky IM, Leary JD. Behavioral and toxicological studies of cyclopentanoid monoterpenes from *Nepeta cataria*. Lloydia 1978;41:367–74.

[4] Mühlbauer RC, Lozano A, Palacio S, Reinli A, Felix R. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. Bone 2003;32:372–80.

[5] Pengelly A. The constituents of medicinal plants: An introduction to the chemistry & therapeutics of herbal medicines. Muswellbrook, NSW, Australia: Sunflower Herbals, 1997.

[6] Santos FA, Rao VSN. Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytother Res 2000;14:240–4.

[7] Buchbauer G. Lavender oil and its therapeutic properties. In: Lis-Balchin M, editor. Lavender the genus ceitaliclavandula/ceitalic, vol. 29. London: Taylor & Francis; 2002. p. 124–39.

[8] Forster HB, Niklas H, Lutz S. Antispasmodic effects of some medicinal plants. Planta Med 1980;40:309–19.

[9] Cupp MJ. Chamomile. In: Cupp MJ, editor. Toxicology and clinical pharmacology of herbal products. Totowa, NJ: Humana Press; 2000 p. 79–83.

[10] Gyllenhall C, Merritt SL, Peterson SD, Block KI, Gochenour T. Efficacy and safety of herbal stimulants and sedatives in sleep disorders. Sleep Med Rev 2000;4:229–51.

[11] Assemi M. Herbs affecting the central nervous system: Gingko, kava, St. John’s wort, and valerian. Clin Obstet Gynecol 2001;44: 824–35.

[12] Houghton PJ. The scientific basis for the reputed activity of valerian. J Pharm Pharmacol 1999;51:505–12.

[13] Hattori A. Camphor in the Edo era—camphor and borneol for medicines. Yakushigaku Zasshi 2000;35:49–54.

[14] Cavadas C, Araujo I, Cotrim MD, Amaral T, Cunha AP, Macedo T, et al. In vitro study on the interaction of *Valeriana officinalis* L. extracts and their amino acids on $GABA_A$ receptors in rat brain. Arzneim-Forsch/Drug Res 1995;45:753–5.

[15] Ebadi MS. Pharmacodynamic basis of herbal medicine. Boca Raton, FL: CRC Press, 2002.

[16] Ferreira F, Santos Ms. Faro C, Pires E, Macedo T. Effect of extracts of *Valeriana officinalis* on (^{3}H)GABA-release in synaptosomes: further evidence for the involvement of free GABA in the Valerian-induced release. Rev Portueguesa Farm 1996;46:74–7.

[17] Mennini T, Bernasconi P, Bombardelli E, Morazzoni P. In vitro study on the interaction of extracts and pure compounds from *Valeriana officinalis* roots with GABA, benzodiazepine and barbiturate receptors in the rat brain. Fitoterapia 1993;64:291–300.

[18] Santos Ms, Ferreira F, Cunha AP, Carvalho AP, Macedo T. An aqueous extract of valerian influences the transport of GABA in synaptosomes. Planta Med 1994;60:278–9.

[19] Johnston GAR. $GABA_A$ receptor pharmacology. Pharmacol Ther 1996;69:173–98.

[20] McKernan RM, Whiting PJ. Which $GABA_A$-receptor subtypes really occur in the brain?. Trends Neurosci 1996;19:139–43.

[21] Chebib M, Johnston GAR. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. J Med Chem 2000; 43:1427–47.

[22] Ortiz JG, Nieves-Natal J, Chavez P. Effects of *Valeriana officinalis* extracts on [^{3}H]Flunitrazepam binding, synaptosomal [^{3}H]GABA

uptake, and hippocampal [^{3}H]GABA release. Neurochem Res 1999; 24:1373–8.

[23] Santos MS, Ferreira F, Faro C, Pires E, Carvalho AP, Cunha AP, et al. The amount of GABA present in aqueous extracts of Valerian is sufficient to account for [^{3}H]GABA release in synaptosomes. Planta Med 1994;60:475–6.

[24] Aoshima H, Hamamoto K. Potentiation of $GABA_A$ receptors expressed in *Xenopus oocytes* by perfume and phytoncid. Biosci Biotech Biochem 1999;63:743–8.

[25] Buchbauer G, Jirovetz L, Jager W, Plank C, Dietrich H. Fragrance compounds and essential oils with sedative effects upon inhalation. J Pharm Sci 1993;82:660–4.

[26] Huang SH, Duke RK, Chebib M, Sasaki K, Wada K, Johnston GAR. Bilobalide, a sesquiterpene trilactone from *Ginkgo biloba*, is an antagonist at recombinant $GABA_A$ receptors. Eur J Pharmacol 2003;464:1–8.

[27] Mihic SJ, Sanna E, Whiting PJ, Harris RA. Pharmacology of recombinant $GABA_A$ receptors. In: Biggio G, Sanna E, Costa E, editors. GABAceinfA/ceinf receptors and anxiety from neurobiology to treatment, vol. 48. New York: Raven Press; 1995. p. 17–39.

[28] Hill-Venning C, Belelli D, Peters JA, Lambert JJ. Subunit-dependent interaction of the general anaesthetic etomidate with the γ-aminobutyric acid type A receptor. Brit J Pharmacol 1997; 120:749–56.

[29] Walters RJ, Hadley SH, Morris KDW. Amin J. Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms. Nat Neurosci 2000;3:1274–81.

[30] Aoshima H, Hossain SJ, Hamamoto K, Yokoyama M, Yamada M, Shingai R. Kinetic analyses of alcohol-induced potentiation of the response of $GABA_A$ receptors composed of α_1 and β_2 subunits. J Biochem 2001;130:703–9.

[31] Priestly CM, Williamson EM, Wafford KA, Sattelle DB. 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*. Brit J Pharmacol 2003;140:1363–72.

[32] Gottesmann C. GABA mechanisms and sleep. Neuroscience 2002; 111:231–9.

[33] Bruneton J. Pharmacognosy, phytochemistry, medicinal plants. Paris, France: Intercept, 1995.

[34] Bos R, Woerdenbag HJ, Hendriks H, Scheffer JJC. Composition of the essential oils from underground parts of *Valeriana officinalis* L. *s.l.* and several closely related taxa. Flavor Frag J 1997;12:359–70.

[35] Howes MR, Houghton PJ. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol Biochem Behav 2003;75:513–27.

[36] Park TJ, Seo HK, Kang BJ, Kim KT. Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors. Biochem Pharmacol 2001;61:787–93.