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Curcumol allosterically modulates GABA(A) receptors in a manner distinct from benzodiazepines

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Yan-Mei Liu^{1,2,3,*}, Hui-Ran Fan^{1,2,3,*}, Jing Ding⁴, Chen Huang³, Shining Deng², Tailin Zhu², Tian-Le Xu³, Wei-Hong Ge¹, Wei-Guang Li^{2,3} & Fei Li²

Inhibitory A type γ -aminobutyric acid receptors (GABA_ARs) play a pivotal role in orchestrating various brain functions and represent an important molecular target in neurological and psychiatric diseases, necessitating the need for the discovery and development of novel modulators. Here, we show that a natural compound curcumol, acts as an allosteric enhancer of GABA_ARs in a manner distinct from benzodiazepines. Curcumol markedly facilitated GABA-activated currents and shifted the GABA concentration-response curve to the left in cultured hippocampal neurons. When co-applied with the classical benzodiazepine diazepam, curcumol further potentiated GABA-induced currents. In contrast, in the presence of a saturating concentration of menthol, a positive modulator for GABA_AR, curcumol failed to further enhance GABA-induced currents, suggesting shared mechanisms underlying these two agents on GABA_ARs. Moreover, the benzodiazepine antagonist flumazenil did not alter the enhancement of GABA response by curcumol and menthol, but abolished that by DZP. Finally, mutations at the β 2 or γ 2 subunit predominantly eliminated modulation of recombinant GABA_ARs by curcumol and menthol, or diazepam, respectively. Curcumol may therefore exert its actions on GABA_ARs at sites distinct from benzodiazepine sites. These findings shed light on the future development of new therapeutics drugs targeting GABA_ARs.

The γ -aminobutyric acid (GABA) system is essential for the orchestration of local networks and the functional interaction between different brain regions¹. As major executors in the GABAergic system, A-type GABA receptors (GABA_ARs) are pentameric protein complexes that form Cl⁻-permeable ion channels that are widely distributed across the central nervous system, and primarily confer fast inhibitory control over neural activity, thus participating in almost every aspect of physiological and pathophysiological brain function². GABA_ARs are made up of 19 known subunits (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π , and ρ 1–3), and many contain two α subunits, two β subunits, and one γ subunit³. Despite the large repertoire resulting from various combinations of these subunits, the main subunit configuration is α 1- β 2- γ 2, at a ratio of 2:2:1, constituting approximately 60% of all GABA_ARs in the brain⁴. There are two GABA-binding sites⁵, formed at two interfaces between α and β subunits. By contrast, the binding site⁶ for benzodiazepines⁷ is formed by one of the α subunits^{6,8,9} and the γ subunit^{6,10–12}. The benzodiazepine as a broad spectrum of positive allosteric modulators of the GABA_AR has been in clinical use for decades and is still among the most widely prescribed drugs for the treatment of insomnia and anxiety disorders.

The clinical use of classical benzodiazepines is limited by their side effects⁷ and the risk of drug dependence^{13,14}. Identification of receptor subtype-selective compounds, and the discovery of novel modulators beyond benzodiazepines, are necessary to overcome these limitations. Indeed, GABA_ARs are also major targets¹⁵ for barbiturates¹⁶, steroids¹⁷, and anaesthetics^{18–26}, all of which are positive modulators. Moreover, given the increasing evidence that targeting GABA_ARs improves treatment in a broad range of neuropsychiatric disorders^{1,27,28}, continued efforts are necessary to discover or develop novel GABA_AR modulators, including agonists and antagonists²⁹.

¹Department of Chinese Materia Medica, College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, 310053, China. ²Department of Children and Adolescent Health Care, Ministry of Education-Shanghai Key Laboratory of Children's Environmental Health, Xin Hua Hospital Affiliated Shanghai Jiao Tong University School of Medicine, Shanghai, 200092, China. ³Collaborative Innovation Centre for Brain Science, and Department of Anatomy, Histology and Embryology, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025, China. ⁴Pharmacy Department, Affiliated Hospital of Taishan Medical University, Taishan 271000, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to W.-G.L. (email: wgli@shsmu.edu.cn) or F.L. (email: feili@shsmu.edu.cn)

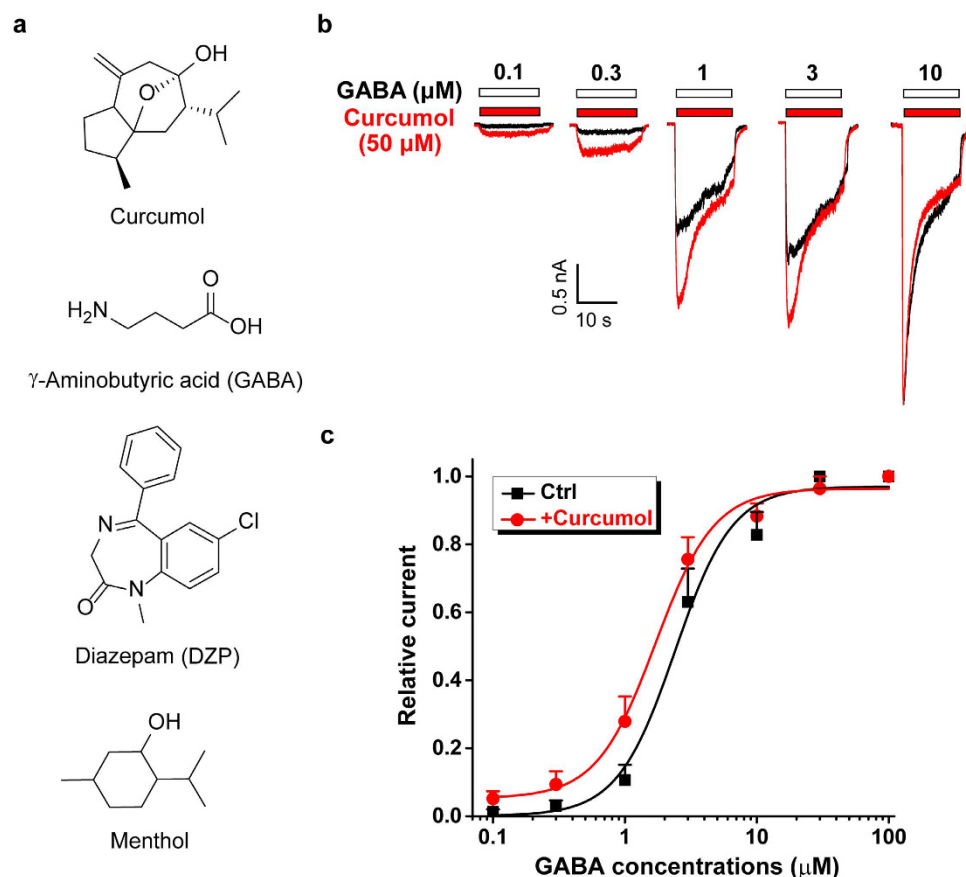


Figure 1. Modulation of GABA response by curcumol in cultured hippocampal neurons. (a) Chemical structures of curcumol and other GABA_AR ligands or modulators used in the present study. (b) Representative traces showing the currents evoked by different concentrations of GABA (black) alone, or curcumol (50 μ M) plus various concentrations of GABA (red) as indicated. (c) Concentration–response curves of GABA for currents evoked in the absence (black squares) or presence (red circles) of 50 μ M curcumol. Current amplitudes were normalized to the maximal response. These values were derived from previously published data³³, regraphed here in a different way to assess the effect of curcumol on GABA concentration–response curve. The EC₅₀ and Hill coefficient values were $2.4 \pm 0.4 \mu$ M, 2.0 ± 0.6 without curcumol and $1.7 \pm 0.2 \mu$ M, 1.9 ± 0.3 with curcumol, respectively. $n = 6$ each group.

Natural compounds isolated from plants are a rich source of novel GABA_AR ligands. Some natural flavonoids, first isolated from plants used as tranquilizers in folkloric medicine, together with their synthetic derivatives, possess selective affinity for the benzodiazepine-binding site of GABA_ARs with a broad spectrum of central nervous system effects³⁰. In addition, a few natural terpenoids containing ether^{31,32} or hydroxyl groups^{33–35} have been identified as positive modulators of GABA_ARs (Fig. 1a), potentiating GABAergic transmission^{33,36} and thereby suppressing aberrant excitability as seen during epileptiform activity^{33,37}. Two compounds isolated from the Chinese medicinal herb *Acorus gramineus*, α - and β -asarone (1-propenyl-2,4,5-methoxybenzol)^{31,32}, act on endogenous and recombinant GABA_ARs, activating the receptor and alleviating epileptic seizures. The widely-used cooling and flavouring agent menthol (5-methyl-2-propan-2-ylcyclohexan-1-ol, Fig. 1a), the best-known monoterpene extracted from the essential oil of the genus *Mentha* of the Lamiaceae family, suppresses hippocampal neuronal excitation and epileptic activity by enhancing GABAergic inhibition³⁷. Menthol also enhances GABA_AR-mediated currents in midbrain periaqueductal grey neurons³⁶, suggesting a broader spectrum of GABA_AR-related pharmacotherapy in future, using menthol and related compounds. Interestingly, menthol has an alike general anaesthetic activity and similar sites of action on the GABA_ARs to the intravenous agent propofol (2,6-di-isopropylphenol), but not to benzodiazepines, steroids or barbiturates³⁴. Curcumol³⁸ [(3S,5S,6S,8aS)-(3-methyl-8-methylidene-5-(propan-2-yl)octahydro-6H-3a,6-epoxyazulen-6-ol)] is a sesquiterpene compound and a major bioactive component of *Rhizoma Curcumae* oil. Notably, it induces minimal activation of GABA_ARs on its own, but facilitates the GABA-activated current in hippocampal neurons and cell lines, which express endogenous and recombinant GABA_ARs³³, respectively. As a result, curcumol suppresses basal and epileptic activity in animals³³, strengthening its pharmacological efficacy as a novel allosteric GABA_AR modulator. However, the molecular mechanisms underlying curcumol modulation on GABA_ARs remain to be established. By comparing the electrophysiological effects of curcumol with other known modulators, and performing mutagenesis analysis on recombinant

GABA_ARs, here we identify that curcuminol as an allosteric modulator of GABA_ARs in a manner distinct from benzodiazepines, but through sites shared with menthol.

Results

Characterization of curcuminol on the GABA concentration-response curve in hippocampal neurons. A previous study³³ showed that curcuminol (Fig. 1a), a bioactive component of *Rhizoma Curcumae* oil^{39–41}, enhanced GABA response in a concentration-dependent manner. In that study³³, we established that at the agonist (i.e. GABA) concentration of 1 μ M, curcuminol facilitated the GABA-induced current with an EC₅₀ of $34.4 \pm 2.9 \mu$ M. To make an obvious and significant effect of curcuminol on GABA_ARs, we chose 50 μ M as the effective concentration in the present study.

We assessed the effects of curcuminol on GABA concentration-response curve in hippocampal neurons by re-examination of the effect of 50 μ M curcuminol on the currents induced by a wide range of GABA concentrations shown in the previous study³³. In contrast to the previous purpose to identify the operational range of GABA concentrations by curcuminol³³, here we perform data re-analysis to generate the concentration-response curves of GABA in the absence and presence of curcuminol. As shown in Fig. 1b,c, the concentration-response curves to GABA were shifted to the left by curcuminol. The EC₅₀ (the agonist concentration that induces the half-maximal response) values in the absence and presence of curcuminol were $2.4 \pm 0.4 \mu$ M and $1.7 \pm 0.2 \mu$ M, respectively. Mechanistically, the 1 μ M GABA used in the following study falls an approximate EC₁₀ and EC₃₀ (the agonist concentrations that give rise to the 10 and 30% of maximal response, respectively) concentration of GABA, in the absence and presence of curcuminol, respectively (Fig. 1c). Meanwhile, the Hill coefficients in the absence or presence of curcuminol were 2.0 ± 0.6 and 1.9 ± 0.3 , respectively. This increase of the apparent affinity to GABA implies a potentially allosteric regulation by curcuminol of GABA-mediated GABA_AR response; however, the precise mechanisms underlying the action of curcuminol on GABA_ARs remain not fully understood.

Interplay of curcuminol and diazepam on GABA-activated currents in hippocampal neurons. To decipher the underlying mechanisms of curcuminol on GABA_ARs, we sought to determine the potential interaction between curcuminol and other known GABA_AR modulators, such as the classical benzodiazepine, diazepam (DZP, Fig. 1a). Cultured hippocampal neurons were exposed to GABA, DZP, and curcuminol, alone or combination with each other (Fig. 2). DZP (1 μ M) alone induced negligible inward currents but significantly potentiated GABA (1 μ M)-evoked currents (Fig. 2a,b), consistent with its allosteric modulatory nature⁷. Likewise, curcuminol (50 μ M) produced minimal currents on its own but substantially enhanced GABA (1 μ M)-induced currents (Fig. 2a,b), consistent with the previous observation³³. We also compared the enhancement of GABA-activated currents by DZP or curcuminol (i.e. $I_{DZP+GABA}$ and $I_{Curcuminol+GABA}$, respectively) with the sum of the independent currents induced by GABA (I_{GABA}) and DZP (I_{DZP}) or curcuminol ($I_{Curcuminol}$), and found that the potentiation of GABA-mediated currents by DZP or curcuminol was more than additive (Fig. 2c,d). This confirmed that curcuminol, like DZP, allosterically potentiates the GABA_AR activation in hippocampal neurons.

Interestingly, curcuminol further increased the current induced by the combination of GABA and DZP (Fig. 2a,b), and the increase ($I_{DZP+Curcuminol+GABA}$) was more than additive ($I_{DZP+GABA} + I_{Curcuminol}$; Fig. 2e), supporting the notion that curcuminol causes an additional enhancement of the DZP-potentiated GABA_AR activation. Consistent with this, DZP also led to a further increase in the current induced by the combination of GABA and curcuminol (Fig. 2a,b), and the increase ($I_{DZP+Curcuminol+GABA}$) was more than additive ($I_{Curcuminol+GABA} + I_{DZP}$; Fig. 2e). Thus, GABA, DZP, and curcuminol act together to facilitate the GABA_AR activation in hippocampal neurons. This suggests that curcuminol, as a positive allosteric modulator of GABA_ARs, likely acts at a site distinct from the benzodiazepine-binding site.

Interplay between curcuminol and menthol on GABA-activated currents in hippocampal neurons.

To understand in more depth molecular mechanisms underlying curcuminol modulation of GABA_ARs, we further investigated the interplay of curcuminol and menthol^{34,37}, both belonging to terpenoid compounds carrying hydroxyl groups (Fig. 1a). Menthol at lower concentrations (up to 100 μ M) did not activate a tangible inward current ($I_{Men} = 0$; data not shown), but significantly potentiated GABA (1 μ M)-evoked currents (Fig. 3a,b), consistent with the previous observation³⁷. Similarly, in an independent set of experiments from that shown in Fig. 2, curcuminol (50 μ M) significantly enhanced the GABA (1 μ M)-induced currents (Fig. 3a,b), and the compound current ($I_{Curcuminol+GABA}$) was more than additive ($I_{Curcuminol} + I_{GABA}$; Fig. 3c). Interestingly, curcuminol-mediated enhancement ($I_{Curcuminol+GABA}$) occluded the further action of menthol (100 μ M) ($I_{Men+Curcuminol+GABA}$; Fig. 3a,b), with menthol unable to improve the current ($I_{Men+Curcuminol+GABA}$) to more than that induced by GABA and curcuminol ($I_{Curcuminol+GABA}$). Conversely, the compound current ($I_{Men+GABA+Curcuminol}$) amplitude to the combination of GABA, curcuminol, and menthol (100 μ M) was much higher than that of GABA and menthol ($I_{Men+GABA}$) (Fig. 3a,b) and, again, more than additive ($I_{Men+GABA} + I_{Curcuminol}$; Fig. 3d). These observations, in contrast to the non-overlapping effects between curcuminol and DZP (1 μ M) (Fig. 2), raise the possibility that curcuminol has a similar mechanism to menthol but not DZP, and that curcuminol holds a much higher efficacy than menthol (100 μ M). Curcuminol would thereby occlude further action of menthol, but would have no similar effects on the modulation by DZP at GABA_ARs (Fig. 2).

To characterize the interplay between curcuminol and menthol more comprehensively, we increased the concentration of menthol up to 3 mM. Menthol (3 mM) alone activated a significant inward current (Fig. 3a, referred to as I_{MEN}) that was blocked by a selective GABA_AR inhibitor, bicuculline methiodide (1 μ M), (data not shown)³⁷, and enhanced by curcuminol (Fig. 3a,b). Moreover, co-application of menthol (3 mM) and GABA enhanced GABA_AR activation (Fig. 3a,b) in a more than additive manner ($I_{MEN+GABA} > I_{MEN} + I_{GABA}$; Fig. 3e). In the simultaneous presence of curcuminol and menthol (3 mM) with GABA, although the overall current ($I_{MEN+Curcuminol+GABA}$) was significantly greater than that induced by GABA and curcuminol ($I_{Curcuminol+GABA}$), there was no difference

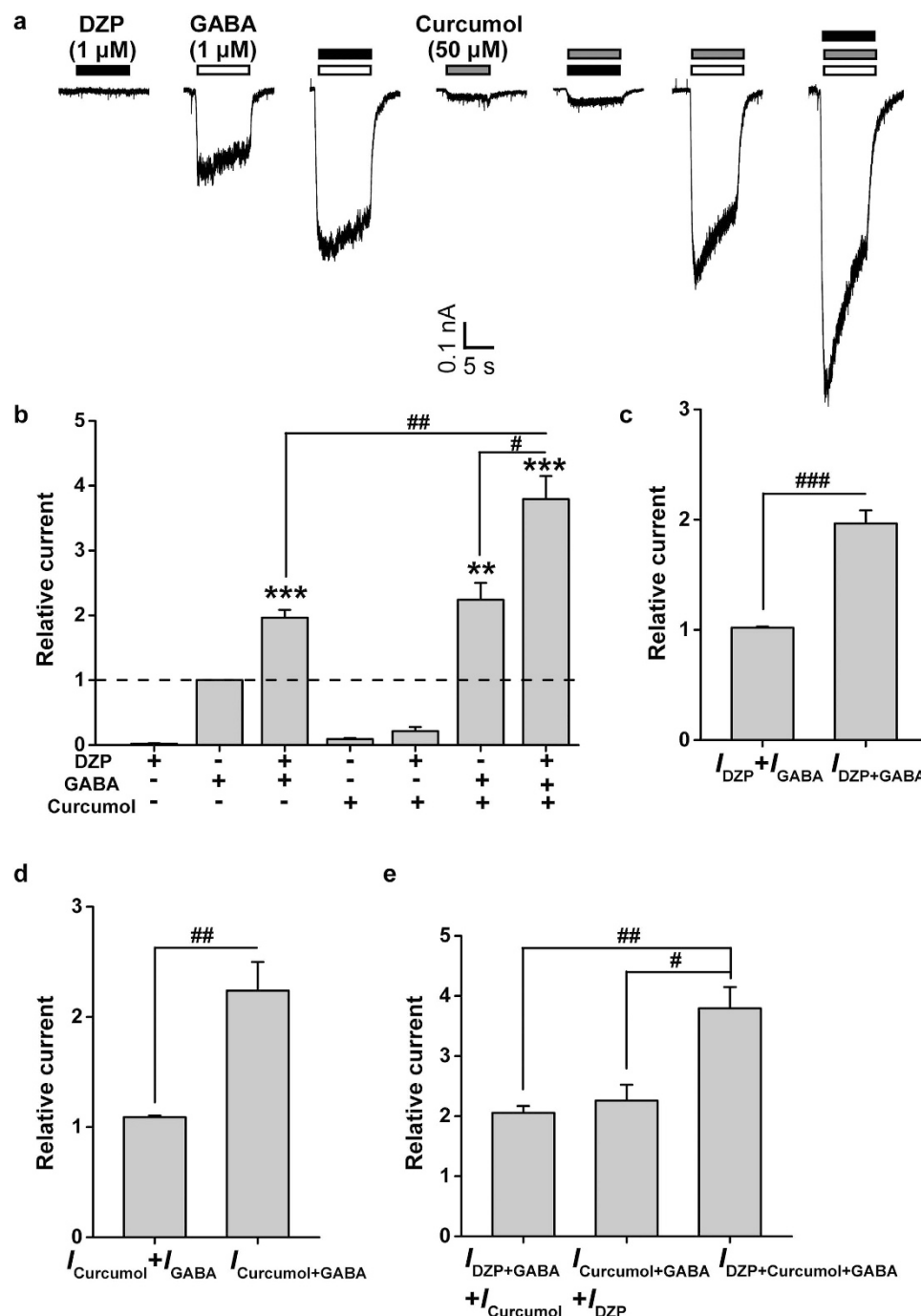


Figure 2. Interplay between diazepam (DZP) and curcumol on GABA-induced currents in cultured hippocampal neurons. (a) Representative traces of GABA (1 μ M)-induced currents in the absence or presence of DZP (1 μ M) or curcumol (50 μ M). (b) Pooled data from (a). (c–e) Histograms showing relative I_{DZP} , I_{GABA} , $I_{DZP+GABA}$, $I_{Curcumol}$, $I_{Curcumol+GABA}$, and $I_{DZP+Curcumol+GABA}$. $I_{Curcumol}$, curcumol-activated current; $I_{Curcumol+GABA}$, current activated by curcumol and GABA; I_{DZP} , diazepam-activated current; $I_{DZP+GABA}$, current activated by DZP and GABA; $I_{DZP+Curcumol+GABA}$, current activated by DZP, curcumol and GABA; I_{GABA} , GABA-activated current. Data represent peak current amplitude normalized to that induced by GABA (1 μ M) alone (dashed line), $n = 6$ each group. ** $P < 0.01$, *** $P < 0.001$, compared with the current induced by GABA alone (dashed line); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared as indicated, paired Student's t -test.

between $I_{MEN+Curcumol+GABA}$ and $I_{MEN+GABA}$ (Fig. 3a,b). This shows that curcumol did not further increase the current induced by GABA and menthol (3 mM) together. In addition, the overall current induced by GABA, curcumol, and menthol (3 mM) did not differ from the sum of $I_{MEN+GABA} + I_{Curcumol}$, or $I_{Curcumol+GABA} + I_{MEN}$ (Fig. 3f). Namely, menthol at higher concentrations saturates an allosteric site for GABA_A modulation and more likely

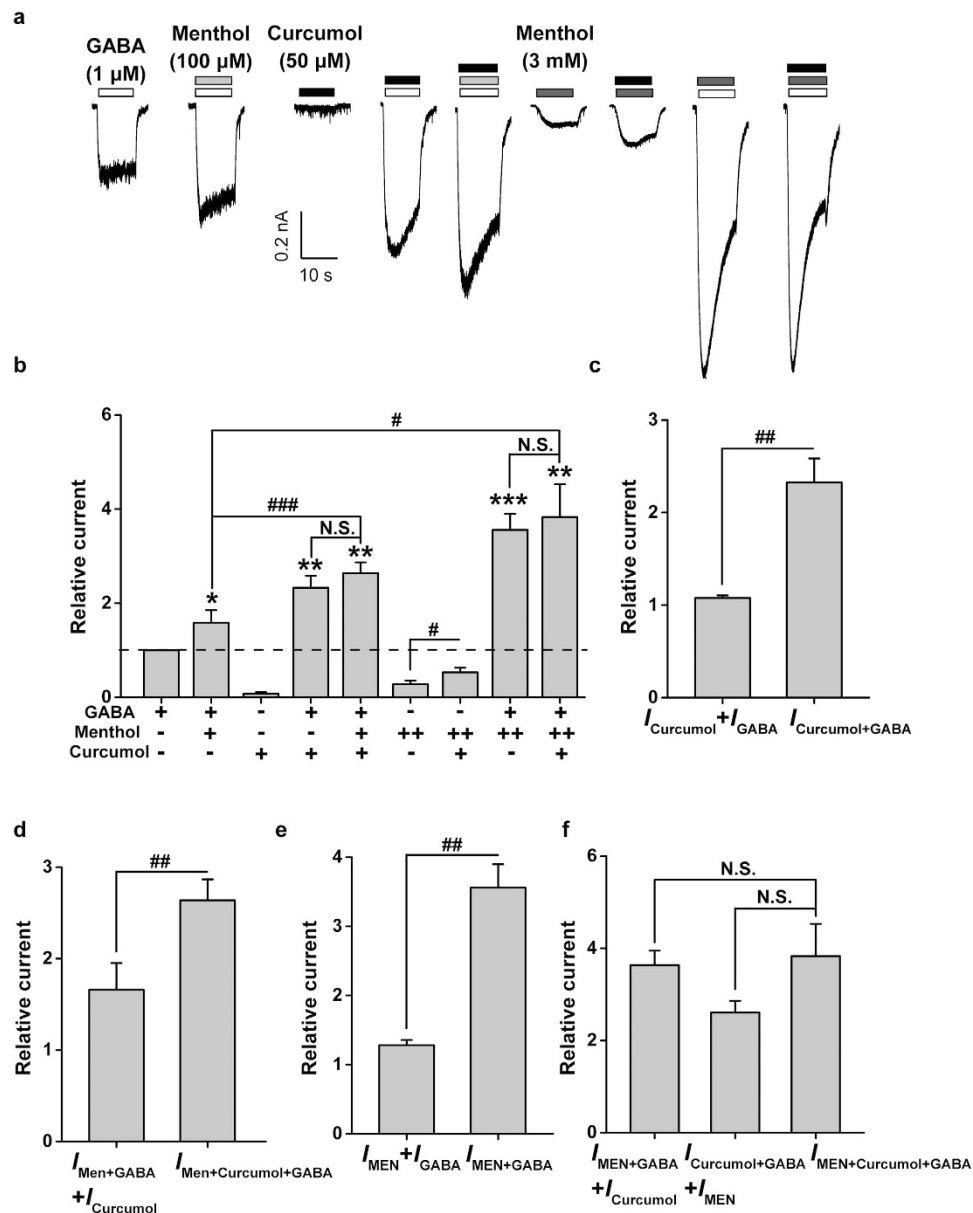


Figure 3. Interplay between menthol and curcuminol on GABA-induced currents in cultured hippocampal neurons. (a) Representative current traces induced by GABA (1 μ M) in the absence or presence of menthol (100 μ M or 3 mM) or curcuminol (50 μ M). (b) Pooled data from (a). Menthol: (+), 100 μ M; (++) , 3 mM. (c–f) Histograms showing relative $I_{Curcuminol}$, I_{GABA} , $I_{Curcuminol+GABA}$, $I_{Men+GABA}$, $I_{Men+Curcuminol+GABA}$, I_{MEN} , $I_{MEN+GABA}$, and $I_{MEN+Curcuminol+GABA}$. $I_{Curcuminol}$, curcuminol-activated current; $I_{Curcuminol+GABA}$, curcuminol plus GABA-activated current; I_{GABA} , GABA-activated current; $I_{Men+GABA}$, menthol (100 μ M) plus GABA-activated current; $I_{Men+Curcuminol+GABA}$, menthol (100 μ M), curcuminol, plus GABA-activated current; I_{MEN} , menthol (3 mM)-activated current; $I_{MEN+GABA}$, menthol (3 mM) plus GABA-activated current; $I_{MEN+Curcuminol+GABA}$, menthol (3 mM), curcuminol, plus GABA-activated current. Data represent peak current amplitude normalized to that induced by GABA (1 μ M) alone (dashed line). n = 5 each group. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with the current induced by GABA alone (dashed line); N.S., not significant, # P < 0.05, ## P < 0.01, ### P < 0.001, compared as indicated, paired Student's t -test.

precludes further action by curcuminol. This suggests that curcuminol and menthol share similar binding sites on GABA_ARs for modulation.

Actions of GABA-activated currents by curcuminol and menthol, but not DZP, are resistant to benzodiazepine antagonist in hippocampal neurons. To underline the differential interplay between curcuminol and menthol or DZP, we then examined whether actions of the above compounds were differentially affected by flumazenil (1 μ M), a benzodiazepine antagonist. When flumazenil (1 μ M) was coapplied with curcuminol and GABA (Fig. 4a), curcuminol still enhanced the GABA-induced current to a comparable extent

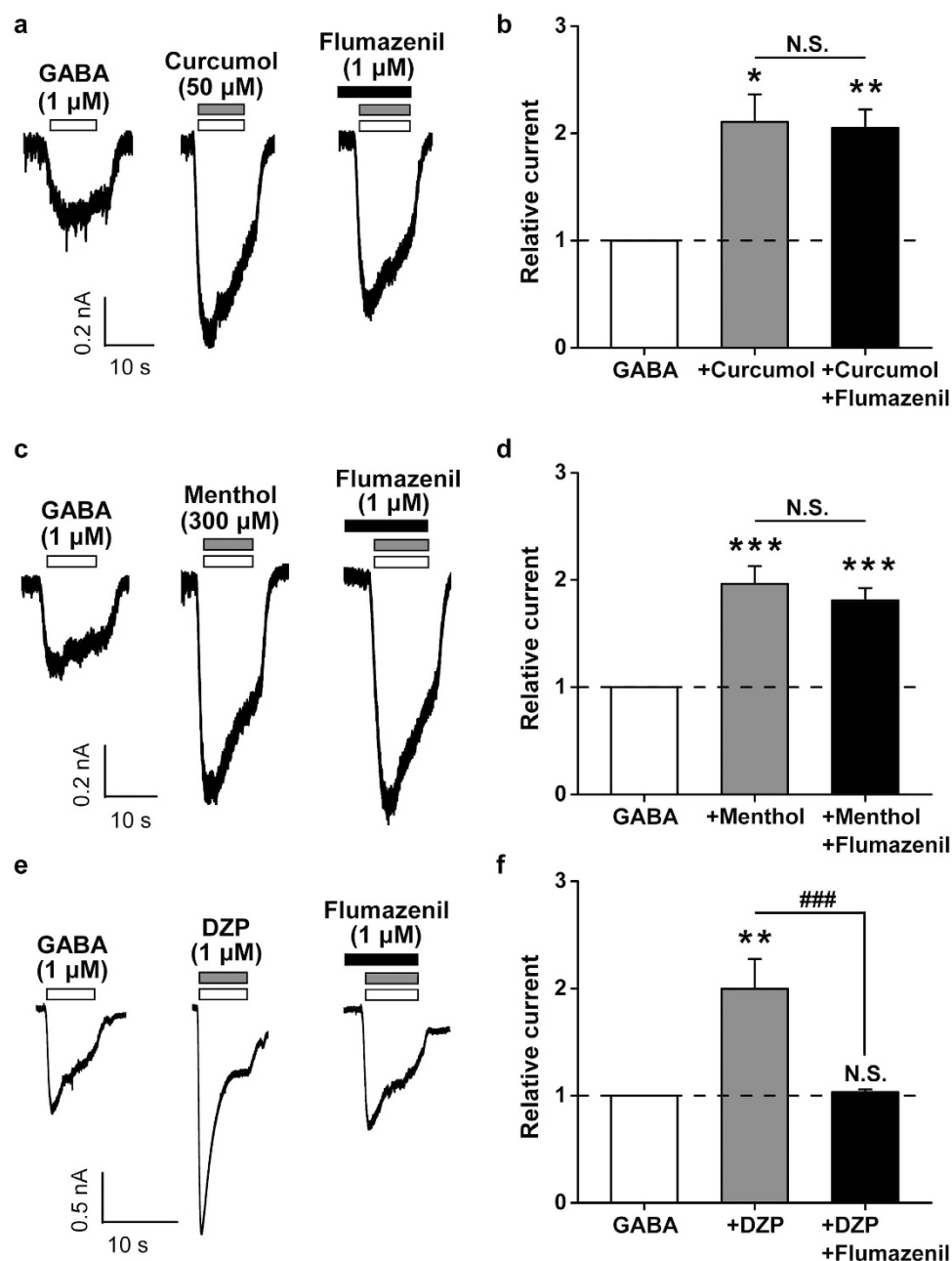


Figure 4. Effects of flumazenil on the modulation of GABA response by curcumin, menthol, or DZP in cultured hippocampal neurons. (a, c, e) Representative current traces induced by GABA (1 μ M) alone, or in the absence or presence of curcumin (100 μ M, a), or menthol (300 μ M, c), or DZP (1 μ M, e), or in the simultaneous presence of flumazenil (1 μ M). (b, d, f) Pooled data from (a), (c) and (e), respectively. Data represent peak current amplitude normalized to that induced by GABA (1 μ M) alone (dashed line). $n = 5-13$ each group. N.S., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with GABA (1 μ M) alone (dashed line), paired Student's t -test; N.S., not significant, ### $P < 0.001$, compared as indicated, unpaired Student's t -test.

($210.6 \pm 25.7\%$ vs. $204.9 \pm 17.4\%$ of GABA-induced currents by curcumin in the absence and presence of flumazenil, respectively, $n = 5-6$ per group, $P > 0.05$, Fig. 4b). Likewise, the effect of menthol was also not altered by flumazenil ($196.3 \pm 16.5\%$ vs. $180.8 \pm 11.7\%$ of GABA-induced currents in the absence and presence of flumazenil, respectively, $n = 10-13$ per group, $P > 0.05$, Fig. 4c,d), which was consistent the previous study performed on *Xenopus* oocytes expressing the $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ subtype of GABA_AR³⁴. By contrast, in the presence of flumazenil, DZP failed to enhance the GABA-induced current in hippocampal neurons ($199.8 \pm 27.6\%$ vs. $103.4 \pm 2.5\%$ of GABA-induced currents by DZP in the absence and presence of flumazenil, respectively, $n = 10$ per group, $P < 0.01$, Fig. 4e,f), verifying flumazenil as a benzodiazepine antagonist. Together, these results strengthen the notion that curcumin and menthol do not share sites of action with benzodiazepines on GABA_ARs.

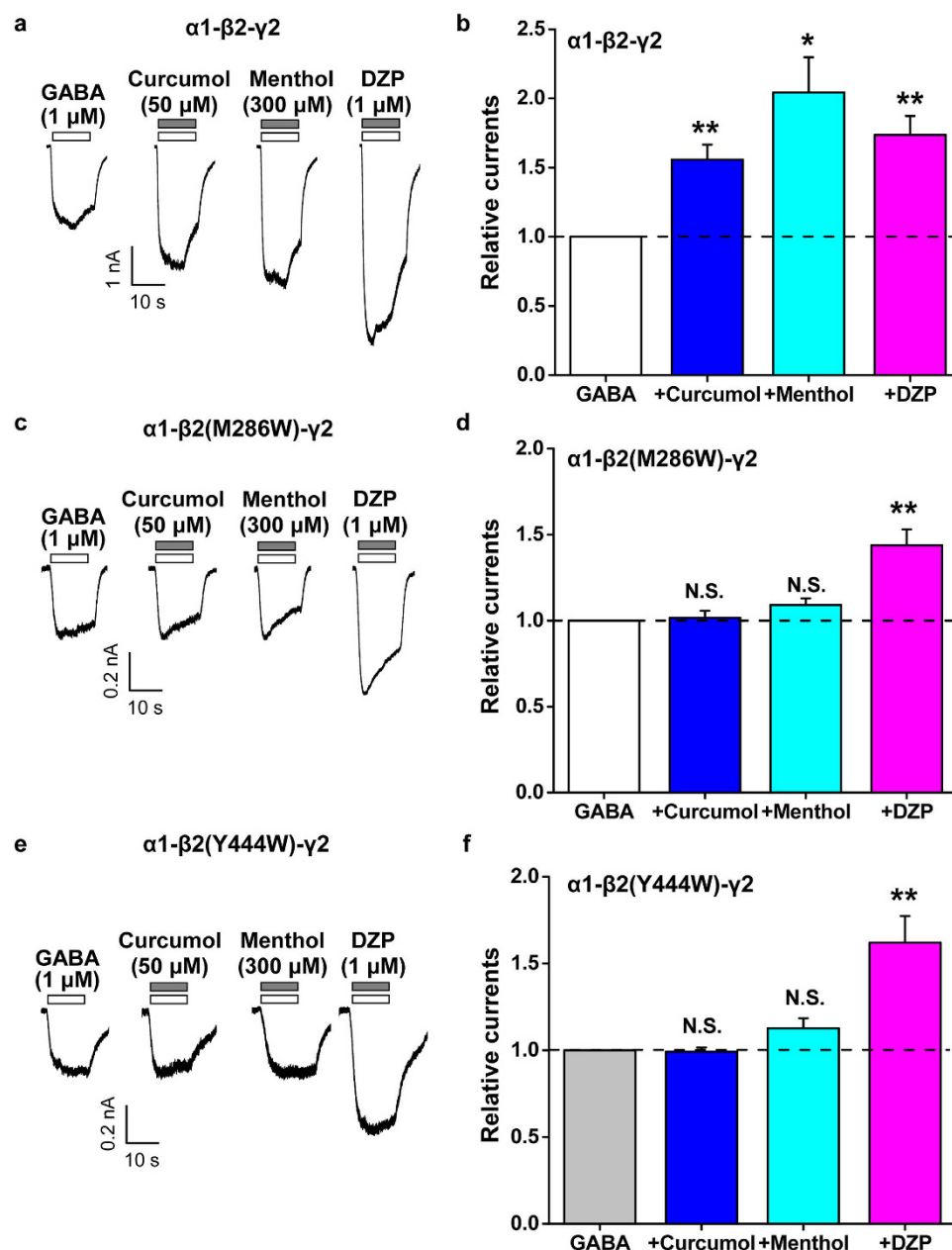


Figure 5. Effects of point mutations in $\beta 2$ subunit of GABA_A R on the modulation of $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ GABA_A R subtype by curcumin, menthol, or DZP. (a, c, e) Representative current traces induced by GABA ($1\text{ }\mu\text{M}$) in the absence or presence of curcumin ($50\text{ }\mu\text{M}$), menthol ($300\text{ }\mu\text{M}$), or DZP ($1\text{ }\mu\text{M}$) in HEK-293T cells that expressed $\alpha 1$, $\beta 2$, $\beta 2\text{-M286W}$, or $\beta 2\text{-Y444W}$, and $\gamma 2$ GABA_A subunits. (b, d, f) Pooled data from (a), (c) and (e), respectively. Data represent peak current amplitude normalized to that induced by GABA ($1\text{ }\mu\text{M}$) alone (dashed line). $n = 3\text{--}8$ each group. N.S., not significant, * $P < 0.05$, ** $P < 0.01$, compared with the current induced by GABA alone (dashed line), paired Student's t -test.

Curcumin shares site of action with menthol, but not DZP, on the $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ subtype of GABA_A R. To investigate binding sites for the modulatory action of curcumin over other known modulators on the GABA_A Rs (Fig. 1a), we turned to confirm the effects of curcumin, menthol, and DZP on recombinant GABA_A Rs expressed in HEK-293T cells. As the $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ subtype constitutes the largest proportion ($\sim 60\%$) of GABA_A Rs in the brain^{4,7} and is primarily responsible for phasic GABAergic inhibition in hippocampal CA1 pyramidal neurons, we therefore used this subtype firstly to examine the actions by different modulators. Curcumin ($50\text{ }\mu\text{M}$), or menthol ($300\text{ }\mu\text{M}$), or DZP ($1\text{ }\mu\text{M}$) each significantly enhanced currents induced by GABA ($1\text{ }\mu\text{M}$) on HEK-293T cells expressing wild-type (WT) $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ GABA_A Rs (Fig. 5a,b). This was analogous with the observation on the cultured hippocampal neurons shown above (Figs 1–4), and consistent with previous reports on the $\alpha 1\text{-}\beta 2\text{-}\gamma 2$ subtype of GABA_A Rs expressed in various recombinant systems^{8,33,34}. These results

therefore lay a foundation on which to examine the specific site(s) responsible for the modulation of GABA_ARs by curcuminol and other modulators.

It has been established that a methionine residue at amino acid position 286 [transmembrane domain (TM) 3] and a tyrosine residue at position 444 (TM4) at the $\beta 2$ subunit are important for the anaesthetic actions^{18–26}, including menthol³⁴, but not benzodiazepines, on the $\alpha 1$ - $\beta 2$ - $\gamma 2$ subtype of GABA_AR. Mutations at either one of these residues to a tryptophan (i.e. M286W or Y444W) both selectively abolished menthol-mediated enhancement of GABA_AR function. Given the structural similarity between curcuminol and menthol (both are terpenoid compounds carrying hydroxyl groups; Fig. 1a), in addition to previous identification of the interplay between curcuminol and menthol over DZP (Figs 2 and 3), we expected that these sites important for menthol would also be essential for the curcuminol action. To investigate this, we exposed these modulators (Fig. 1a) to HEK-293T cells expressing mutant [$\alpha 1$ - $\beta 2$ (M286W)- $\gamma 2$ or $\alpha 1$ - $\beta 2$ (Y444W)- $\gamma 2$] GABA_ARs. Previous studies suggested that the GABA concentration–response relationships (i.e. the agonist concentration that induces the half-maximal response, EC₅₀ and Hill coefficient) for both mutant receptors are similar to those for the WT GABA_AR^{19,20,34}. Therefore, GABA (1 μ M) was also used to screen for modulation by curcuminol (50 μ M), menthol (300 μ M), and DZP (1 μ M). We found no enhancement of either type of mutant receptor current by menthol (Fig. 5c–f), consistent with the previous study in *Xenopus* oocytes expressing these mutant receptors³⁴. Notably, the modulation by curcuminol was also abolished by inclusion of the mutations in the $\beta 2$ subunits (Fig. 5c–f). By contrast, the enhancement of mutant $\beta 2$ -M286W or $\beta 2$ -Y444W currents by DZP (Fig. 5c–f) was not significantly different from the WT $\alpha 1$ - $\beta 2$ - $\gamma 2$ GABA_AR (173.7 \pm 13.6%, 143.8 \pm 9.2%, and 162.0 \pm 15.3% of GABA-induced currents by DZP on the WT, $\beta 2$ -M286W, and $\beta 2$ -Y444W GABA_ARs, respectively, $n = 4–6$ per group, $P > 0.05$ vs. WT). These results were comparable with the previous report studied in *Xenopus* oocytes³⁴, which showed that flunitrazepam, another type of benzodiazepine, also reserved its allosterically modulatory effect. The lack of mutation effects on these sites to benzodiazepines³⁴ (Fig. 5c–f) agrees with a previous study showing that the α subunit adjacent to the $\gamma 2$ subunit determines the sensitivity to benzodiazepines in the recombinant receptors⁸. Together, these results collectively point to a notion that curcuminol is an allosteric modulator for GABA_ARs in a manner distinct from benzodiazepines.

Variant mechanisms underlying actions of curcuminol over menthol or DZP on the $\alpha 5$ - $\beta 2$ - $\gamma 2$ subtype of GABA_AR. Next, we extended the mechanistic study of curcuminol over menthol or DZP to another GABA_AR subtypes. While the $\alpha 1$ -containing GABA_ARs primarily govern the phasic GABAergic inhibition^{4,42}, the $\alpha 5$ -containing are the major isoforms underlying tonic inhibition^{43–45} in hippocampal neurons. Accordingly, the effects of curcuminol over menthol or DZP were examined on the HEK-293T cells expressing either WT or mutant $\alpha 5$ - $\beta 2$ - $\gamma 2$ GABA_ARs. As expected, curcuminol (50 μ M), or menthol (300 μ M), or DZP (1 μ M) each significantly potentiated the currents induced by GABA (1 μ M) on HEK-293T cells expressing WT $\alpha 5$ - $\beta 2$ - $\gamma 2$ GABA_ARs (Fig. 6a,b), all of which are similar with the $\alpha 1$ - $\beta 2$ - $\gamma 2$ subtype (Fig. 5a,b).

Then, we exposed curcuminol, menthol, and DZP, respectively, to HEK-293T cells expressing the mutant [$\alpha 5$ - $\beta 2$ (M286W)- $\gamma 2$ or $\alpha 5$ - $\beta 2$ (Y444W)- $\gamma 2$] GABA_ARs. In line with the $\alpha 1$ - $\beta 2$ - $\gamma 2$ subtype of GABA_AR (Fig. 5c–f), the modulation by curcuminol was also abolished by inclusion of either the M286W (Fig. 6c,d) or Y444W (Fig. 6e,f) mutations in the $\beta 2$ subunit of the $\alpha 5$ - $\beta 2$ - $\gamma 2$ subtype of GABA_AR. Interestingly, the enhancement of the $\alpha 5$ - $\beta 2$ - $\gamma 2$ GABA_AR response by menthol was eliminated in the $\beta 2$ -M286W (Fig. 6c,d), but not $\beta 2$ -Y444W (Fig. 6e,f)-containing receptors. As expected, the enhancement of GABA-induced currents in $\beta 2$ -M286W or $\beta 2$ -Y444W mutants by DZP (Fig. 5c–f) was not significantly different from the WT $\alpha 5$ - $\beta 2$ - $\gamma 2$ GABA_AR (152.1 \pm 13.9%, 187.8 \pm 11.5%, and 176.0 \pm 16.5% of GABA-induced currents by DZP on the WT, $\beta 2$ -M286W, and $\beta 2$ -Y444W GABA_ARs, respectively, $n = 4–8$ per group, $P > 0.05$ vs. WT). The differential responsiveness to curcuminol over menthol or DZP in the $\alpha 5$ - $\beta 2$ - $\gamma 2$ GABA_AR mutants bring up variant mechanisms underlying the actions of these modulators.

A mutation in $\gamma 2$ subunit of GABA_AR resistant to benzodiazepine preserves the actions of curcuminol and menthol. Finally, to underpin the differential mechanisms conferring the modulatory actions of curcuminol over menthol or DZP (Fig. 1a), we then examined the effects of these modulators on the mutant GABA_ARs resistant to benzodiazepine modulation. It has been established that a phenylalanine at position 77 in the $\gamma 2$ subunit is essential for the binding of benzodiazepine and the resultant regulation of GABA_ARs^{6,46}. Consistent with the previous report studied in *Xenopus* oocytes⁴⁶, inclusion of the F77Y mutation (Phe \rightarrow Tyr) in the $\gamma 2$ subunit indeed abolished the enhancement of GABA-induced currents by DZP (1 μ M) in $\alpha 1$ -containing GABA_ARs (Fig. 7a,b). Similarly, the $\alpha 5$ - $\beta 2$ - $\gamma 2$ (F77Y) GABA_AR also became insensitive to DZP (Fig. 7c,d). Notably, the effect of curcuminol was completely preserved ($\alpha 1$ -containing: 155.7 \pm 10.9% and 188.1 \pm 18.3% of GABA-induced currents by curcuminol on the WT and $\gamma 2$ -F77Y GABA_ARs, respectively, $n = 4–5$ per group, $P > 0.05$, Figs 5b and 7b; $\alpha 5$ -containing: 170.1 \pm 10.0% and 169.9 \pm 11.7% of GABA-induced currents by curcuminol on the WT and $\gamma 2$ -F77Y GABA_ARs, respectively, $n = 7–8$ per group, $P > 0.05$, Figs 6b and 7d). Similarly, the effect of menthol on the GABA-induced response was also largely retained ($\alpha 1$ -containing: 204.3 \pm 25.4% and 225.5 \pm 31.1% of GABA-induced currents by menthol on the WT and $\gamma 2$ -F77Y GABA_ARs, respectively, $n = 3–4$ per group, $P > 0.05$, Figs 5b and 7b; $\alpha 5$ -containing: 190.3 \pm 18.9% and 247.3 \pm 28.5% of GABA-induced currents by menthol on the WT and $\gamma 2$ -F77Y GABA_ARs, respectively, $n = 5–8$ per group, $P > 0.05$, Figs 6b and 7d). Thus, the mutation in the $\gamma 2$ subunit of GABA_AR resistant to benzodiazepine by no means affect the actions of curcuminol and menthol. In summary, our results collectively establish the notion that curcuminol exerts its facilitatory actions on GABA_ARs at sites distinct from benzodiazepine sites (Fig. 8).

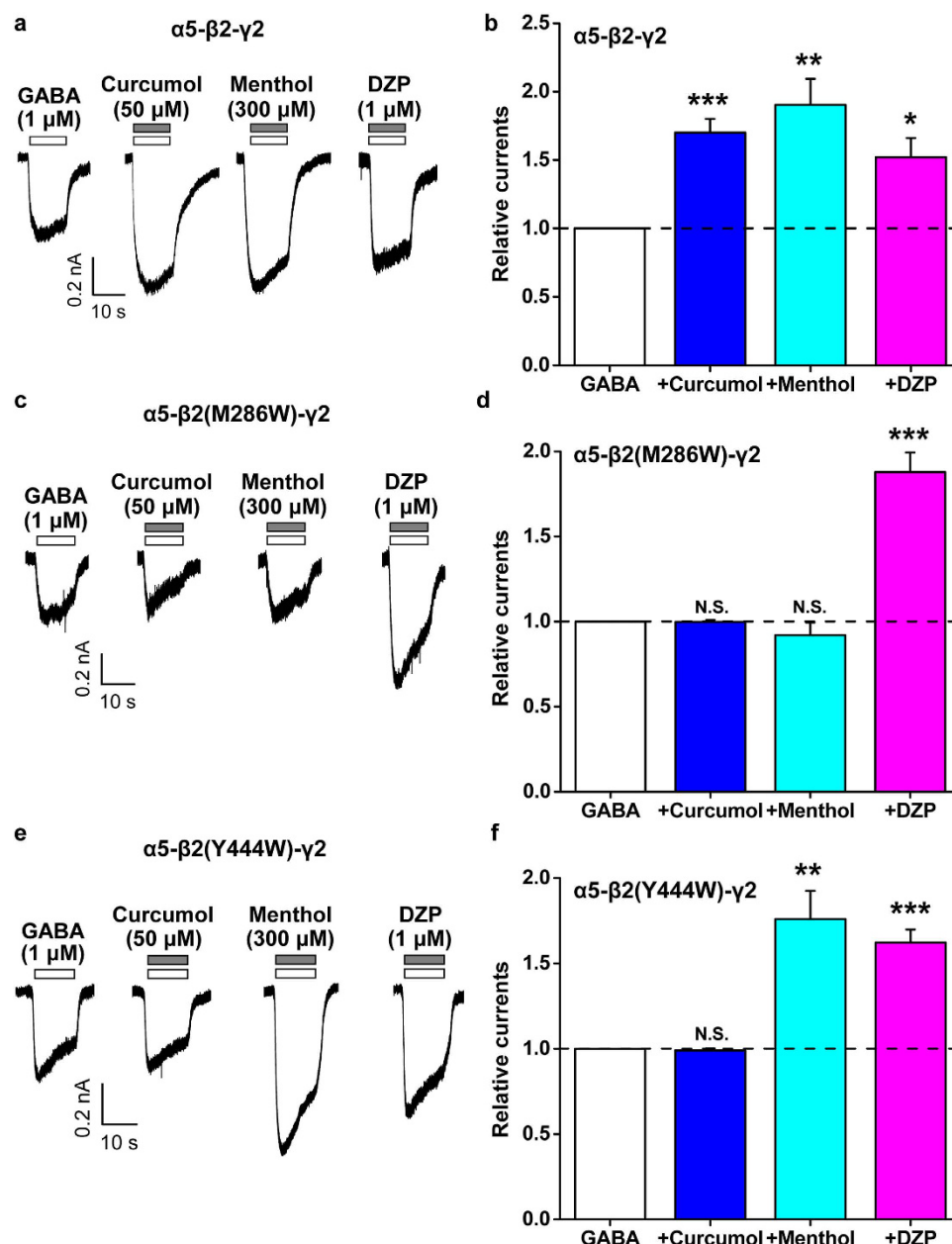


Figure 6. Effects of point mutations in $\beta 2$ subunit of GABA_AR on the modulation of $\alpha 5\text{-}\beta 2\text{-}\gamma 2$ GABA_AR subtype by curcumol, menthol, or DZP. (a, c, e) Representative current traces induced by GABA (1 μM) in the absence or presence of curcumol (50 μM), menthol (300 μM), or DZP (1 μM) in HEK-293T cells that expressed $\alpha 5$, $\beta 2$, $\beta 2\text{-M286W}$, or $\beta 2\text{-Y444W}$, and $\gamma 2$ GABA_AR subunits. (b, d, f) Pooled data from (a), (c) and (e), respectively. Data represent peak current amplitude normalized to that induced by GABA (1 μM) alone (dashed line). $n = 6\text{--}8$ each group. N.S., not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the current induced by GABA alone (dashed line), paired Student's t -test.

Discussion

In the present study, we have shown that curcumol (Fig. 1a), a natural compound and major bioactive component of *Rhizoma Curcumae* oil, acts as an allosteric modulator of GABA_ARs (Fig. 1b,c) in a manner different from that of the classical benzodiazepines. Curcumol significantly potentiated the GABA_AR activation in neurons in a way that did not overlap with modulation by DZP, a well-characterized benzodiazepine, but acted together with DZP to enhance receptor function (Fig. 2). By contrast, curcumol occluded the effects of menthol, another type of GABA_AR modulator, at the concentration of 100 μM , and was occluded by this compound at the concentration up to 3 mM, indicative of a shared binding site between curcumol and menthol (Fig. 3). Moreover, the benzodiazepine antagonist flumazenil had no impact on the enhancements of GABA response by curcumol and menthol, but abolished that by DZP (Fig. 4). Finally, while single mutations (M286W or Y444W) in the $\beta 2$ subunit abolished the effects of curcumol and menthol, but not DZP (Figs 5 and 6), single mutation (F77Y) in the GABA_AR $\gamma 2$

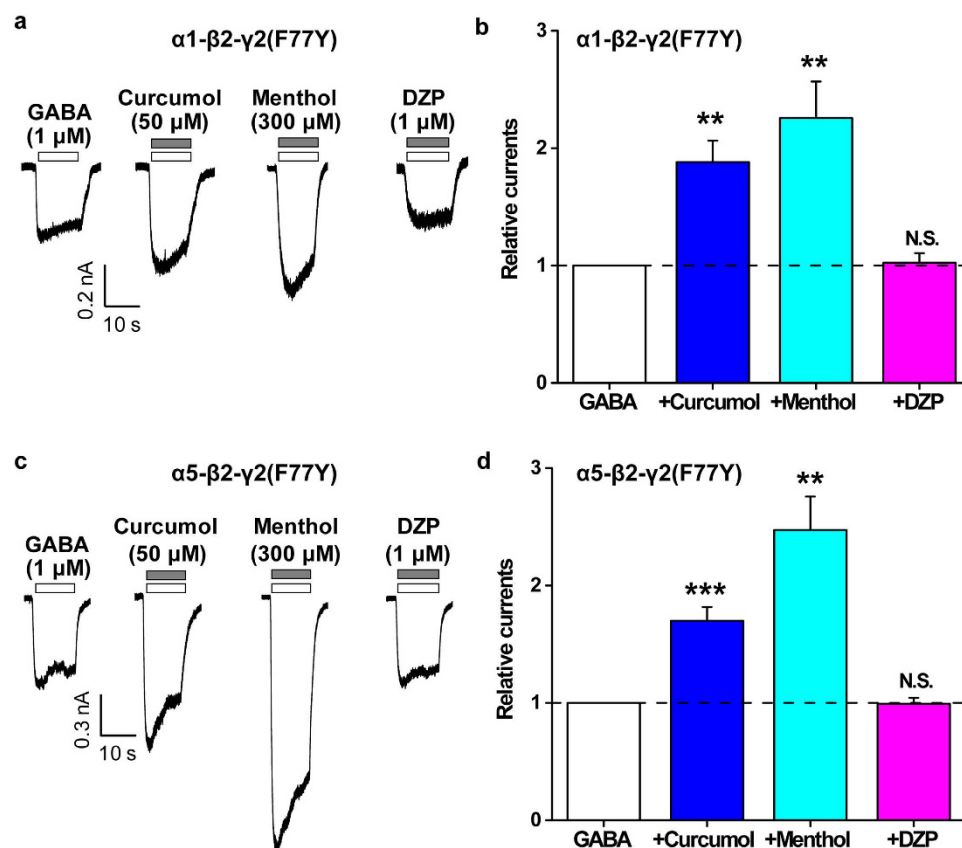


Figure 7. Effects of point mutations in $\gamma 2$ subunit of GABA_AR on the modulation of $\alpha 1-\beta 2-\gamma 2$ or $\alpha 5-\beta 2-\gamma 2$ GABA_AR by curcumin, menthol, or DZP. (a, c) Representative current traces induced by GABA (1 μ M) in the absence or presence of curcumin (50 μ M), menthol (300 μ M), or DZP (1 μ M) in HEK-293T cells that expressed $\alpha 1$ or $\alpha 5$, $\beta 2$, and $\gamma 2$ -F77Y GABA_AR subunits. (b, d) Pooled data from (a) and (c), respectively. Data represent peak current amplitude normalized to that induced by GABA (1 μ M) alone (dashed line). $n = 4-8$ each group. N.S., not significant, ** $P < 0.01$, *** $P < 0.001$, compared with the current induced by GABA alone (dashed line), paired Student's t -test.

subunit abolished the effects of DZP, but not curcumin nor menthol (Fig. 7). Curcumin therefore exerts its actions on GABA_ARs at sites distinct from those of benzodiazepines (Fig. 8). These findings shed more light on the modulation of GABA_ARs and could guide the development of new drugs targeting this receptor.

In line with the multifaceted physiological and pathophysiological roles of GABA_ARs in the central nervous system, the pharmacology^{9,47} and the drug development⁴⁸ on these receptors have also advanced considerably in recent decades. In addition to the natural agonist GABA⁵, positive GABA_AR modulators include benzodiazepines^{6,7}, barbiturates¹⁶, steroids¹⁷, and anaesthetics¹⁸⁻²⁶, each of which has specific binding sites on GABA_ARs. Several lines of evidence from the present study support that curcumin shares mechanisms with anaesthetics in the allosteric modulation of GABA_ARs. *First*, although curcumin and DZP enhanced each other's allosteric modulation (Fig. 2), curcumin and menthol reciprocally and concentration-dependently occluded each other's effects (Fig. 3), suggesting that curcumin acts on GABA_ARs *via* a mechanism different from that of benzodiazepines, but similar to that of menthol. *Second*, menthol and curcumin are both terpenoid compounds (monoterpene and sesquiterpene, respectively) with a functional hydroxyl group (Fig. 1a), a characteristic stereochemical configuration that differs from that of DZP, providing the structural basis of ligands for curcumin action independent of benzodiazepine binding sites. It is noteworthy that the structure-effect relationship of menthol indicates the importance of the hydroxyl group in these ligands^{34,37}. Likewise, curdione [(3S,6E,10S)-6,10-dimethyl-3-propan-2-ylcyclohex-6-ene-1,4-dione], an analogue of curcumin, predominantly lacks the hydroxyl group and exhibits greatly reduced potency at the GABA_AR³³. *Third*, mutagenesis analysis of the GABA_AR demonstrated that the TM3 and TM4 regions in the $\beta 2$ subunits are important for the potentiating effects of curcumin and menthol, but not DZP. Together with a previous study³⁴ showing that menthol shares general anaesthetic activity and GABA_AR site of action with the intravenous agent propofol, but not with benzodiazepines, steroids or barbiturates, we determined that curcumin likely represents a new member of the anaesthetic family for allosteric modulation of GABA_ARs.

Belonging to the non-classical anaesthetic subclass of GABA_AR modulators, curcumin not only shares an obvious chemical scaffold with menthol and propofol, but also contains new information about the structure-activity relationship for this particular form of GABA_AR pharmacology¹⁸⁻²⁶. As discussed earlier, the hydroxyl group in these compounds^{33,34,37} is essential for the positive modulation of GABA_ARs. The ortho positioning of an

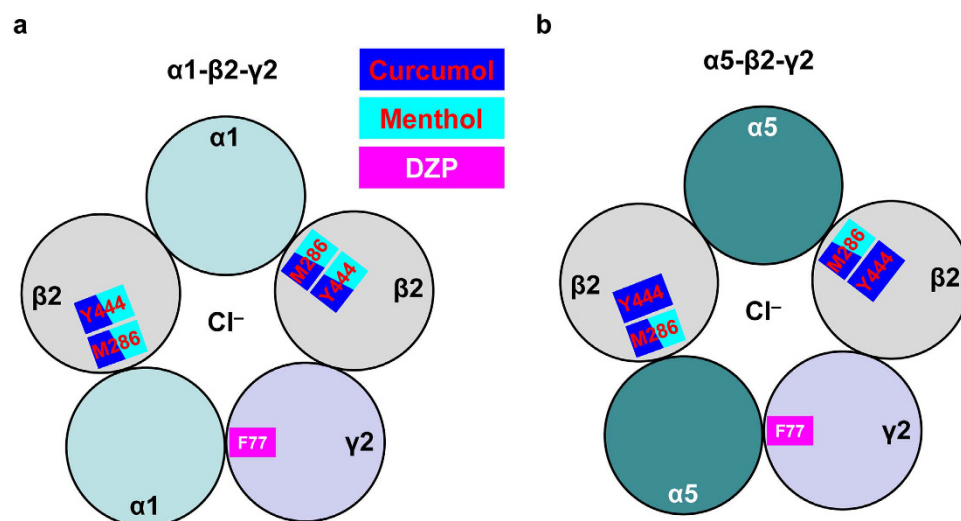


Figure 8. A hypothetical scheme for the modulation of GABA_A receptors by curcumol, menthol, or DZP through different mechanisms. (a) For the α1-β2-γ2 GABA_AR, while curcumol and menthol but not DZP act the receptor through the sites of Met-286 (M286) and Tyr-444 (Y444) in the β2 subunit, DZP but not curcumol nor menthol acts the receptor through Phe-77 (F77) in the γ2 subunit. (b) For the α5-β2-γ2 GABA_AR, while curcumol acts the receptor through the sites of M286 and Y444, menthol acts the receptor through the site of Phe-77 (F77) in the γ2 subunit. Please see the text for more details.

aliphatic chain is also a prerequisite for the activity of propofol or menthol analogues, including both the allosteric modulation^{19,34,49} and direct activation of GABA_ARs⁵⁰. Accordingly, curcumol shares equivalent positioning of an isopropyl adjacent to their respective hydroxyl groups (Fig. 1a), which likely plays a major part in the interaction with GABA_ARs. Notably, curcumol preferentially enhances receptor function, which is different from propofol and menthol that hold both efficacies of allosterically enhancing and directly activating GABA_ARs. In the present study, together with the previous report³³, curcumol at the concentrations even up to its water solubility limit (~300 μM)³⁸ induced only minimal direct activation of GABA_ARs. Moreover, curcumol was more potent than menthol, but probably less than propofol^{19,34}. Curcumol (50 μM) could significantly occlude the action of menthol (100 μM, Fig. 3). These pharmacological efficacy differences would be ascribed to the backbone structure of these compounds: propofol is a phenol (pK_a 11.0, planar ring structure) and menthol is a neutral cyclohexanol (chair structure), but curcumol is an epoxy azulene (a more complex structure). A better understanding of the structure–function relationship of curcumol interaction with GABA_ARs will aid the design of new drugs with higher efficacy and specificity for GABA_ARs.

Curcumol does not always run parallel with menthol on the modulation of GABA_ARs (Fig. 8). In the α5-β2 (Y444W)-γ2 mutated GABA_ARs, while the action of curcumol was eliminated, that of menthol kept intact (Fig. 6e,f). These effects were α subunit specific, as in the α1-β2(Y444W)-γ2 mutated GABA_ARs, the actions of curcumol and menthol were both abolished (Fig. 5e,f). In addition, these effects were dependent on the specific residue(s) in the β2 subunit. In the β2-M286W mutated GABA_ARs, both α1- (Fig. 5c,d) and α5-containing subtypes (Fig. 6c,d) became unresponsive to curcumol in addition to menthol. The more consensus involvement of β2-M286 residue located at TM3 region in the GABA_AR modulation implies a more direct role of this site^{19,34} in conferring the anaesthetic modulation of GABA_ARs^{19,34}. This is also reminiscent of an observation that GABA-induced inter-subunit conformational movements in the α1-TM1-β2-TM3 transmembrane subunit interface are necessary to gate the GABA_AR channels^{21,25}. Of note, the β2-Y444 residue located at TM4 region is also important for anaesthetic modulation^{20,34}, of which the dynamic structural arrangements^{15,25} are still being actively investigated. It is definitely meaningful to further dissect these subtle variances, including the possibility that different subunit interfaces are being used in the α1-β2-γ2 and α5-β2-γ2 GABA_ARs for anaesthetic modulation, which would be helpful for identification of receptor subtype-selective compounds for drug development in the future. In fact, many compounds, including propofol, etomidate, avermectin, and many others have been reported to mediate their effects through the same anaesthetic site¹⁵. Not only, multiple propofol-binding sites^{18–26,51} have also been identified. Nevertheless, the present identification of curcumol working in a similar way to menthol through acting at anaesthetic sites distinct from the benzodiazepine site will inspire more structural and functional studies using this novel compound.

Curcumol preferentially enhances GABA-induced GABA_AR activation, its prominent feature over other known anaesthetic modulators (i.e. propofol and menthol). However, it is unlikely to open the chloride channel considerably in the absence of GABA, which gives this compound its intriguing potential to be an ideal candidate GABA_AR drug. This self-limiting property of curcumol for GABA_AR modulation is also reminiscent of the widely-prescribed benzodiazepines in current therapeutic use. In contrast to barbiturates, benzodiazepines^{6,7} do not directly activate GABA_ARs in the absence of GABA (Fig. 2). Nevertheless, the clinical use of benzodiazepines is currently limited because their various pharmacological effects are not clearly separable by dosing.

For instance, although the anxiolytic actions of benzodiazepines are observed at lower doses than their sedative actions, sedation is still a problem if benzodiazepines are used as daytime anxiolytics. Benzodiazepines also have addictive properties and are liable to be abused^{13,14}, which limits their long-term use, and physical dependence and tolerance are areas of concern⁷. Considering this, curcumin holds a potential promise for the future development of novel GABA_AR drugs. Importantly, curcumin not only potentiates GABA-induced GABA_AR activation, but also amplifies the modulation of GABA_ARs in the presence of benzodiazepines (i.e. DZP) (Fig. 2). Therefore, as a non-classical anaesthetic modulator, curcumin and its derivatives might represent an alternative or supplementary strategy to alleviate or remove the side-effects that limit long-term and high-dose administration of benzodiazepines. However, the assumption remains under-developed yet, which needs to be carefully investigated in the future.

Curcumin is a natural compound isolated from *Rhizoma Curcumae* oil. Used alone or mixed in a specific type of traditional Chinese medicine, knowledge of its pharmacological effects on the central nervous system is increasing. *Rhizoma Curcumae* (rhizome of *Curcuma*; Ezhu) has been used as a condiment and home remedy in China for thousands of years, illustrating its lack of prominent toxicity in human. *Rhizoma Curcumae* oil has been suggested to possess pharmacological efficacy in a number of domains, including neuroprotection³⁹, cognitive enhancement⁴⁰, and anti-seizure efficacy⁴¹. Of the three main ingredients in *Rhizoma Curcumae* oil (curcumin, curcumin, and curdione), curcumin is the most potent GABA_AR modulator, and probably confers, at least in part, the pharmacological effects reported above. Moreover, like most naturally derived substances, curcumin is lipophilic and readily crosses the blood–brain barrier⁵², with the maximal concentration of curcumin after intravenous injection of *Rhizoma curcuma* oil up to 108.85 ± 65.91 , 92.38 ± 17.63 $\mu\text{g/g}$ in the liver and brain, equivalent to 458.43 ± 278.87 and 390.86 ± 74.59 μM (both the densities of liver and brain tissue were assumed to be 1.0 g/ml), respectively. Using the radioactive [³H]-curcumin, a previous study⁵³ demonstrated that curcumin can be rapidly and completely absorbed orally in rats; it emerged in the blood at 5 min and peaked at 15 min, respectively, after the oral administration. In addition, tissue distribution (including the penetration into the brain), drug stability and metabolism, expressing as the area under concentration time curve of curcumin, under oral administration all were comparable with that by intravenous injection⁵³, supporting a more easily administration way for using this drug. Based on the pharmacokinetics of curcumin, together with the pharmacological effects on GABA_ARs, it is not surprising that curcumin is capable of targeting against the central nervous system to treat neurological diseases. Indeed, curcumin alone decreased basal locomotor activity and chemically induced seizure activity in mice³³, confirming its effectiveness as a GABA_AR modulator to target the central function. However, despite curcumin belonging to the anaesthetics class of GABA_AR modulators, its anaesthetic effects remain unexplored. Of note, whether the long-term use of curcumin would produce dependence or tolerance, as with benzodiazepines, remains to be determined in the future studies. Nevertheless, the present study has contained new information about the pharmacological nature of curcumin on the central nervous system, and provides a primary basis for further in-depth studies regarding the pharmacological development of curcumin and its related drugs.

In summary, we have identified the natural compound curcumin as an allosteric modulator of GABA_ARs. Curcumin possesses an intriguing self-limiting efficacy at GABA_ARs, in addition to its mechanisms being similar to anaesthetics but independent on benzodiazepine binding sites. This work therefore suggests a novel approach to the development of drugs targeting GABA_ARs.

Methods

Animals. Animal procedures reported in the present study were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine, Shanghai, China. All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were housed under standard laboratory conditions (12/12 h light/dark, temperature 22–26 °C, air humidity 55–60%) with food and water *ad libitum*. Animal procedures were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University School of Medicine, and approved by the Institutional Animal Care and Use Committee (Department of Laboratory Animal Science, Shanghai Jiao Tong University School of Medicine) (Policy Number DLAS-MP-ANIM. 01–05).

Cell culture. Primary cultures of mouse hippocampal neurons were prepared according to previously described techniques³³. In brief, 15-day-old embryonic C57BL/6J mice were anesthetized with halothane. Brains were removed rapidly and placed in ice-cold Ca²⁺- and Mg²⁺-free phosphate buffered solution. Tissues were dissected and incubated with 0.05% trypsin-EDTA for 10 min at 37 °C, followed by trituration with fire-polished glass pipettes, and plated on poly-D-lysine-coated 35 mm culture dishes at a density of 1×10^6 cells per dish. Neurons were cultured with Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and maintained at 37 °C in a humidified 5% CO₂ atmosphere incubator. Cultures were fed twice a week and used for electrophysiological recording 10–20 days after plating. For neuron cultures, glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine (20 $\mu\text{g/ml}$; Sigma-Aldrich) and uridine (20 $\mu\text{g/ml}$; Sigma-Aldrich).

Human embryonic kidney (HEK)-293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1 mM L-glutamine, 10% foetal bovine serum, 50 units/ml penicillin, and 50 $\mu\text{g/ml}$ streptomycin (all from Invitrogen).

Site-directed mutagenesis. Mutations of receptor cDNA were generated with the QuikChange[®] mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's protocol using high-pressure-liquid-chromatography-purified or PAGE-purified oligonucleotide primers (Sigma-Genosys, The Woodlands, TX). All mutants were verified by DNA sequence analysis.

Functional expression of the recombinant GABA_ARs. The rat $\alpha 1$, $\beta 2$, and $\gamma 2$ subunit cDNA of GABA_AR were obtained from Dr. Yu Tian Wang (University of British Columbia, Vancouver, BC, Canada). The rat $\alpha 5$ subunit cDNA was kindly provided by Dr. David H. Farb (Boston University School of Medicine, Boston, Massachusetts, USA). Transient transfection of HEK-293T cells was carried out using HilyMax liposome transfection reagent (Dojindo Laboratories). Cotransfection with a green fluorescent protein expression vector, pEGFP-C3, was used to enable identification of transfected cells for patch clamp recording by monitoring the fluorescence of green fluorescent protein. Electrophysiological measurements were performed 24–48 h after transfection.

Electrophysiology. Whole-cell recordings were made using an Axon 700A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Membrane currents were sampled and analysed using a Digidata 1440 interface and a personal computer running Clampex and Clampfit software (Version 10, Axon Instruments). In voltage clamp mode, the membrane potential was held at -60 mV for whole-cell current recording. All electrophysiological experiments were carried out at room temperature ($23 \pm 2^\circ\text{C}$).

The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 N-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), and 10 glucose (pH 7.4 with Tris-base, 325–330 mOsm/L). The pipette solution was composed of (in mM): 120 KCl, 30 NaCl, 1 MgCl₂, 0.5 CaCl₂, 5 ethylene glycol tetraacetic acid (EGTA), 2 Mg-ATP, 10 HEPES, pH 7.2 adjusted with Tris-base.

Chemicals and drugs. The chemicals used in the present study curcuminol [(3S,5S,6S,8aS)-3-methyl-8-methylidene-5-(propan-2-yl)octahydro-6H-3a,6-epoxyazulen-6-ol], menthol [5-methyl-2-propan-2-ylcyclohexan-1-ol], and diazepam (DZP) [7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one] were purchased from Sigma-Aldrich (St. Louis, MO). Curcuminol, menthol, and DZP were initially dissolved as concentrated stock solutions in dimethyl sulfoxide and subsequently diluted to the desired concentration in the standard external solution. The final concentration of dimethyl sulfoxide was lower than 0.1% and was confirmed to be ineffective alone at the same concentration in control experiments (data not shown). Other drugs were either first dissolved in deionized water and then diluted to a final concentration in standard external solution just before use or dissolved directly in the standard external solution. Drugs were applied using a rapid application technique termed the “Y-tube” method as described previously^{54–56}. The tip of the drug tube was positioned 50–100 μM away from the patched cells. This system allows a complete exchange of external solution surrounding a cell within 20 ms. Throughout the experiment, the bath was superfused continuously with the standard external solution.

Data analysis. Values are expressed as the mean \pm S.E.M. Groups are compared using Student's *t* test. $P < 0.05$ was considered to be statistically significant. *P* and *n* represent the value of significance and the number of neurons or cells, respectively. Clampfit 10.5 (Molecular Devices) was used for data analysis. The smooth concentration-response curves of curcuminol on facilitation of the GABA response in hippocampal neurons were drawn according to a modified Michaelis-Menten equation by the method of least squares (the Newton-Raphson method) after normalizing to the maximal GABA response: $I = I_{\text{max}} \times C^h / (C^h + EC_{50}^h)$, where *I* is the normalized value of the current, I_{max} is the maximal response, *C* is the drug concentration, EC_{50} is the concentration which induces the half-maximal response and *h* is the apparent Hill coefficient.

References

- Rudolph, U. & Mohler, H. GABA_A receptor subtypes: Therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. *Annu Rev Pharmacol Toxicol* **54**, 483–507 (2014).
- Olsen, R. W. & Sieghart, W. GABA_A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* **56**, 141–148 (2009).
- Olsen, R. W. & Sieghart, W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* **60**, 243–260 (2008).
- McKernan, R. M. & Whiting, P. J. Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci* **19**, 139–143 (1996).
- Lummis, S. C. Locating GABA in GABA receptor binding sites. *Biochem Soc Trans* **37**, 1343–1346, doi: 10.1042/BST0371343 (2009).
- Sigel, E. & Buhr, A. The benzodiazepine binding site of GABA_A receptors. *Trends Pharmacol Sci* **18**, 425–429 (1997).
- Rudolph, U. & Knoflach, F. Beyond classical benzodiazepines: novel therapeutic potential of GABA_A receptor subtypes. *Nat Rev Drug Discov* **10**, 685–697 (2011).
- Minier, F. & Sigel, E. Positioning of the alpha-subunit isoforms confers a functional signature to gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **101**, 7769–7774 (2004).
- Mohler, H. The rise of a new GABA pharmacology. *Neuropharmacology* **60**, 1042–1049 (2011).
- Pritchett, D. B. *et al.* Importance of a novel GABA_A receptor subunit for benzodiazepine pharmacology. *Nature* **338**, 582–585 (1989).
- Sigel, E., Baur, R., Trube, G., Mohler, H. & Malherbe, P. The effect of subunit composition of rat brain GABA_A receptors on channel function. *Neuron* **5**, 703–711 (1990).
- Gunther, U. *et al.* Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **92**, 7749–7753 (1995).
- Tan, K. R. *et al.* Neural bases for addictive properties of benzodiazepines. *Nature* **463**, 769–774 (2010).
- Calixto, E. GABA withdrawal syndrome: GABA_A receptor, synapse, neurobiological implications and analogies with other abstinences. *Neuroscience* **313**, 57–72 (2016).
- Puthenkalam, R. *et al.* Structural studies of GABA_A receptor binding sites: which experimental structure tells us what? *Front Mol Neurosci* **9**, 44 (2016).
- Macdonald, R. L. & Olsen, R. W. GABA_A receptor channels. *Annu Rev Neurosci* **17**, 569–602 (1994).
- Hosie, A. M., Wilkins, M. E., da Silva, H. M. & Smart, T. G. Endogenous neurosteroids regulate GABA_A receptors through two discrete transmembrane sites. *Nature* **444**, 486–489 (2006).
- Mihic, S. J. *et al.* Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* **389**, 385–389 (1997).

19. Krasowski, M. D., Nishikawa, K., Nikolaeva, N., Lin, A. & Harrison, N. L. Methionine 286 in transmembrane domain 3 of the GABAA receptor beta subunit controls a binding cavity for propofol and other alkylphenol general anesthetics. *Neuropharmacology* **41**, 952–964 (2001).
20. Richardson, J. E. *et al.* A conserved tyrosine in the beta2 subunit M4 segment is a determinant of gamma-aminobutyric acid type A receptor sensitivity to propofol. *Anesthesiology* **107**, 412–418 (2007).
21. Bali, M., Jansen, M. & Akabas, M. H. GABA-induced intersubunit conformational movement in the GABAA receptor alpha 1M1-beta 2M3 transmembrane subunit interface: experimental basis for homology modeling of an intravenous anesthetic binding site. *J Neurosci* **29**, 3083–3092 (2009).
22. Nury, H. *et al.* X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature* **469**, 428–431 (2011).
23. Yip, G. M. *et al.* A propofol binding site on mammalian GABAA receptors identified by photolabeling. *Nat Chem Biol* **9**, 715–720 (2013).
24. Sauguet, L. *et al.* Structural basis for potentiation by alcohols and anaesthetics in a ligand-gated ion channel. *Nat Commun* **4**, 1697 (2013).
25. Jayakar, S. S. *et al.* Multiple propofol-binding sites in a gamma-aminobutyric acid type A receptor (GABAAR) identified using a photoreactive propofol analog. *J Biol Chem* **289**, 27456–27468 (2014).
26. Maldifassi, M. C., Baur, R. & Sigel, E. Functional sites involved in modulation of the GABAA receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital. *Neuropharmacology* **105**, 207–214 (2016).
27. Braat, S. & Kooy, R. F. The GABAA receptor as a therapeutic target for neurodevelopmental disorders. *Neuron* **86**, 1119–1130 (2015).
28. Knabl, J. *et al.* Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature* **451**, 330–334 (2008).
29. Fernandez, F. *et al.* Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome. *Nat Neurosci* **10**, 411–413 (2007).
30. Wasowski, C. & Marder, M. Flavonoids as GABAA receptor ligands: the whole story? *J Exp Pharmacol* **4**, 9–24 (2012).
31. Huang, C. *et al.* alpha-asarone from *Acorus gramineus* alleviates epilepsy by modulating A-type GABA receptors. *Neuropharmacology* **65**, 1–11 (2013).
32. Zaugg, J. *et al.* Positive GABA(A) receptor modulators from *Acorus calamus* and structural analysis of (+)-dioxosarcoguaiacol by 1D and 2D NMR and molecular modeling. *J Nat Prod* **74**, 1437–1443 (2011).
33. Ding, J. *et al.* Curcuminol from *Rhizoma Curcumae* suppresses epileptic seizure by facilitation of GABA(A) receptors. *Neuropharmacology* **81**, 244–255 (2014).
34. Watt, E. E. *et al.* Menthol shares general anesthetic activity and sites of action on the GABA(A) receptor with the intravenous agent, propofol. *Eur J Pharmacol* **590**, 120–126 (2008).
35. Hall, A. C. *et al.* Modulation of human GABAA and glycine receptor currents by menthol and related monoterpenoids. *Eur J Pharmacol* **506**, 9–16 (2004).
36. Lau, B. K., Karim, S., Goodchild, A. K., Vaughan, C. W. & Drew, G. M. Menthol enhances phasic and tonic GABAA receptor-mediated currents in midbrain periaqueductal grey neurons. *Br J Pharmacol* **171**, 2803–2813 (2014).
37. Zhang, X. B. *et al.* A-type GABA receptor as a central target of TRPM8 agonist menthol. *PLoS One* **3**, e3386 (2008).
38. Wang, L. *et al.* Subunit-specific inhibition of glycine receptors by curcuminol. *J Pharmacol Exp Ther* **343**, 371–379 (2012).
39. Dohare, P., Garg, P., Sharma, U., Jagannathan, N. R. & Ray, M. Neuroprotective efficacy and therapeutic window of curcuma oil: in rat embolic stroke model. *BMC Complement Altern Med* **8**, 55 (2008).
40. Sun, C. Y. *et al.* Effect of *Rhizoma curcumae* oil on the learning and memory in rats exposed to chronic hypoxia and the possible mechanisms. *Acta Physiologica Sinica* **60**, 228–234 (2008).
41. Wang, Y. & Zhao, X. J. Experimental study on the antiepileptic properties of *Rhizoma curcumae* oil in different epilepsy models. *Pharmacol Clin Chin Materia Medica* **20**, 11–12 (2004).
42. Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W. & Sperk, G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* **101**, 815–850 (2000).
43. Caraiscos, V. B. *et al.* Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **101**, 3662–3667 (2004).
44. Serwanski, D. R. *et al.* Synaptic and nonsynaptic localization of GABAA receptors containing the alpha5 subunit in the rat brain. *J Comp Neurol* **499** (2006).
45. Glykys, J., Mann, E. O. & Mody, I. Which GABA(A) receptor subunits are necessary for tonic inhibition in the hippocampus? *J Neurosci* **28**, 1421–1426 (2008).
46. Buhr, A., Baur, R. & Sigel, E. Subtle changes in residue 77 of the gamma subunit of alpha1beta2gamma2 GABAA receptors drastically alter the affinity for ligands of the benzodiazepine binding site. *J Biol Chem* **272**, 11799–11804 (1997).
47. Johnston, G. A. GABA(A) receptor channel pharmacology. *Curr Pharm Des* **11**, 1867–1885 (2005).
48. Froestl, W. An historical perspective on GABAergic drugs. *Future Med Chem* **3**, 163–175 (2011).
49. Krasowski, M. D., Hong, X., Hopfinger, A. J. & Harrison, N. L. 4D-QSAR analysis of a set of propofol analogues: mapping binding sites for an anesthetic phenol on the GABA(A) receptor. *J Med Chem* **45**, 3210–3221 (2002).
50. Mohammadi, B. *et al.* Structural requirements of phenol derivatives for direct activation of chloride currents via GABA(A) receptors. *Eur J Pharmacol* **421**, 85–91 (2001).
51. Moraga-Cid, G., Yevenes, G. E., Schmalzing, G., Peoples, R. W. & Aguayo, L. G. A Single phenylalanine residue in the main intracellular loop of alpha1 gamma-aminobutyric acid type A and glycine receptors influences their sensitivity to propofol. *Anesthesiology* **115**, 464–473 (2011).
52. Zhang, R. *et al.* Tissue distribution of curcuminol in rats after intravenous injection of zedoary turmeric oil fat emulsion. *Asian J Pharmacodynamics Pharmacokinetics* **9**, 51–57 (2009).
53. Su, C. Y., Liu, X. Y., Xu, H. X. & Zhu, X. Y. The metabolism of 3H-curcuminol in normal rats and tumour bearing mice. *Acta Pharmaceutica Sinica* **15**, 257–262 (1980).
54. Murase, K., Randic, M., Shirasaki, T., Nakagawa, T. & Akaike, N. Serotonin suppresses N-methyl-D-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. *Brain Res* **525**, 84–91 (1990).
55. Dong, X. P. & Xu, T. L. Radix paeoniae rubra suppression of sodium current in acutely dissociated rat hippocampal CA1 neurons. *Brain Res* **940**, 1–9 (2002).
56. Li, Y., Wu, L. J., Legendre, P. & Xu, T. L. Asymmetric cross-inhibition between GABAA and glycine receptors in rat spinal dorsal horn neurons. *J Biol Chem* **278**, 38637–38645 (2003).

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Author Contributions

T.L.X., W.H.G., W.G.L., and F.L. designed the project. Y.M.L., H.R.F., J.D., C.H., S.D., and T.Z. performed cell culture. Y.M.L., J.D., and C.H. carried out electrophysiological recordings. Y.M.L., J.D., and W.G.L. performed data analysis. Y.M.L., W.G.L., and F.L. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

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