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ORIGINAL ARTICLE

Sesquiterpenes and sesquiterpenoids harbor modulatory allosteric potential and affect inhibitory $GABA_A$ receptor function in vitro

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Abstract

Naturally occurring compounds such as sesquiterpenes and sesquiterpenoids (SQTs) have been shown to modulate $GABA_A$ receptors ($GABA_A$Rs). In this study, the modulatory potential of 11 SQTs at $GABA_A$Rs was analyzed to characterize their potential neurotropic activity. Transfected HEK293 cells and primary hippocampal neurons were functionally investigated using electrophysiological whole-cell recordings. Significantly different effects of β-caryophyllene and α-humulene, as well as their respective derivatives β-caryolanol and humulol, were observed in the HEK293 cell system. In neurons, the concomitant presence of phasic and tonic $GABA_A$R configurations accounts for differences in receptor modulation by SQTs. The in vivo presence of the γ_2 and δ subunits is important for SQT modulation. While phasic $GABA_A$ receptors in hippocampal neurons exhibited significantly altered GABA-evoked current amplitudes in the presence of humulol and guaiol, negative allosteric potential at recombinantly expressed $\alpha_1\beta_2\gamma_2$ receptors was only verified for humolol. Modeling and docking studies provided support for the binding of SQTs to the neurosteroid-binding site of the $GABA_A$R localized between transmembrane segments 1 and 3 at the ($^+\alpha$)-($^-\alpha$) interface. In sum, differences in the modulation of $GABA_A$R isoforms between SQTs were identified. Another finding is that our results provide an indication that nutritional digestion affects the neurotropic potential of natural compounds.

KEYWORDS
allosteric modulation, $GABA_A$ receptor, patch clamp recording

Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$R, γ-aminobutyric acid type A receptor; GFP, green fluorescent protein; HEK293, human embryonic kidney cells; M1-4, transmembrane domains 1–4; PTX, picrotoxinin; RRID, Research Resource Identifier; SQTs, sesquiterpenes and sesquiterpenoids.

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1 | INTRODUCTION

$GABA_A$Rs represent the major inhibitory ligand-gated chloride ion channels in the central nervous system. $GABA_A$Rs mediate fast phasic synaptic inhibition as well as tonic perisynaptic and extrasynaptic inhibition, and are expressed in brain areas, for example, cortex, hippocampus, and olfactory bulb. They contribute to the balance between excitatory and inhibitory neurotransmission processes by inhibiting incoming action potentials through chloride ion influx (Mortensen et al., 2012; Sieghart, 2006).

$GABA_A$Rs are heteropentameric receptors that belong to the superfamily of Cys-loop receptors also including glycine receptors, the $5HT_3$ receptor, and nicotinic acetylcholine receptors (nAChRs). Common to all Cys-loop receptors is their large N-terminal domain followed by four transmembrane domains (M1-4) with M2 representing the ion channel pore, and a short extracellular C-terminus. The large intracellular loop between M3 and M4 is of the highest variability between Cys-loop receptors bearing binding sites for other structural proteins (Kasaragod & Schindelin, 2019).

$GABA_A$R subunits are encoded by 19 genes (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3, π) (Simon et al., 2004). Most $GABA_A$Rs are formed by α, β, and γ/δ subunits with the $\alpha_1\beta_2/_3\gamma_2$ configurations representing the most abundant receptor subtypes in the brain (Fritschy & Mohler, 1995). Recent structures revealed an arrangement of α−β−α−β−γ/δ in a clockwise manner (Laverty et al., 2017; Masiulis et al., 2019). The large extracellular N-terminus harbors the ligand-binding site for GABA at the interface of two adjacent subunits (α and β). The ion channel can be blocked by the antagonist picrotoxinin (PTX) which binds to residues located in M2 constricting the ion channel pore to about 1.5 Å. Following the binding of another antagonist, bicuculline, to the orthosteric-binding site, the $GABA_A$Rs turn into a closed conformation similar to the PTX-bound state (Masiulis et al., 2019). Diazepam, a benzodiazepine, is a potent allosteric modulator of GABAergic function binding specifically to the orthosteric-binding site at the interface of α_1 and γ_2 subunits. Diazepam is an established drug used as a muscle relaxant and a sedative substance (Rudolph & Knoflach, 2011). Recently, a second binding site for benzodiazepines was demonstrated responsible for the observed biphasic $GABA_A$R potentiation at higher diazepam concentrations (Olsen, 2018; Walters et al., 2000). This binding site is localized between the transmembrane domains at the interface of subunits β (β_3^+) and α (α_1^-). Similarly, neurosteroid binding has been demonstrated within a hydrophobic-binding pocket between adjacent transmembrane domains (Alvarez & Estrin, 2015; Alvarez & Pecci, 2018; Miller et al., 2017).

Several studies describe modulation of $GABA_A$Rs by natural compounds taken up via nutrition or used in aroma therapy, however, most lack receptor subtype specificity (Johnston et al., 2006). Positive and negative allosteric modulation of $GABA_A$Rs has been shown, for instance, for flavonoids, terpenoids, phenols, and polyacetylenic alcohols, bearing convulsive, anticonvulsive, sedative or anxiolytic potential. Because of the partly high hydrophobicity of terpenoids, binding to the transmembrane subunit interfaces or modulation of the lipid surrounding have been postulated as underlying mechanisms (Manayi et al., 2016; Silva et al., 2019). Among different terpene subtypes, volatile bicyclic monoterpenoids carrying a hydroxy group revealed positive allosteric modulators at $\alpha_1\beta_2\gamma_2$ but also at $\alpha_1\beta_2$ $GABA_A$Rs (Kessler et al., 2012, 2014). Modulation of GABAergic function has also been ascribed to sesquiterpenes and sesquiterpenoids (SQTs) (Kasaragod et al., 2019; Manayi et al., 2016).

In this study, we investigated the modulatory effects of 11 SQTs occurring in different plants like hop and chamomile on $GABA_A$R configurations present in the human brain. Therefore, the $GABA_A$Rs expressed in transfected HEK293 cells and primary hippocampal neurons were characterized by electrophysiological whole-cell recordings. Our functional data were accompanied by structural modeling and molecular docking studies to further investigate possible binding modes of the SQTs studied. We found significant differences in the modulation of $GABA_A$R isoforms between different SQTs as well as between transfected cells and hippocampal neurons. The results also indicate that structural changes because of digestion and biotransformation processes may affect the neurotropic potential of natural compounds.

2 | MATERIALS AND METHODS

2.1 | Chemical Information

GABA, $ZnCl_2$, picrotoxinin, diazepam, and gaboxadol were acquired from Sigma-Aldrich. Stock solutions of GABA (1 M) and $ZnCl_2$ (100 mM) were prepared in water. Picrotoxinin (50 mM), diazepam (10 mM), and gaboxadol (100 mM) were dissolved in ethanol. Guaiol, α-humulene, α-bisabolol, β-caryophyllene, and nootkatone were purchased from Sigma-Aldrich. β-Caryolanol and humulol were synthesized according to Heinlein & Buettner, (2012). Spathulenol, α-bisabolone oxide A, α-bisabolol oxide A, and α-bisabolol oxide B were isolated by a combination of different extraction and isolation steps. In short, dried chamomile flower heads were extracted with dichloromethane and the volatile fraction was isolated by means of solvent-assisted flavor evaporation (Engel et al., 1999). Centrifugal partition chromatography resulted in the direct isolation of α-bisabolone oxide A. Silica gel chromatography and size exclusion chromatography provided further purification of spathulenol, α-bisabolol oxide A, and α-bisabolol oxide B. Additionally, α-bisabolol oxide A was isolated by preparative two-dimensional GC. SQT stock solutions (100 mM) were prepared in ethanol. All stock solutions were stored at −20°C. Fresh solutions were prepared from stock solutions on the day of recording (final concentration of ethanol 0.6%).

2.2 | Extraction and isolation of SQTs from chamomile and hop for GC-MS analyses

Dried hop cones (2.43 g) and dried chamomile flower heads (2.62 g), both purchased from a local company (Wurdies Kräuter GmbH & Co.

KG), were finely chopped for 1 min with a mini chopper (Kenwood CH180, Kenwood Limited). Solvent extraction was performed with dichloromethane (DCM, 50 ml, 30 min). After drying over sodium sulfate, the extracts were applied to solvent-assisted flavor evaporation (SAFE) at 60°C in a vacuum (Engel et al., 1999). The distillates were concentrated to 100 µl by Vigreux and subsequent micro distillation at 50°C (Bemelmans, 1979). As the last step, the distillates were diluted 1:10 (DCM) for GC-MS analysis.

2.3 | GC-MS analyses of the hop and chamomile distillates

The analyses were performed on a GC 6,890 (Agilent Technologies, Santa Clara, CA, USA) connected to a MSD 5,973 (Hewlett-Packard, Palo Alto, CA, USA), equipped with a GERSTEL MPS 2 multipurpose sampler and a GERSTEL CIS 3 injection system (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). An uncoated fused silica capillary pre-column (3 m × 0.53 mm i.D.) was fixed to a DB-5 capillary column (30 × 0.25 mm i.D., film thickness 0.25 µm; both from Agilent J&W Scientific). The oven program was started at 60°C and was raised at 3°C/min until 246°C. The carrier gas (helium) flow was set to 1 ml/min. A split of 20:1 was used for the application of the sample (2 µl). EI mass spectra were recorded in full scan mode (40.0–400.0 amu) using 70 eV.

2.4 | Cell lines

HEK293 human embryonic kidney cells (CRL-1573, RRID:CVCL_0045, ATCC; passages 33 lot 70016364 and 34 lot 61714301) were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum, 200 mM GlutaMAX, 100 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific) under standard growth conditions at 37°C and 5% CO_2. Following thawing, cells used for experiments were between passages 6 and 20. The cell line is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee.

2.5 | Transfection

HEK293 cells were transiently transfected using a modified calcium-phosphate precipitation method. Plasmid DNAs of α_1, α_4, α_6, β_2, β_3, γ_2, δ subunits, and GFP were mixed with the following combinations and ratios: α_1:β_2:GFP 1:1:1, α_1:β_2:γ_2:GFP 1:1:0.5:1, α_4:β_3:δ:GFP 1:1:0.5:1, α_6:β_3:δ:GFP 1:1:0.5:1. DNA was mixed with 0.1× TE buffer, 2.5 M $CaCl_2$, and 2× HBS buffer (50 mM HEPES, 12 mM glucose, 10 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.98) and applied to the cells. Cell medium was exchanged after 6 hr to reduce transfection stress. Electrophysiological recordings were performed 24–48 hr post-transfection.

2.6 | Neuronal preparation and culture

Hippocampal neurons were prepared from mouse embryos at embryonic day 17 (E17) from pregnant female wild-type CD1 mice (Strain code: 022, RRID:IMSR_CRL:022, Charles River). Female pregnant mice were subjected to a 10-min-deep CO_2 anesthesia. Experiments were authorized by the local veterinary authority and Committee on the Ethics of Animal Experiments (Regierung von Unterfranken, license FBVVL 568/200-324/13). In short, hippocampi from all embryos (n = 10–16) were pooled and trypsinated for 30 min by incubation in 0.5 mg/ml trypsin, 0.2 mg/ml EDTA, and 10 µg/µl DNase I in PBS for 30 min at 37°C. After adding 10% fetal calf serum, neurons were dissociated by trituration, counted, and seeded on poly-L-lysine-coated coverslips. Hippocampal neurons were cultured under standard growth conditions at 37°C and 5% CO_2 in a neurobasal medium containing 2 mM GlutaMAX and 2% (v/v) B27 supplement (Thermo Fisher Scientific). Neurons were used for patch clamp experiments after 18–21 days in culture.

2.7 | Electrophysiology

Whole-cell recordings of transfected HEK293 cells and hippocampal neurons were obtained using the patch clamp technique. A Sutter P97 horizontal puller (Sutter Instrument) was used to pull recording pipettes (3–5 MΩ) from thin-walled borosilicate capillaries (TW150-F4; World Precision Instruments, Sarasota, FL, USA). Recordings were obtained with an EPC10 USB amplifier operated with Patchmaster software (HEKA Elekronik). Currents were low-pass filtered at 2.9 kHz and digitized at 20 kHz. Cells were held at −60 mV. An Octaflow II system (ALA Scientific Instruments) was used to apply agonist solutions directly on patched cells for 50 ms with a pressure of 1 bar. The intracellular buffer contained (in mM) 120 CsCl, 20 $N(Et)_4Cl$, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES; pH 7.3, adjusted with CsOH (295 ± 1.5 mOsm/L) and the extracellular solution consisted of (in mM) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES pH 7.3, adjusted with NaOH (320 ± 1.5 mOsm/L). All measurements were performed at 21°C.

2.8 | Molecular modeling and docking

The docking study was performed at two different locations at the transmembrane domain of the $GABA_A$-receptor: the diazepam-binding site in the $\beta^+\alpha^-$-interface and the pregnanolone-binding site in the αα-interface. For the former, a CryoEM structure of the human full-length $\alpha_1\beta_3\gamma_2$-$GABA_A$-receptor was used (PDB: 6HUP) (Masiulis et al., 2019), for the latter (i.e., the pregnanolone-binding site) the crystal structure of an $\alpha_5\beta_3$-chimera (PDB: 5O8F) (Miller et al., 2017). The preparation of the structures as well as the setup of the docking calculations with AutoDock (Morris et al., 1998) are described in detail in the Supplemental Information.

2.9 | Statistical analysis

GraphPad Prism 9.0.0 (GraphPad Software) was used to calculate mean values, standard deviation, standard error of the mean, and statistical significance. The two-tailed paired *t* test was used to estimate significance values with $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. Data were not assessed for normality, no test for outliers was conducted, and no sample size calculation was done. The sample size is based on previous studies of a similar design (Milanos et al., 2018).

2.10 | Study and experimental design

This study was not a pre-registered study. Therefore, no randomization was performed to allocate subjects in the study. Moreover, this study did not include animal experiments. Hippocampal cultures were obtained from pooled hippocampi of embryos from pregnant female CD1. Three independent cultures were used for the electrophysiological recordings from mature neurons between DIV 18–21 in culture. No blinding of the experimenter was performed in the electrophysiological data acquisition and analysis.

Exclusion criteria for transfected HEK293 cells used for electrophysiological measurements: We defined exclusion criteria as transfected cells might not have picked up all plasmids (3 to express a defined type of $GABA_AR$). Among the cells measured, one cell did not show a picrotoxinin block arguing that the expression of the $GABA_AR$ was not achieved. Thus, this cell was excluded from analysis (8 of 9 cells were used for analysis). For neurons, not all neurons in the hippocampal culture represent GABAergic cells. Picrotoxinin blocks GABAergic neurons and was used as a selection criterion. Synaptic $GABA_ARs$ were verified by potentiation with diazepam. Gaboxadol, a superagonist at tonic $GABA_ARs$, was used to show that cells express tonic $GABA_ARs$. Using these criteria, we excluded two cells from our initial recordings from hippocampal neurons not exhibiting a picrotoxin block and concomitantly no diazepam potentiation.

3 | RESULTS

3.1 | Positive and negative allosteric modulation of $GABA_ARs$ of the α1β2 subtype by SQTs

In this study, the modulatory effect of 11 different SQTs was investigated on $GABA_ARs$ (Figure 1a). β-Caryophyllene and α-humulene are major compounds of the volatile fraction of hop. In previous studies it was shown, that they are transformed into β-caryolanol and humulol, respectively, when applied to an in vitro digestion model (Heinlein & Buettner, 2012) (Figure 1b, left side). (*E*)-β-Farnesene, spathulenol, α-bisabolol, α-bisabolol oxide A, α-bisabolol oxide B, and α-bisabolone oxide A are major constituents of the volatile fraction of chamomile. (Figure 1b, right side). Guaiol is a major constituent in valerian (*Valeriana officinalis* L.) and was chosen because of its structural similarity to spathulenol. SQTs were diluted in ethanol, which resulted in final ethanol concentrations of 0.6% for single modulator measurements and 1% for co-application of two modulators in the patch solutions.

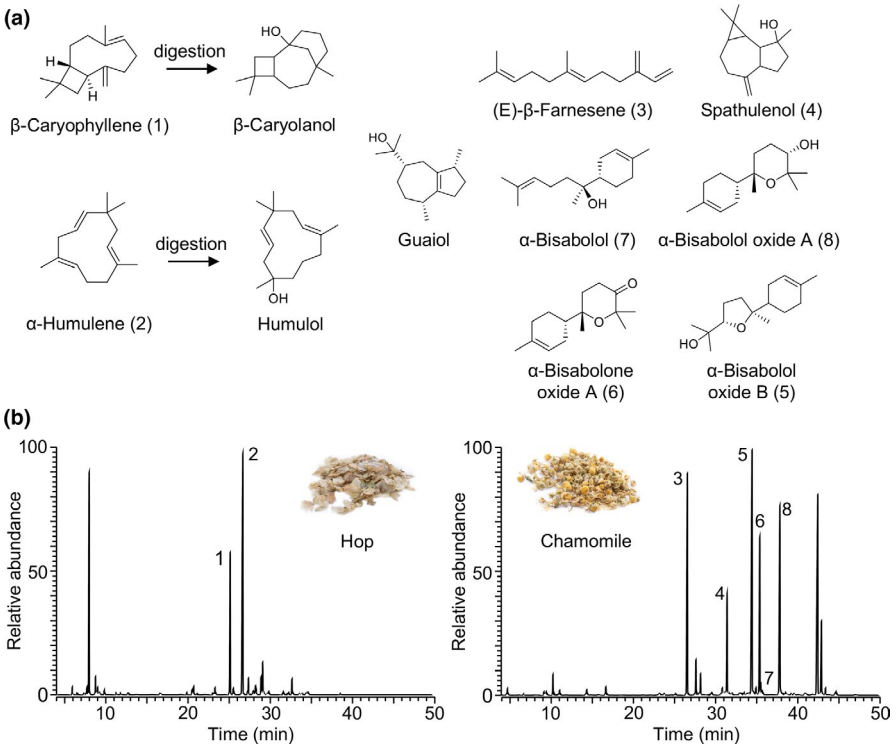

FIGURE 1 Structures of SQTs and chromatograms of distillates obtained from hop and chamomile. (a) Chemical structures of all modulators used in this study. (b) Total ion chromatograms of the distillates of hop (left) and chamomile (right) obtained after solvent-assisted flavor evaporation. The target SQTs are marked with the numbers according to a

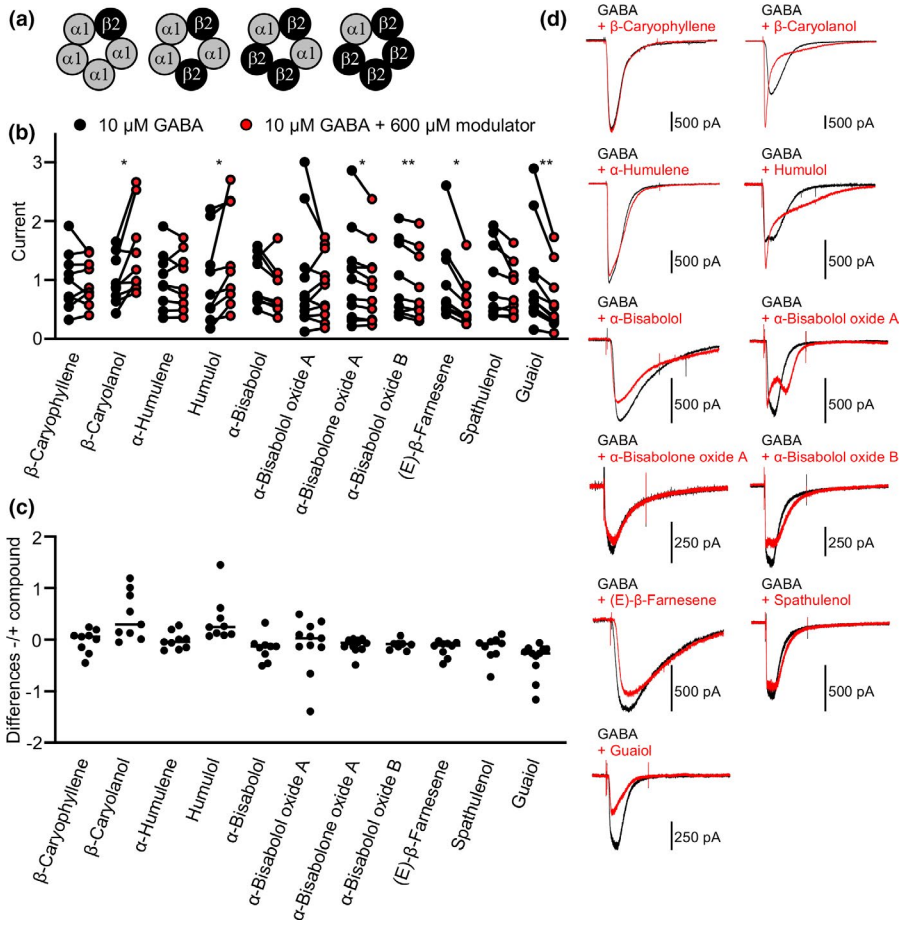

FIGURE 2 Positive and negative allosteric modulation of $\alpha_1\beta_2$ $GABA_A$Rs by SQTs. (a) Possible receptor configurations for $\alpha_1\beta_2$ $GABA_A$Rs. (b) Normalized currents of patch clamp recordings from transfected HEK293 cells during application of 10 μM GABA (black dots) or with co-application of 600 μM of the appropriate modulator (red dots). Each pair of dots represents one cell. Patch clamp experiments were performed on at least 3 days with independent cell batches and transfections, total number of cells recorded was $n = 9–12$. $^*p \leq .05$, $^{**}p \leq .01$. (c) Differences between each current pair in b are shown. (d) Example current traces for each recording are shown in b. Black traces, 10 μM GABA; red traces, 10 μM GABA + 600 μM modulator

A matching ethanol concentration was used for control measurements without a modulator.

Previous studies demonstrated that GABAergic modulation by terpenoids is most probably independent of the presence of the γ_2 subunit in the pentameric $GABA_A$R complex (Kessler et al., 2014). Therefore, the modulatory effect of SQTs was first analyzed on heteromeric $GABA_A$Rs composed of α_1 and the β_2 subunits. Cells were co-transfected with a green fluorescent protein (GFP) to distinguish transfected from untransfected cells during patch clamp experiments by using a fluorescence microscope.

Multiple receptor configurations in the pentameric receptor complex are possible resulting from $\alpha_1\beta_2$ subunit transfection (Figure 2a). Their effects on agonist potency are unknown, but expression differences seem to influence receptor stoichiometry, with 2:3 being the most likely one (Wagoner & Czajkowski, 2010). For analysis of SQT modulation, 10 μM GABA (referring to $EC_{10\text{-}30}$, (Milanos et al., 2018) was applied for 50 ms, followed by a 50 ms co-application with 600 μM of the appropriate modulator (Figure 2b, c). A concentration of 600 μM for the SOTs was chosen because of previous results on other terpenes starting with modulation at concentrations of 100 μM and being most effective between 300 μM and 1 mM (van Brederode et al., 2016; Kessler et al., 2014; Milanos et al., 2018). Obtained maximum current (I_{max}) values were divided by the mean GABA current for better comparability. Co-application with β-caryolanol (146 ± 24%, $p = .017$) and humulol (138 ± 29%, $p = .033$) resulted in significantly increased I_{max}, whereas α-bisabolone oxide A (89 ± 22%, $p = .023$), α-bisabolol oxide B (90 ± 20%, $p = .009$), (*E*)-β-farnesene (83 ± 19%, $p = .010$), and guaiol (62 ± 16%, $p = .0039$) significantly reduced GABA-induced I_{max} values (Table 1). Representative current traces for these recordings are shown in Figure 2d. The significantly different effects of β-caryophyllene and α-humulene and their respective derivatives β-caryolanol and humulol suggest that the additional hydroxy group may account for the observed differences in receptor modulation. Furthermore, α-bisabolone oxide A, α-bisabolol oxide B, (*E*)-β-farnesene, and guaiol exhibited a negative allosteric modulatory effect independent of the presence of a hydroxy group. As a single concentration of 600 μM SQTs was used for recordings, we

TABLE 1 Electrophysiological data obtained from $\alpha_1\beta_2$ $GABA_A$Rs expressed in HEK293 cells

Compound	*c* [μM]	I_{rel} ± *SEM* [%]	I_{abs} ± *SEM* [nA]	*p*-values	*n*
GABA	10	100 ± 17	1.84 ± 0.30	0.7600	9
β-Caryophyllene	600	98 ± 13	1.80 ± 0.24		
GABA	10	100 ± 14	1.19 ± 0.16	0.0172*	9
β-Caryolanol	600	146 ± 24	1.73 ± 0.28		
GABA	10	100 ± 16	2.56 ± 0.42	0.7578	9
α-Humulene	600	98 ± 16	2.51 ± 0.41		
GABA	10	100 ± 25	1.33 ± 0.33	0.0336*	9
Humulol	600	138 ± 29	1.84 ± 0.38		
GABA	10	100 ± 14	0.71 ± 0.10	0.0720	9
α-Bisabolol	600	83 ± 14	0.59 ± 0.10		
GABA	10	100 ± 27	0.67 ± 0.18	0.5244	10
α-Bisabolol oxide A	600	90 ± 17	0.60 ± 0.11		
GABA	10	100 ± 26	0.33 ± 0.09	0.0236*	12
α-Bisabolone oxide A	600	89 ± 22	0.29 ± 0.07		
GABA	10	100 ± 21	0.75 ± 0.16	0.0097**	9
α-Bisabolol oxide B	600	90 ± 20	0.67 ± 0.15		
GABA	10	100 ± 24	0.39 ± 0.09	0.0101*	9
(*E*)-β-Farnesene	600	83 ± 19	0.32 ± 0.07		
GABA	10	100 ± 21	0.68 ± 0.14	0.0746	9
Spathulenol	600	83 ± 15	0.56 ± 0.10		
GABA	10	100 ± 25	0.58 ± 0.15	0.0039**	10
Guaiol	600	62 ± 16	0.36 ± 0.09		
GABA	10	100 ± 13	1.03 ± 0.13		9
β-Caryolanol +Humulol	500 + 500	108 ± 9	1.11 ± 0.10	0.4388	
β-Caryolanol +α-Bisabolol	500 + 500	56 ± 7	0.57 ± 0.07	0.0004***	
β-Caryolanol +Guaiol	500 + 500	52 ± 7	0.53 ± 0.07	0.0002***	

Note: c, concentration; *SEM*, standard error of the mean; levels of significance with *p*-values *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$; *n*, number of recorded cells.

cannot exclude maximal positive or negative allosteric modulation at lower or higher concentrations of the GABAergic modulators.

In our initial screen, a modulator concentration of 600 μM was used. As it is known that hydrophobic chemicals can bind to plastic tubes (Heinlein et al., 2014) as used in the application system of the electrophysiological setup, samples of the applied solutions were taken before and after passage through the application system. These samples were analyzed by GC-MS to reveal concentration differences between the start and end of the recording session (Supporting information Figure S1). Our data revealed a reduction of the used substances by a mean of 52% (~300 μM released by the application system) while transported through the tubing system, suggesting that the real modulatory effect is probably much more pronounced. With a reduction of the concentration of the substances released by the perfusion system, a concentration ~300 μM should still be sufficient to modulate $GABA_A$ receptors (Milanos et al., 2018).

3.2 | Modulation of the GABAergic response by co-applied SQTs is difficult to predict

As some SQTs modulate $GABA_A$Rs of the $\alpha_1\beta_2$ subtype positively and others modulate negatively, we hypothesized additive or neutralizing effects for co-applications of two modulators. β-Caryolanol showed the largest positive modulation. It was co-applied with humulol, another positive modulator to investigate additive effects. A second round of co-application analyzed competing effects between SQTs, respectively. Here, β-caryolanol together with the negative modulators α-bisabolol and guaiol, were used (Figure 3a, b). 10 μM GABA were co-applied with 500 μM of each modulator. Interestingly, co-application of β-caryolanol and humulol showed no additive modulatory effect (108 ± 9%, $p = .43$). In contrast, co-application of β-caryolanol and α-bisabolol (56 ± 7%, $p = .0004$) or guaiol (52 ± 7%, $p = .0002$; Table 1) showed significantly enhanced negative modulation of the maximal GABA-induced chloride current.

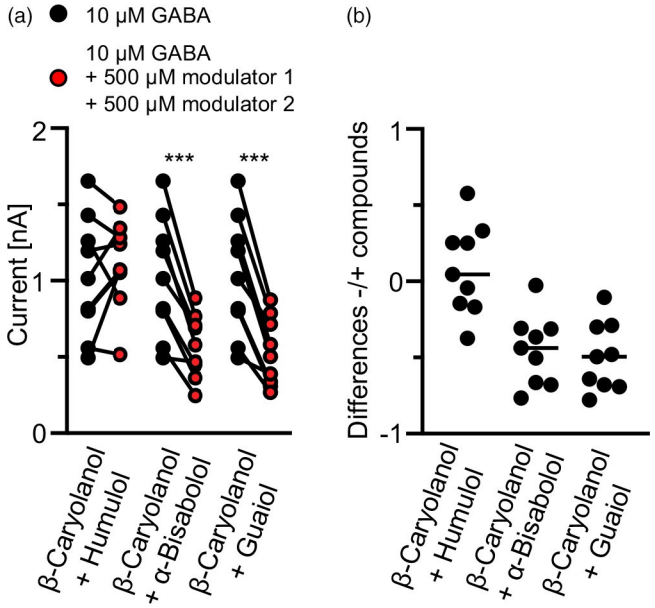

FIGURE 3 Co-application of two SQTs generates competitive rather than additive modulation at $GABA_A$Rs. (a) Currents acquired by patch clamp recordings from HEK293 cells expressing $\alpha_1\beta_2$ $GABA_A$Rs. Currents were recorded during the application of 10 μM GABA (black dots) or with co-application of two modulators with a concentration of 500 μM per modulator (red dots). Each pair of dots represents one patched HEK293 cell. Experiments from 3 recording sessions using independent cell batches and transfections are shown, number of cells recorded was 9 for every condition, significance level ***$p \leq .001$. (b) Differences between each current pair in a are shown

These data argue that the modulatory effect upon co-application of multiple SQTs is difficult to predict and might result from different affinities to the receptor protein.

3.3 | The $GABA_A$R subunit γ_2 plays a major role in the modulation by SQTs

SQTs modulate $\alpha_1\beta_2$ $GABA_A$Rs in transfected HEK293 cells, but they might have a different effect in vivo, where multiple GABA receptor configurations are present in the same neuron (phasic receptors containing the γ_2 subunit and tonic receptors containing a δ subunit). Among possible $GABA_A$R subunit combinations, $\alpha_1\beta_2\gamma_2$ is the most abundant receptor configuration in the brain (Figure 4a) (Whiting, 2003). Patch clamp recordings were performed on hippocampal neurons at day in vitro 18–21 (DIV18-21) to check if endogenous $GABA_A$R configurations are affected by SQTs. No significant effects on I_{max} were observed for β-caryophyllene (94 ± 22%), β-caryolanol (79 ± 21%), and α-humulene (93 ± 19%) when co-applied with 10 μM GABA (Figure 4b–g). However, a significant decrease in current for 10 μM GABA + humulol (78 ± 19%, $p = .0056$, Table 2) as well as a significant increase for guaiol (120 ± 27%, $p = .0008$). These data are in contrast to the effects observed for $\alpha_1\beta_2$ receptors in transfected HEK293 cells and might argue for differences in GABAergic modulation by $GABA_A$Rs containing γ or also possibly δ subunits or other α/β subunits than α_1/β_2. At GABA concentrations of 10 μM, tonic $GABA_A$ receptors including the δ subunit are saturated and may less contribute to the observed effects than synaptic receptor compositions including the γ subunit (Karim et al., 2013; Mortensen et al., 2012). Therefore, $GABA_A$R modulation was generally tested in hippocampal neurons by diazepam, a positive allosteric modulator, the antagonist picrotoxinin, or the agonist gaboxadol. Diazepam (138 ± 33%, $p \leq 0.0001$) and gaboxadol (176 ± 41%, $p = .0025$) application resulted in significantly increased currents and hint for the presence of γ and δ containing receptors (Chua & Chebib, 2017). In contrast, GABAergic currents were significantly reduced by co-application of GABA and picrotoxinin to 41%–59% (Figure 4c, f, h–j). These data suggest that the presence of different $GABA_A$R configurations in one neuron harboring γ and δ subunits may play a role in $GABA_A$R modulation by SQTs. $GABA_A$Rs containing the γ_2 subunit enable phasic inhibition, receptors harboring a δ subunit mediate tonic inhibition (Olsen & Sieghart, 2009).

To investigate the SQT modulation on phasic $GABA_A$Rs, HEK293 cells were transfected with $\alpha_1\beta_2\gamma_2$ receptors to see if the presence of the γ_2 subunit is responsible for the observed effects in hippocampal neurons (Figure 5a). Similar to the result obtained from whole-cell recordings on primary neurons, β-caryophyllene (90 ± 37%), β-caryolanol (72 ± 27%), α-humulene (97 ± 28%), and guaiol (100 ± 32%) had no significant effect on the currents evoked by 10 μM GABA, whereas co-application of humulol (84 ± 26%, $p = .016$, Table 3) significantly reduced maximum currents (Figure 5b–g). In transfected HEK293 cells with the $\alpha_1\beta_2\gamma_2$ subunits, diazepam (111 ± 35%) elicited increased currents ($p = .0596$). Application of $ZnCl_2$ (92 ± 36%) had no significant effect, verifying the presence of the γ_2 subunit in the receptor complexes. Picrotoxinin blocked GABA-induced currents by 55%–68%. Representative traces are shown in Figure 5h–j. Taken together, these results indicate that the γ_2 subunit plays a major role in the modulation of $GABA_A$Rs by SQTs.

3.4 | SQTs exhibited rather negative allosteric modulatory potential at tonic $GABA_A$Rs

Next, tonic $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ $GABA_A$Rs expressed in HEK293 cells were analyzed (Figure 6a, h). Here again, 10 μM GABA together with different SQTs were co-applied. Application of 10 μM GABA together with the SQTs saturates tonic receptors (Karim et al., 2013; Mortensen et al., 2012). Therefore, the modulatory potential obtained might differ from SQT effects under non-saturating conditions. All SQTs significantly reduced the maximal current response at $\alpha_4\beta_3\delta$ receptors (Figure 6b–g, Table 3), with guaiol (65 ± 23%, $p = .0075$) having the strongest effect. Similar results were obtained for $\alpha_6\beta_3\delta$ receptors with β-caryophyllene (76 ± 26%, $p = .0029$) and guaiol (75 ± 33%, $p = .0078$) significantly reducing GABA-induced I_{max} values. $\alpha_4\beta_3\delta$ receptors subtypes were significantly blocked by co-application of GABA and picrotoxinin while picrotoxinin block was less pronounced for $\alpha_6\beta_3\delta$ not reaching significance ($p = .0558$) in one block of measurements. The presence of the expression of tonic receptors was verified by

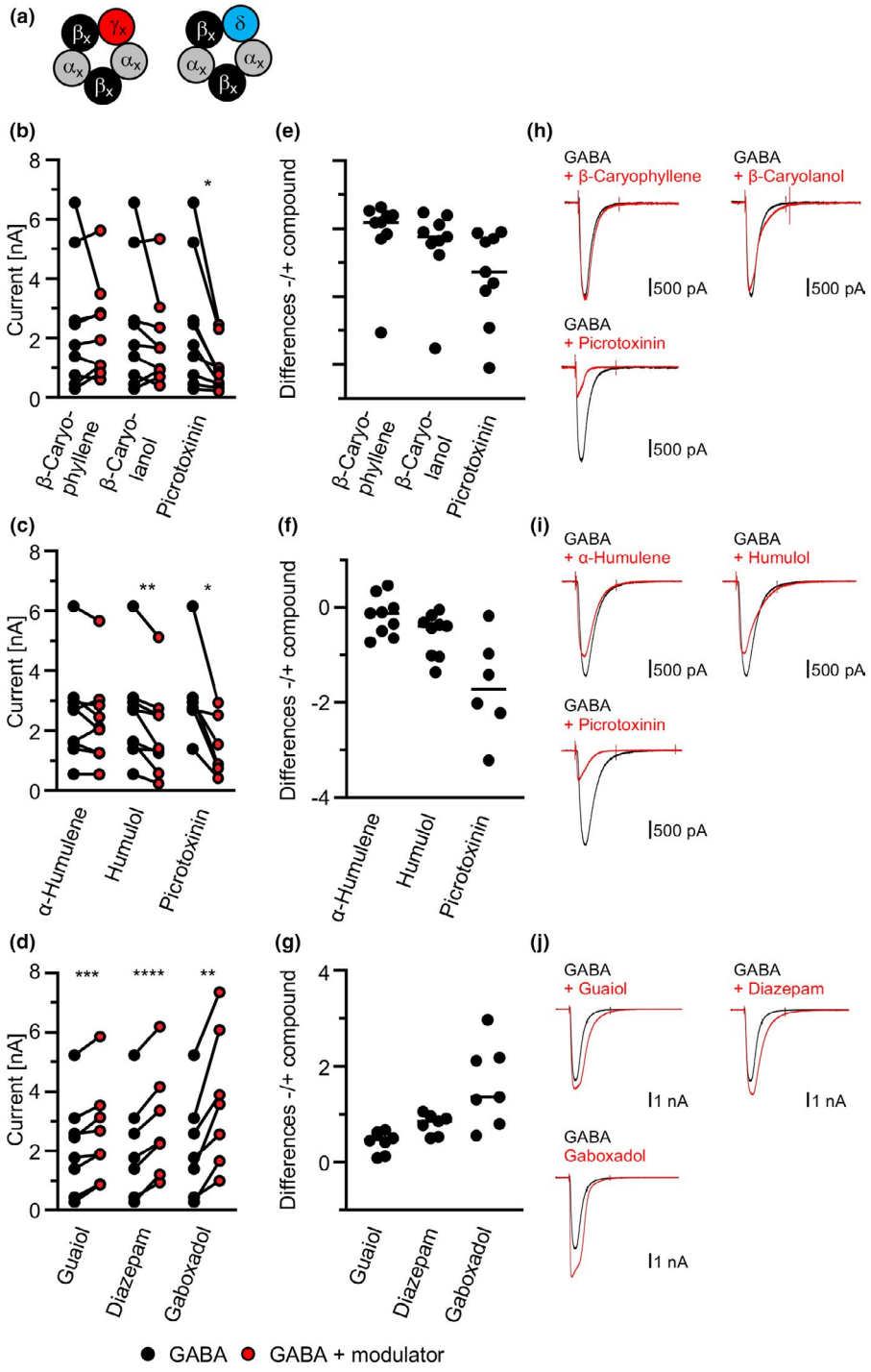

FIGURE 4 The modulatory potential of SQTs at $GABA_A$Rs differs between transfected cells and primary hippocampal neurons. (a) Possible receptor configurations for $GABA_A$Rs in hippocampal neurons. Subunits marked with *x* have multiple subtypes contributing to various receptor combinations, with $\alpha_1\beta_2\gamma_2$ being the major isoform. (b–d) Absolute currents of patch clamp recordings during application of 10 μM GABA (black dots) or with co-application of 600 μM modulator (red dots). Furthermore, 10 μM GABA was co-applied with 10 μM diazepam or 100 μM picrotoxinin. 1 mM gaboxadol was applied without co-application of GABA. Each pair of dots represents one patched hippocampal neuron at DIV18-21 in culture. Patch clamp experiments were performed on at least 3 days with cells isolated from different mice ($n = 3$), number of recorded cells varied between 6 and 9 (see also Table 2), significance level $^*p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$, $^{****}p \le .0001$. (e–g) Differences between each current pair in b-d are shown. (h–j) Representative current traces for each modulator in b–d are shown. Black traces: 10 μM GABA; red traces: 10 μM GABA +600 μM modulator/ 10 μM diazepam/ 100 μM picrotoxinin; 1 mM gaboxadol

gaboxadol which increased current amplitudes for $\alpha_6\beta_3\delta$ receptors. In sum, δ-subunit containing $GABA_A$Rs are most probably rather negatively modulated by SQTs.

3.5 | SQTs with their lipophilic scaffold fit well to the binding sites of neurosteroids and diazepam localized in the transmembrane segment of $GABA_A$Rs

A binding site for SQTs is not described, yet. SQTs are highly hydrophobic. Hence, they might favor a localization within the transmembrane segment of the $GABA_A$R in the lipid bilayer neighborhood. The low-affinity-binding site for diazepam and the neurosteroid-binding site have been described between transmembrane domains of $GABA_A$Rs (Masiulis et al., 2019; Miller et al., 2017). Molecular modeling and docking studies were performed for five SQTs investigated in detail in this study, namely β-caryophyllene, β-caryolanol, α-humulene, humulol, and guaiol. β-Caryolanol and humulol are derivatives of β-caryophyllene and α-humulene (Figure 1a) and differed in modulatory potential at $\alpha_1\beta_2$ $GABA_A$Rs compared to the non-metabolized substances (Figure 2). To determine whether and how the SQTs prefer binding

TABLE 2 Electrophysiological data obtained from hippocampal neurons

Compound	*c* [μM]	$I_{rel} \pm SEM$ [%]	$I_{abs} \pm SEM$ [nA]	*p*-values	*n*
GABA	10	100 ± 30	2.39 ± 0.72		9
β-Caryophyllene	600	94 ± 22	2.25 ± 0.54	0.7294	
β-Caryolanol	600	79 ± 21	1.90 ± 0.51	0.2557	
Picrotoxinin	100	41 ± 11	0.98 ± 0.28	0.0163*	
GABA	10	100 ± 21	2.54 ± 0.54		9
α-Humulene	600	93 ± 19	2.36 ± 0.49	0.2238	
Humulol	600	78 ± 19	1.97 ± 0.49	0.0056**	
Picrotoxinin	100	59 ± 16	1.51 ± 0.42	0.0119*	6
GABA	10	100 ± 26	2.16 ± 0.56		8
Guaiol	600	120 ± 27	2.59 ± 0.58	0.0008***	
Diazepam	10	138 ± 33	2.92 ± 0.69	<0.0001****	7
Gaboxadol	1,000	176 ± 41	3.73 ± 0.87	0.0025**	

Note: c, concentration; *SEM*, standard error of the mean; levels of significance with *p*-values $^{*}p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$, $^{****}p \le .0001$; *n*, number of recorded cells.

to the low-affinity diazepam site or the neurosteroid site, a docking to the complete αβ- or αα-interface, respectively, was first performed. Since two possible interfaces can be formed between the α- and the β-domain, namely $\beta_3^+\alpha_1^-$ (diazepam site) and $\alpha_1^+\beta_3^-$ (an alternative site), both were studied together. Although all substances were found in the diazepam site, often showing an extensive and reasonable overlap with the benzodiazepine-scaffold, α-humulene, and β-caryophyllene leave several hydrogen bonding groups of the pocket unsaturated. The hydroxylated compounds humulol (S-humulol but not R-humulol; both might be present in the humulol fraction) and β-caryolanol form hydrogen bonds to the β_3M2 or M3 side chains (β_3^+) within the diazepam-binding site, guaiol to helix α_1M1 (α_1^-), all fitting nicely into this pocket. With exception of R-humulol, the binding modes of the SQTs in the alternative $\alpha_1^+\beta_3^-$-site are less preferred.

In the case of docking to the entire αα-interface, all compounds could be found in the location analogous to the diazepam site, but only for the three compounds with hydroxy groups binding modes were obtained in the neurosteroid-binding site. A focused docking to this particular site showed that α-humulene and β-caryophyllene cannot form particularly favorable interactions here, whereas humulol, β-caryolanol, and also guaiol are able to form at least one of the two polar interactions of the endogenous ligand pregnanolone to the receptor backbone and fit well with their lipophilic scaffold (Figure 7). Thus, binding to both, the diazepam- and the pregnanolone-binding site can be suggested, with the poses in the first one scored better.

4 | DISCUSSION

$GABA_A$Rs facilitate inhibitory neurotransmission in the central nervous system. They present a potential target for anticonvulsive and anesthetic drugs (Chua & Chebib, 2017; Krall et al., 2015; Kreuzer et al., 2019; Laverty et al., 2017; Zhu et al., 2018). Moreover, naturally occurring substances, such as terpenoids, are able to modulate GABAergic function (Manayi et al., 2016). In this study, SQTs isolated from chamomile were found to harbor allosteric potential at $GABA_A$Rs in transfected cell lines and primary neurons. Previously, we have identified odorous monoterpene structures from *Sideritis* extracts with a preference of a bicyclic character in combination with the presence of a hydroxy group harboring positive allosteric potential at $GABA_A$Rs of the $\alpha_1\beta_2$ configuration in vitro (Kessler et al., 2012, 2014). Terpenoids represent a diverse group of natural compounds formed by the condensation of isoprene units with the potential to modulate $GABA_A$Rs and thus discussed for treatment of CNS disorders (Manayi et al., 2016). Valerenic acid, a sesquiterpenoid from *Valeriana officinalis* L., is used to treat anxiety and sleep disorders. It binds to the $GABA_A$Rs dependent on the β isotype present in the complex (Khom et al., 2007). For bilobalide, present in *Gingko biloba*, different actions at the $GABA_A$R have been described. Anticonvulsant but also antagonistic action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$Rs similar to bicuculline and picrotoxin have been found (Huang et al., 2003; Kiewert et al., 2007). Here, we studied the neurotropic potential of SQTs assumed to be taken up by nutrition and digested in the human body. Biotransformation processes might alter the modulatory potential, for example, gastrointestinal processes were identified to modulate the chemical composition of ingested aroma constituents thus changing bioactivity of essential oils (Heinlein & Buettner, 2012). However, about the underlying mechanism of the gut–brain axis there is little systematized effort.

α-humulene, a monocyclic, and β-caryophyllene, a bicyclic sesquiterpene, are major components of the volatile fraction of hop but also part of many other essential oils. As shown in previous studies, metabolization processes taking place during intake and digestion of these compounds lead to changes in the structure. Both, α-humulene and β-caryophyllene are hydroxylated because of the low pH in the gastric phase of digestion which

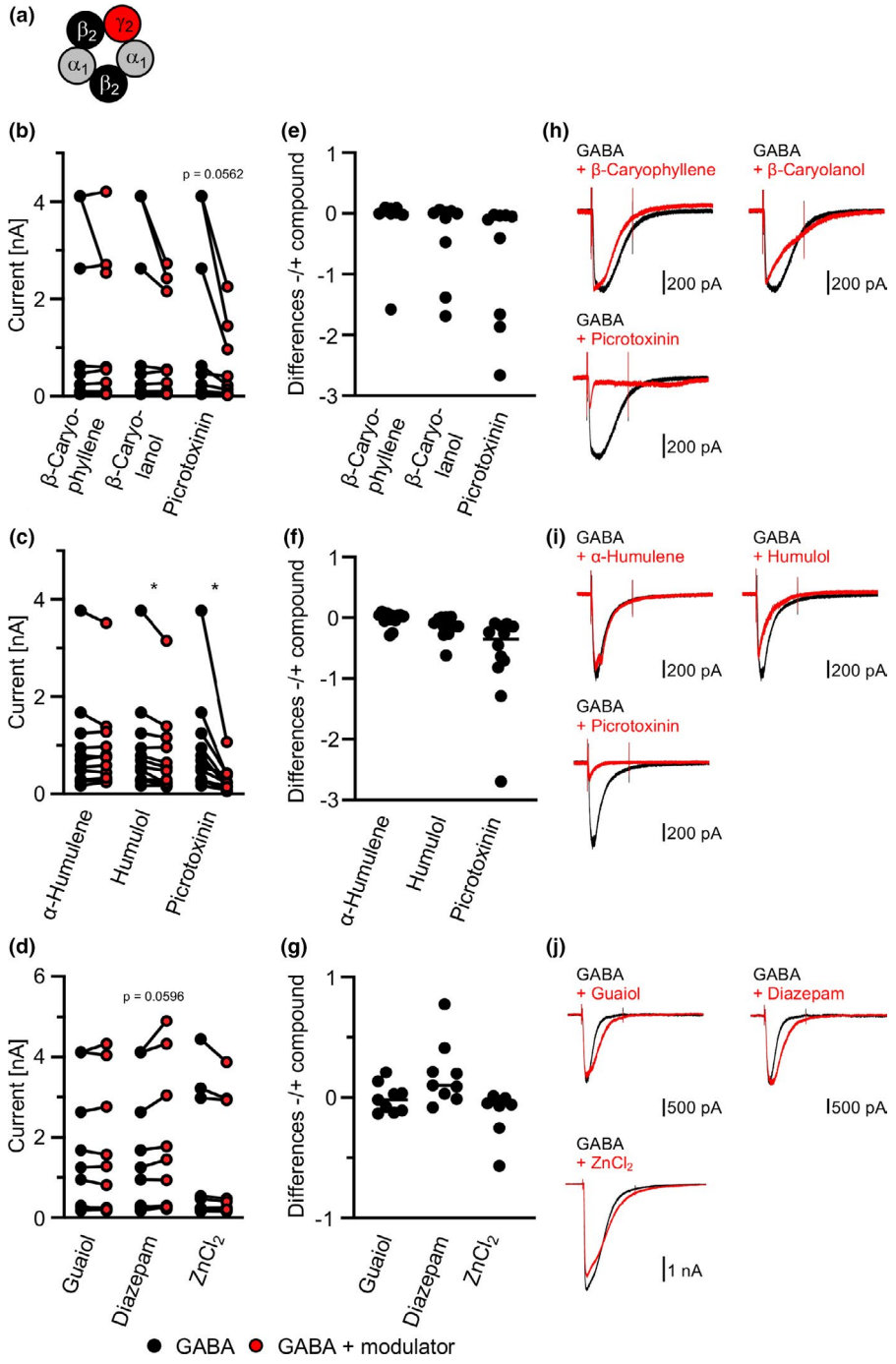

FIGURE 5 SQT modulation of phasic $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs. (a–d) Absolute currents of patch clamp recordings during application of 10 μM GABA (black dots) or with co-application of 600 μM modulator (red dots), recorded from HEK293 cells transfected with $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs. 10 μM GABA was co-applied with 10 μM diazepam, 10 μM $ZnCl_2$, or 100 μM picrotoxinin. Each pair of dots represents one patched HEK293 cell. Patch clamp experiments were performed on at least 3 days with different cell batches, total numbers of recorded cells (n = 8–12) are shown in Table 3. $^*p \le .05$, $^{**}p \le .01$. (e-g) Differences between each current pair in b-d are shown. (h-j) Example traces for each modulator in b-d are shown. Black traces: 10 μM GABA; red traces: 10 μM GABA +600 μM modulator/ 10 μM diazepam/ 10 μM $ZnCl_2$/ 100 μM picrotoxinin

may affect the modulatory potential of the substance (Heinlein & Buettner, 2012). Indeed, in transfected HEK293 cells with $GABA_A$Rs of the $\alpha_1\beta_2$ subtype, the hydroxylated metabolites humulol and β-caryolanol showed positive allosteric potential by significantly increasing the GABAergic current upon co-application of GABA in a low concentration. In contrast, the parent compounds α-humulene and β-caryophyllene had no effect on GABAergic current. These data are in line with previous reports showing positive allosteric modulation of the GABAergic currents by terpenes and terpenoids harboring a hydroxy group (Kessler

TABLE 3 Electrophysiological data obtained from $\alpha1\beta2\gamma2$, $\alpha_4\beta_3\delta$, and $\alpha_6\beta_3\delta$ $GABA_A$Rs

$GABA_A$R configuration	Compound	*c* [μM]	$I_{rel} \pm SEM$ [%]	$I_{abs} \pm SEM$ [nA]	*p*-values	*n*
$\alpha_1\beta_2\gamma_2$	GABA	10	100 ± 42	1.38 ± 0.58		9
	β-Caryophyllene	600	90 ± 37	1.24 ± 0.51	0.4508	
	β-Caryolanol	600	72 ± 27	0.99 ± 0.37	0.1230	
	Picrotoxinin	100	45 ± 19	0.62 ± 0.26	0.0562	
$\alpha_1\beta_2\gamma_2$	GABA	10	100 ± 31	0.93 ± 0.29		12
	α-Humulene	600	97 ± 28	0.90 ± 0.26	0.4655	
	Humulol	600	84 ± 26	0.78 ± 0.25	0.0162*	
	Picrotoxinin	100	32 ± 9	0.30 ± 0.08	0.0137*	
$\alpha_1\beta_2\gamma_2$	GABA	10	100 ± 31	1.72 ± 0.52		9
	Guaiol	600	100 ± 32	1.71 ± 0.54	0.8838	
	Diazepam	10	111 ± 35	1.91 ± 0.59	0.0596	
	$ZnCl_2$	10	92 ± 36	1.41 ± 0.55	0.1079	8
$\alpha_4\beta_3\delta$	GABA	10	100 ± 38	0.98 ± 0.38		9
	β-Caryophyllene	600	80 ± 33	0.78 ± 0.32	0.0118*	
	β-Caryolanol	600	72 ± 32	0.70 ± 0.31	0.0049**	
	Picrotoxinin	100	27 ± 8	0.26 ± 0.08	0.0421*	
$\alpha_4\beta_3\delta$	GABA	10	100 ± 27	0.78 ± 0.21		10
	α-Humulene	600	77 ± 21	0.60 ± 0.16	0.0248*	
	Humulol	600	69 ± 17	0.53 ± 0.13	0.0199*	
	Picrotoxinin	100	32 ± 9	0.25 ± 0.07	0.0178*	
$\alpha_4\beta_3\delta$	GABA	10	100 ± 31	1.27 ± 0.39		9
	Guaiol	600	65 ± 23	0.83 ± 0.29	0.0075**	
	Gaboxadol	1,000	90 ± 30	1.15 ± 0.38	0.3296	
$\alpha_6\beta_3\delta$	GABA	10	100 ± 30	0.52 ± 0.16		9
	β-Caryophyllene	600	76 ± 26	0.39 ± 0.14	0.0029**	
	β-Caryolanol	600	96 ± 35	0.50 ± 0.18	0.6095	
	Picrotoxinin	100	24 ± 6	0.12 ± 0.03	0.0225*	
$\alpha_6\beta_3\delta$	GABA	10	100 ± 33	0.17 ± 0.06		8
	α-Humulene	600	86 ± 27	0.15 ± 0.05	0.2121	
	Humulol	600	98 ± 30	0.17 ± 0.05	0.8655	
	Picrotoxinin	100	43 ± 16	0.07 ± 0.03	0.0558	
$\alpha_6\beta_3\delta$	GABA	10	100 ± 37	0.50 ± 0.18		8
	Guaiol	600	75 ± 33	0.37 ± 0.16	0.0078**	
	Gaboxadol	1,000	166 ± 69	0.83 ± 0.34	0.0918	

Note: *c*, concentration; *SEM*, standard error of the mean; levels of significance with *p*-values $^*p \le .05$, $^{**}p \le .01$; *n*, number of recorded cells.

et al., 2014). Guaiol, however, a sesquiterpenoid also bearing one hydroxy group like β-caryolanol and humulol exhibited a negative allosteric effect at the same receptor type. Attempts to predict additive or neutralizing effects using co-application of two sesquiterpenes harboring positive allosteric potential or with contrary potential failed. Thus, a prediction of the neurotropic activity seems to be difficult from the observed functional data following single application. Contrary actions at $GABA_A$Rs have been observed for bilobalide but also for other substances such as ethanol, arguing for affinity, subtype-specific, and possibly cell-specific effects (Forstera et al., 2016; Kiewert et al., 2007).

The lipophilicity of SQTs does allow binding to the lipid environment of $GABA_A$Rs. Structural similarities to diazepam and neurosteroids point also to binding in a hydrophobic pocket formed between transmembrane domains of the $GABA_A$R (Laverty et al., 2017; Masiulis et al., 2019). Computational docking studies at the neurosteroid and the diazepam-binding sites, which are localized in a lipophilic surrounding, indicated possible binding modes in both sites. Nevertheless, the diazepam site as well as the alternative $\alpha_1^+\beta_3$-site also contain some polar amino acids. As these would presumably interact with water molecules in the unbound state, binding at these sites would be expected to result in a strong desolvation penalty,

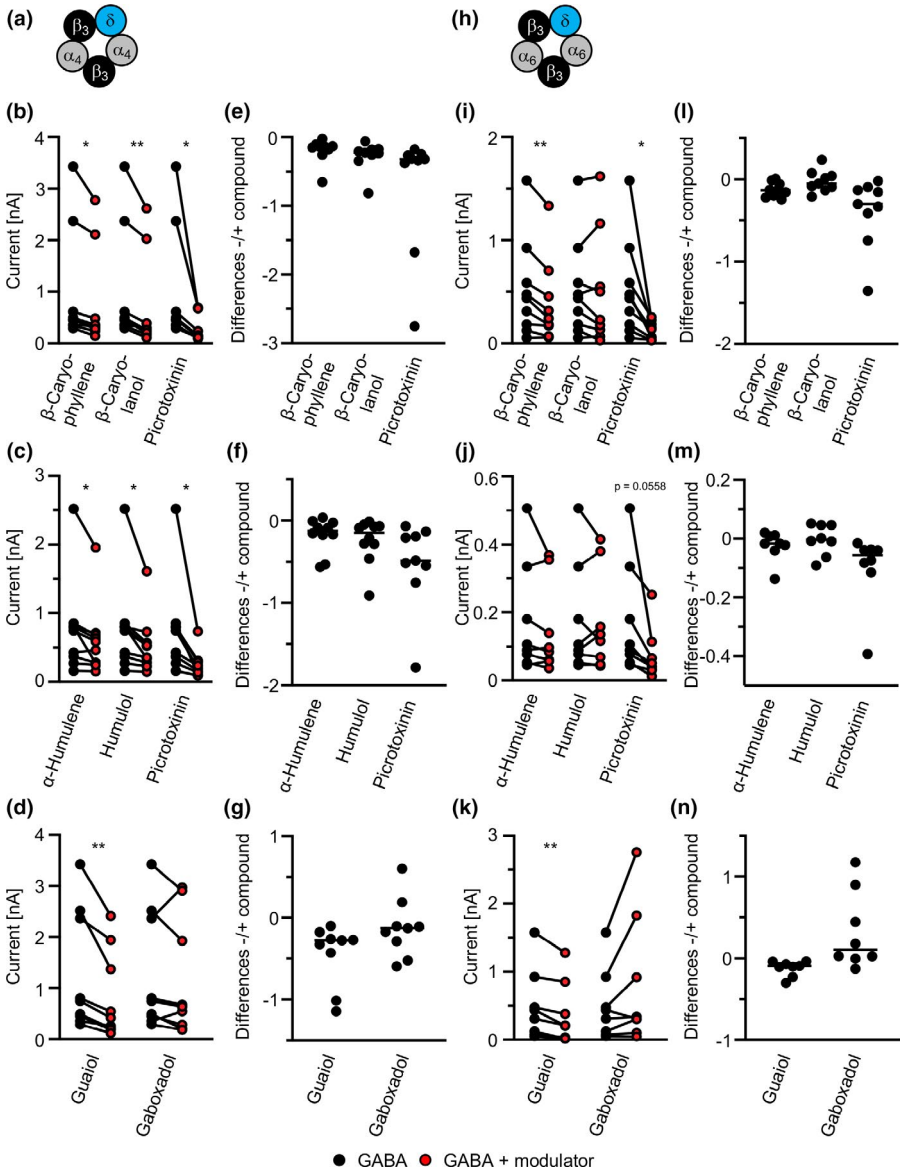

FIGURE 6 Modulation of tonic $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ $GABA_A$Rs by SQTs. (a–g) Measurements on $\alpha_4\beta_3\delta$ $GABA_A$Rs. (h–n) Measurements on $\alpha_6\beta_3\delta$ $GABA_A$Rs. B–d, i–k) Absolute currents of patch clamp recordings from HEK293 cells transfected with $\alpha_4\beta_3\delta$ or $\alpha_6\beta_3\delta$ $GABA_A$Rs, respectively, during application of 10 μM GABA (black dots) or following co-application of 600 μM modulator (red dots). Furthermore, 10 μM GABA was co-applied with 100 μM picrotoxinin. 1 mM gaboxadol was applied without co-application of GABA. Each pair of dots represents one patched HEK293 cell. Patch clamp experiments were performed on at least 3 days with different cell batches, number of recorded cells were between 8 and 10. $^*p \le .05$, $^{**}p \le .01$. (e–g, l–m) Differences between each current pair in b–d, i–k are shown

which is not sufficiently considered by the scoring functions. Even though humulol and β-caryolanol can form polar interactions here, the binding modes of these otherwise completely hydrophobic molecules always show an unfavorably positioned methyl group, for instance, near the negatively charged Asp272 of helix β_3M3. The pregnanolone-binding site, on the other hand, is composed entirely of lipophilic amino acids except at the extreme ends, where the endogenous ligand forms polar interactions with the receptor. In addition, this binding site is rather surface-exposed. This is reflected by a comparison of buried surfaces: whereas diazepam is completely buried and shows no solvent accessible surface area in the complex, for pregnanolone only 69% of the ligand surface area are buried upon binding to the receptor. However, the remaining 31% of the apolar ligand surface are not directed toward the aqueous solution, but rather toward the lipid bilayer, which is energetically attractive. This difference in burial is markedly reflected in the scoring, which favors direct interactions, but does not consider favorable exposure to a lipophilic environment. Accordingly, better scores of the poses in the diazepam-binding site should not be overinterpreted. Rather, the structure of the neurosteroid-binding site would better match the scaffold of hydroxylated SQTs, which is also supported by the observation that the hydroxylated SQTs, when docked to the entire αα-interface, were located in the neurosteroid-binding site, whereas the non-hydroxylated ones were not found at that location. Hence, binding to the lipophilic interface forming the neurosteroid-binding site is possibly preferred over the diazepam-binding site, especially for humulol and β-caryolanol. In addition, it is known that the hydroxy group of neurosteroids is crucially involved in their GABA-potentiation effects (Miller et al., 2017), which could possibly explain the effects of SQTs. Nevertheless, an interaction to other modulatory proteins of the $GABA_A$R cannot be excluded.

Previous data on monoterpenoids suggested positive allosteric potential at $GABA_A$Rs independent of the γ_2 subunit (Kessler et al., 2014). Our initial comparisons of α-humulene and β-caryophyllene versus their respective derivatives humulol and β-caryolanol, suggested a similar pattern. However, other tested

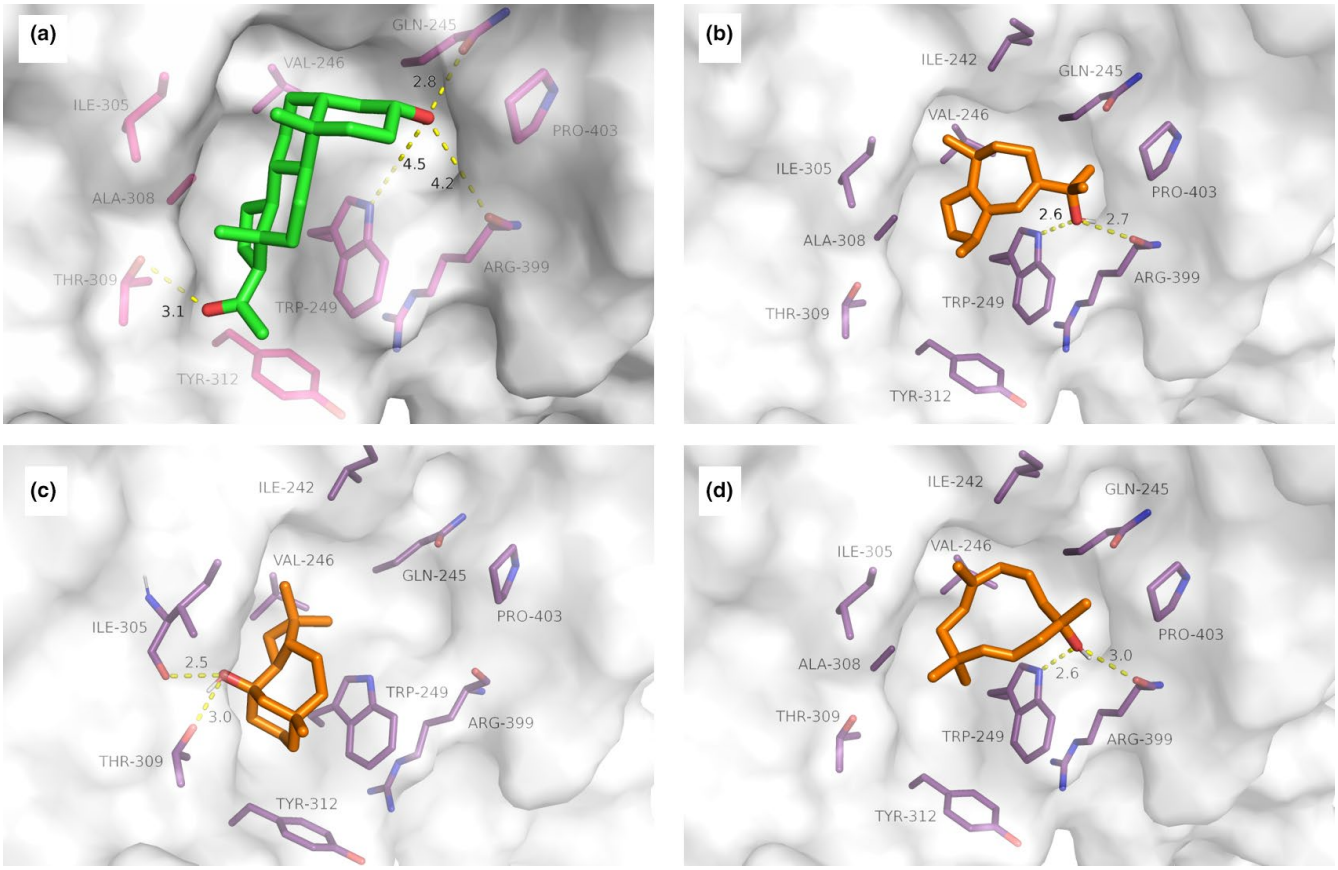

FIGURE 7 Molecular docking results at the neurosteroid-binding site localized in the TM domain of the $GABA_AR$. As a reference, the experimentally observed binding mode of pregnanolone in the αα-interface (PDB: 5O8F) is shown in (a). Binding modes proposed by docking of the SQTs guaiol (b), β-caryolanol (c), and S-humulol (d) are illustrated based on the top-scoring docking solutions

sesquiterpenoids also harboring a hydroxy group, for example, bisabolol, bisabolol oxide B, and spathulenol did not follow this pattern. Therefore, we investigated sesquiterpene modulation at isolated primary neurons of the hippocampus rich in GABAergic interneurons (Pelkey et al., 2017). The main receptor configuration is the $\alpha_1\beta_2\gamma_2$ subtype (Olsen & Sieghart, 2008). Those $GABA_ARs$ enable phasic inhibition whereas subtypes including the α4, α6, or δ subunit regulate long-lasting inhibition and thereby control the excitability of the neurons (Lee & Maguire, 2014). The neurotropic activity of the SQTs was different on primary hippocampal neurons in comparison to the controlled over-expression of the $\alpha_1\beta_2$ subtype in HEK293 cells. While β-caryolanol showed no longer a significant modulatory effect, humulol turned into a negative and guaiol into a positive allosteric modulator. Control measurements using the superagonist gaboxadol and the benzodiazepine agonist diazepam confirmed the presence of phasic as well as tonic $GABA_ARs$ in the hippocampal neurons. Back to controlled over-expression, the presence of the γ_2 subunit in the $\alpha_1\beta_2\gamma_2$ subunit composition led to the same observation as in hippocampal neurons with humulol as a negative allosteric modulator. Hence, the presence of the γ_2 subunit in the majority of $GABA_ARs$ (65%) in primary neurons is an important component for sesquiterpene modulation. At tonic $GABA_ARs$ in the $\alpha_4\beta_3\delta$ configuration, β-caryophyllene and α-humulene as well as the sesquiterpenoids humulol and β-caryolanol but also guaiol exhibited negative allosteric modulation. However, all data were obtained under GABA-saturating conditions. At $\alpha_6\beta_3\delta$ receptors, β-caryophyllene and guaiol but not β-caryolanol, humulene and humulol decreased GABAergic currents. As both tonic receptor configurations ($\alpha_4\beta_3\delta$, $\alpha_6\beta_3\delta$) harbor β3, the observed effects are most probably independent of the β subunit and therefore dissimilar to modulation of $GABA_ARs$ by valerenic acid remarkably dependent on the β subunit isotype ($\beta_3<\beta_2«\beta_1$) (Khom et al., 2007).

Positive allosteric modulation of tonic $GABA_ARs$ has been reported for monoterpenoid structures, verbenol and myrtenol, measured on hippocampal slices (van Brederode et al., 2016). In contrast to monoterpenes, our results show the negative modulatory potential of SQTs at $GABA_ARs$ mainly at tonic receptor subtypes. Bilobalide, a sesquiterpene lactone, antagonizes $GABA_ARs$ similar to bicuculline and picrotoxinin and the SQTs used here, but also exerts anticonvulsant activity via a presynaptic route maintaining the GABA levels and thus the GAD (glutamate decarboxylase) activity in the hippocampus and cerebral cortex (Sasaki et al., 2000). The action of sesquiterpenes at postsynaptic $GABA_ARs$ but also at presynaptic sites regulating the activity of the GABA synthesizing enzyme GAD has been supported recently for the malaria drugs artemisinins. Artemisinins modulate $GABA_ARs$ via targeting the binding epitope of the scaffold protein gephyrin by hindering the critical interaction of gephyrin and $GABA_ARs$

and thus decreasing GABAergic inhibition (Kasaragod et al., 2019). Moreover, sesquiterpenes interact with PDXK (pyridoxal kinase), an enzyme regulating the synthesis of GABA via GAD activity in presynaptic terminals (Kasaragod et al., 2020). Both of the abovementioned actions, however, require a longer presence of the SQTs enabling uptake into the cell to facilitate their action.

Based on a qualitative analysis of the binding modes obtained from our docking studies, preferential binding of the hydroxy group carrying SQTs to the neurosteroid-binding site and the diazepam site seems possible. Furthermore, we cannot exclude an indirect interaction of SQTs with associated proteins of the $GABA_AR$ or interactions with presynaptic enzymes and thereby modulating GABAergic activity. In our approach, the SQTs were co-applied together with the neurotransmitter GABA enabling monitoring of short and direct effects on receptor opening and closing only. Together with the recently observed shared potential of SQTs at postsynaptic and presynaptic sites, short-term and long-term aspects at GABAergic neurotransmission in the presence of SQTs should be investigated in more detail in the future, combined with affinity and binding studies to differentiate direct from indirect actions of SQTs on GABAergic inhibition processes. A better understanding of these mechanisms will further define their therapeutic potential for CNS disorders.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

CV and AB participated in research design. Electrophysiology was performed by DJ. GC-MS and isolation of SQTs was performed by BS. DJ, CV, BS, and HL carried out data analysis. MZ and CS carried out modeling and docking analysis. CV, DJ, and BS contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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