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Modulation of GABAergic Synaptic Currents and Current Responses by α-Thujone and Dihydroumbellulone

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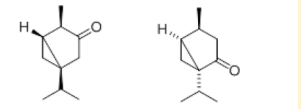
ABSTRACT: α-Thujone (**1a**), a constituent of wormwood, has been suspected to cause adverse psychoactive reactions in addicted drinkers of absinthe. While the content of **1a** in absinthe is too low for such effects, at higher doses it can indeed induce seizures and inhibit $GABA_A$ receptors ($GABA_ARs$). The effect of **1a** on GABAergic synaptic currents and the mechanisms by which it modulates $GABA_ARs$ remain unknown. To address these issues, cultured hippocampal neurons were used to investigate the action of **1a** on GABAergic miniature inhibitory postsynaptic currents (mIPSCs) and on responses to exogenous GABA applications. Since lipophilic compounds often show nonspecific actions related to their hydrophobicity, the action of **1a** was compared to that of dihydroumbellulone (**2**), a configurationally pseudoenantiomeric constitutional isomer. α-Thujone (**1a**) reduced mIPSC frequency and amplitude and also moderately affected their kinetics, indicating both pre- and postsynaptic mechanisms. Analysis of current responses to exogenous GABA revealed that **1a** reduced their amplitude, affecting their onset, desensitization, and deactivation, suggesting an effect on receptor gating. In contrast, **2** caused only a weak or negligible effect on GABAergic currents, supporting the effects of **1a** on GABAergic inhibition as being due to specific interactions with $GABA_ARs$.



Few natural products are more controversial than the monoterpene ketone α-thujone (**1a**), a compound found in absinthe. The consumption of absinthe, an alcoholic beverage very popular in late 19th century France,[1] was linked to a psychoactive syndrome characterized by hallucinations and seizures (absinthism).[2] This was, in turn, correlated to the presence of **1a**, a major constituent of some chemotypes of wormwood (*Artemisia absinthium* L.), a major ingredient of absinthe.[3] The association between α-thujone and absinthism has always been controversial and seems nowadays substantially disproved,[1,4] as is the suggestion that **1a**, on account of an alleged structural similarity with Δ^9-THC, can behave as a biologically active analogue of the psychotropic constituent of marijuana (*Cannabis sativa* L.).[5] α-Thujone (**1a**) shows a certain binding affinity to CB1 cannabinoid receptors, but it is substantially unable to activate them.[6] On the other hand, modern research has confirmed that **1a**, at dosages unlikely to be reached by the consumption of absinthe, can indeed induce seizures, one of the hallmarks of absinthism.[7,8] This effect can be alleviated by benzodiazepines,[8] a class of $GABA_AR$ positive modulators, and **1a** has been shown to down-regulate GABAergic currents, suggesting an interaction with $GABA_ARs$.[8] An inhibitory effect of **1a** on $GABA_A$ receptors was also observed in the *Xenopus laevis* oocyte expression model,[9] whereas potent inhibition of another cys-loop receptor, $5\text{-}HT_3$, was further demonstrated.[10] On the other hand, monoterpenes structurally unrelated to **1a**, such as menthol[9] and thymol,[11] have also been shown to exert a modulatory action on $GABA_ARs$, with no substantial difference in affinity being observed between α-thujone (**1a**) and its C-1 epimer, β-thujone (**1b**).[9] These observations show that **1a** is a modulator of $GABA_ARs$, potentially capable of inducing seizures at high and nondietary dosages, but do not rule out the involvement of lipophilic stickiness rather than a specific protein binding in its action. Also unclear is the overall relevance of **1a** to synaptic GABAergic transmission, the major form of physiological activity of $GABA_ARs$.

To address these issues, cultured hippocampal neurons were used to investigate the comparative effects of **1a** and its closely related isomer, dihydroumbellulone (**2**), on GABAergic miniature inhibitory postsynaptic currents (mIPSCs) and on current responses evoked by rapid GABA applications. Compared to α-thujone (**1a**), the structure of **2** is characterized by the translocation of the carbonyl to the adjacent methylene carbon and by a different configuration of the ring fusion, configurationally enantiomeric to that of β-thujone (**1b**) and pseudoenantiomeric with that of α-thujone (**1a**). While maintaining an overall structural similarity to the thujones, these changes should be sufficient to be translated into differences in bioactivity when specific small-molecule−protein interactions are involved.

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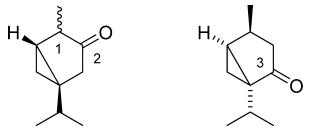

1a: β-Methyl
1b: α-Methyl

2

RESULTS AND DISCUSSION

mIPSCs were recorded in the whole-cell configuration of the patch-clamp technique at the membrane voltage of −70 mV (Figure 1a). In control conditions, the mean mIPSC amplitude was −52.80 ± 3.63 pA (n = 16), and their frequency was 0.97 ± 0.11 Hz (n = 16). The action of **1a** and **2** was evaluated for each cell by dividing the current amplitude measured in the presence of a compound by the control value measured for the same cell. At 100 μM, α-thujone (**1a**) reduced the mIPSC amplitude significantly (relative amplitude 0.64 ± 0.01, n = 4, p < 0.05, Figure 1b). A concentration increase to 300 μM caused only a slightly larger reduction of the relative amplitude (0.59 ± 0.02, n = 7, p < 0.05, Figure 1b; relative amplitudes at 100 and 300 μM were not significantly different), suggesting saturation of this effect. Dihydroumbellulone (**2**) at a concentration of 300 μM induced only a modest, but still significant, reduction of the mIPSC amplitude (relative amplitude 0.90 ± 0.03, n = 5, p < 0.05, Figure 1b). The impact of **1a** and **2** on mIPSC amplitudes is further illustrated by the cumulative plots presented in Figure 1e. α-Thujone (**1a**) caused a large and dose-dependent decrease in mIPSC frequency (Figure 1c). At 100 μM, a more than 2-fold frequency reduction was observed (relative frequency 0.41 ± 0.02, n = 4, p < 0.05), which further decreased at 300 μM (0.26 ± 0.07, n = 7, p < 0.05; an overall 4-fold decrease). mIPSC frequency also showed a trend of decreasing in the presence of 300 μM **2**, but this effect did not reach statistical significance (0.75 ± 0.08, n = 5, p > 0.05, Figure 1c). Notably, concomitant reduction of mIPSC amplitude and frequency by **1a** caused a strong reduction of the total charge transfer (see Experimental Section; relative total charge transfer 0.27 ± 0.07, n = 4, p < 0.05 and 0.15 ± 0.06, n = 4, p < 0.05 for 100 and 300 μM **1**), while the effect of **2** on this parameter did not reach statistical significance (0.66 ± 0.14, n = 5, p > 0.05 for 300 μM **2**).

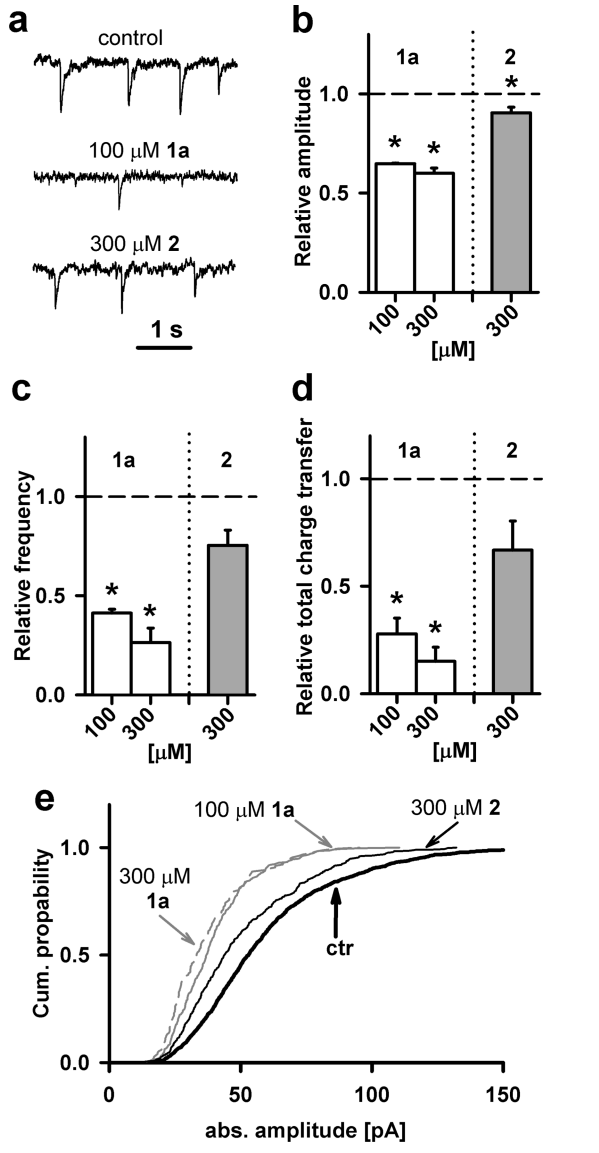

Figure 1. α-Thujone (**1a**) reduces amplitude of the mIPSCs. (A) Typical mIPSC traces recorded at −70 mV in control conditions (upper trace), in the presence of 100 μM **1a** (middle trace), and in the presence of 300 μM dihydroumbellulone (**2**) (lower trace). (b) Statistics of effects of **1a** and **2** on mIPSC amplitudes. (c) Effect of **1a** and **2** on mIPSC frequency. (d) Statistics for the effects of **1a** and **2** on the total charge transfer. (e) Cumulative histograms for all mIPSC amplitude values measured at the concentrations of **1a** and **2** indicated on the graph and for control conditions. In (b)−(d), the statistics of relative values are shown with respect to the controls measured on the same cells. Asterisks indicate statistically significant differences.

The efficiency of synaptic signal integration depends not only on the amplitude of synaptic currents but also on their time course. The effects of **1a** and **2** were analyzed therefore for two key parameters of mIPSC kinetics, namely, the rise time (assessed as 10−90% amplitude rise time) and the decay time course, determining these parameters for averaged mIPSC (see Experimental Section). In control conditions, the rise time was 0.86 ± 0.05 ms (n = 14, p < 0.05), and it was markedly slowed in the presence of **1a** (relative rise time 1.38 ± 0.10, n = 4, p < 0.05 and 1.48 ± 0.20, n = 5, p < 0.05 for 100 and 300 μM **1**, Figure 2a, b). On the other hand, **2** did not affect the kinetics of mIPSC onset significantly (relative rise time 0.95 ± 0.09, n = 5, p > 0.05; Figure 2b). The decaying phase of mIPSCs could be fitted satisfactorily with a biexponential function with the weighted time constant of τ_{mean} = 20.36 ± 1.34 ms (n = 13). Within the concentration range investigated, neither compound exhibited any significant effect on τ_{mean} (relative decay 1.03 ± 0.21, n = 4; 1.01 ± 0.14, n = 4; 1.06 ± 0.13, n = 5 for 100 μM **1a**, 300 μM **1a**, and 300 μM **2**, Figure 2c, d). Taken together, these results show that **1a** can affect markedly mIPSC amplitude, frequency, and onset, having, however, no effect on their decay time course. Compound **2** exerted much weaker effects on the mIPSC amplitude and frequency.

Analysis of the effect of **1a** and **2** on mIPSCs provides information on the overall effect of these compounds on GABAergic synaptic transmission. However, as discussed elsewhere,[12−14] this analysis cannot provide an unambiguous insight into the underlying pharmacological mechanism(s). To shed light on the action of these compounds on specific $GABA_AR$ properties (binding and gating), the information obtained from mIPSCs was therefore complemented with the analysis of current responses to a rapid agonist application. First, responses elicited by low GABA concentration (3 μM, applied for 8 s with

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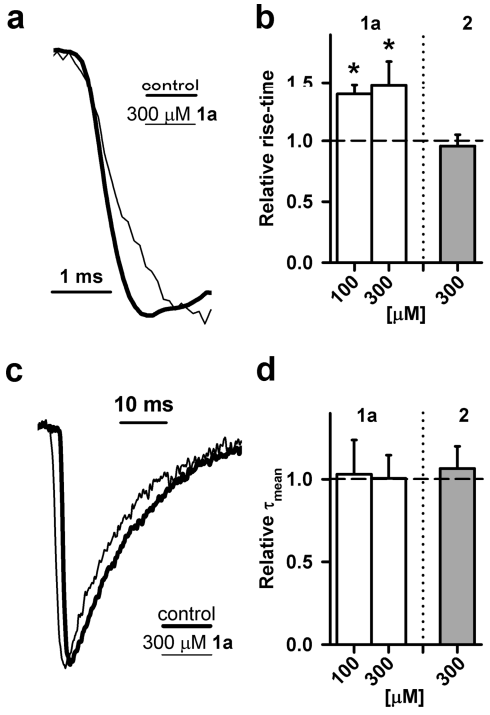

Figure 2. α-Thujone (**1a**) slows the onset of mIPSCs but does not affect their decay. (a) Example of normalized rising phase of mIPSCs recorded from the same neuron in control conditions (thick line) and in the presence of 300 μM **1a** (thin line). (b) Statistics of the action of **1a** and **2** on the mIPSC 10−90% rise time. (c) Typical normalized and superimposed averaged mIPSCs determined in control conditions (thick line) and in the presence of **1a** (thin line). For the sake of clarity, the traces were shifted horizontally with respect to each other. (d) Statistics of the action of **1a** or **2** on the mean decay time constant (τ_{mean}). Asterisks indicate statistically significant differences.

the Bio-Logic system; see Experimental Section) were analyzed, which provide key information on basic receptor properties considered.[14] These recordings were performed in the whole-cell configuration of the patch-clamp technique, setting the membrane voltage at −40 mV to avoid excessively large current amplitudes.[15] The mean current amplitude in control conditions was −1844 ± 162.05 pA (n = 50). High concentrations of **1a** clearly reduced the amplitude of these currents (relative amplitudes were 0.63 ± 0.05, n = 13, p < 0.05 and 0.71 ± 0.09, n = 10, for 100 and 300 μM α-thujone, p < 0.05, Figure 3a, b). However, at lower concentrations of **1a** (10−30 μM), the effect was modest and amplitudes were not significantly different from controls (Figure 3b). On the other hand, **2** did not cause any significant changes in the amplitude of current responses even at 300 μM concentrations (Figure 3b). Since **1a** exerted a marked effect on the onset kinetics of mIPSC, it was important to check if this compound could also modulate the rising phase of the current responses. Interestingly, **1a** strongly slowed the onset kinetics of these currents in a dose-dependent manner (relative rise time 2.33 ± 0.49, n = 13, p < 0.05 and 2.96 ± 0.67, n = 10, p < 0.05 for 100 and 300 μM; Figure 3c, d), while **2** at concentrations up to 1 mM failed to significantly affect the current onset (Figure 3d). As shown in Figure 3e, f, **1a** could also decrease the extent of current fading ($(I_{Peak} - I_{End})/I_{Peak}$; see Experimental Section) in a dose-dependent manner (control: 0.31 ± 0.01, n = 37; 10 μM **1a**: 0.30 ± 0.03, n = 4, p > 0.05; 30 μM **1a**: 0.28 ± 0.05, n = 10; 100 μM **1a**: 0.21 ± 0.02, n = 13, p < 0.05; 300 μM **1a** 0.18 ± 0.03, n = 10, p < 0.05; Figure 3f).

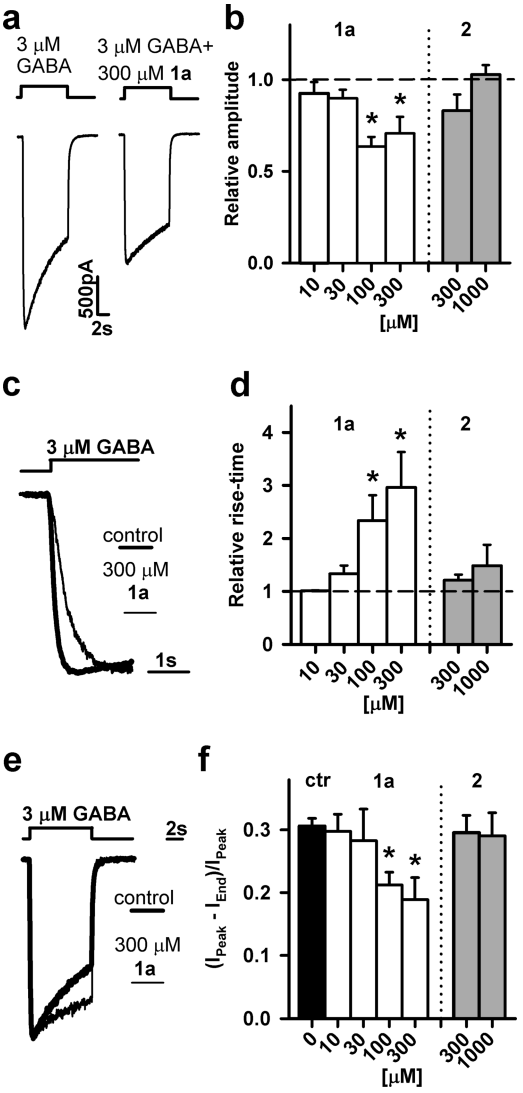

Figure 3. α-Thujone (**1a**), but not dihydroumbellulone (**2**), reduces the amplitude of currents evoked by low GABA concentration (3 μM). (a) Representative current responses evoked by application of 3 μM GABA in control conditions (left) and in the presence of 300 μM **1a** (right) recorded from the same neuron. (b) Statistics of the effect of **1a** and **2** on current amplitudes. (c) Normalized and superimposed initial parts of current traces recorded in control conditions (thin line) and in the presence of 300 μM **1a** (thick line). (d) Statistics of the onset kinetics for currents recorded in the presence of **1a** and **2**. (e) Normalized and superimposed initial parts of current traces recorded in control conditions (thin line) and in the presence of 300 μM **1a** (thick line). Note a markedly stronger fading in the presence of **1a**. (f) Statistics of the extent of fading for currents recorded in the presence of **1a** and **2**. Insets above the current traces in (a), (c), and (d) indicate GABA application. Asterisks indicate statistically significant differences.

Since fading is most likely associated with receptor desensitization (but to some extent also with binding),[14] these observations provide the first direct proof that α-thujone (**1a**) can affect both binding and gating of $GABA_A$Rs. No significant effect of **2** (300 μM and 1 mM) on current fading was found (Figure 3f). Since **1a** and **2** have very similar polarities, these observations suggest that the action of **1a** on $GABA_A$Rs is due to a specific interaction and not simply to lipophilic interactions or perturbation of membrane fluidity.

To further evaluate the effect of **1a** on $GABA_A$Rs, the analysis of current responses was extended to currents evoked

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by 30 μM GABA, a concentration close to the Ec_{50} value for this agonist[13,16,17] and for which a particularly strong dependence of current amplitude and kinetics on receptor binding properties is expected. At this GABA concentration, the current onset occurs within a few milliseconds[14] and is beyond resolution of the multibarrel system (Bio-Logic). Therefore, at this as well as at higher GABA concentrations, the ultrafast, piezoelectric-driven perfusion system was used (exchange time 100−150 μs; see Experimental Section), and recordings were performed in the excised-patch mode. The mean amplitude in control conditions was −141.25 pA (n = 9) and was reduced significantly by a 300 μM concentration of **1a** (relative amplitude 0.81 ± 0.05, n = 9, p < 0.05). Moreover, the 10−90% rise time, which was 2.06 ± 0.20 ms (n = 9) in the control, was prolonged significantly by 300 μM **1a** (relative rise time 1.38 ± 0.13, n = 9, p < 0.05). At saturating GABA concentrations, the binding step, which was completed much faster than the conformational transitions between fully bound states (gating), becomes rate limiting and largely determines the time course of current responses.[14] To evaluate the effect of **1a** on gating, its impact on current responses to a 10 mM GABA application for different time intervals was analyzed. The mean amplitude of current responses recorded in the excised patch mode at the membrane voltage of −40 mV was −913 ± 276 pA (n = 13). In accordance with the observations on the responses evoked by lower concentrations of GABA (Figures 3 and 4), it was found that 300 μM **1a** significantly reduced the amplitude (relative amplitude 0.69 ± 0.04, n = 7, p < 0.05; Figure 4a, b), while no effect was observed with **2** (0.94 ± 0.04, n = 4, p > 0.05; Figure 4b).The rise time of these responses (0.45 ± 0.08 ms, n = 11, in control conditions) was slowed by 300 μM **1a** (relative rise time 1.29 ± 0.11, n = 7, p > 0.05; Figure 4c, d), but not by the same concentration of **2** (0.98 ± 0.06, n = 4, p > 0.05). Remarkably, these data are qualitatively similar to those obtained for synaptic currents. At all GABA concentrations assayed, repeatedly evoked responses in the presence of **1a** (the standard application protocol consisted of three subsequent sweeps) showed a constant amplitude, strongly suggesting the lack of use-dependent inhibition.

Next, the deactivation kinetics (time course of the current following agonist removal) of responses evoked by short (3 ms) applications of a saturating neurotransmitter (Figure 5a) was assessed. Current deactivation was fitted with a sum of three exponents (see Experimental Section), and in control conditions, the mean deactivation time constant was τ_{mean}= 45.34 ± 12.66 ms (n = 4). Surprisingly, contrary to mIPSC, **1a** significantly prolonged the deactivation kinetics (relative τ_{mean} = 1.35 ± 0.13, n = 4, p < 0.05; Figure 5a, b). Moreover, the change in deactivation time constant was mainly due to reduction in the value of the fastest time constant (τ_1 = 1.91 ± 0.39 ms for control and 3.22 ± 0.84 ms in the presence of **1**, p < 0.05; Figure 5) and in the respective percentage of this deactivation component (A_1 = 0.5 ± 0.02 for control and 0.32 ± 0.13, n = 4, for **1a**; the effect on this parameter did not reach statistical significance, p > 0.05, Figure 5c).

The deactivation phase of current responses is known to depend critically on the kinetics of desensitization,[18] and it was therefore important to evaluate the effect of **1a** on this feature of $GABA_A$Rs. Toward this aim, current responses to prolonged (500 ms) application of a saturating (10 mM) GABA concentration were recorded. Under these conditions, the current fading (Figure 5d) represents the accumulation of receptors in the desensitized conformations. Within a time window up to 50 ms (most relevant to the synaptic transmission),

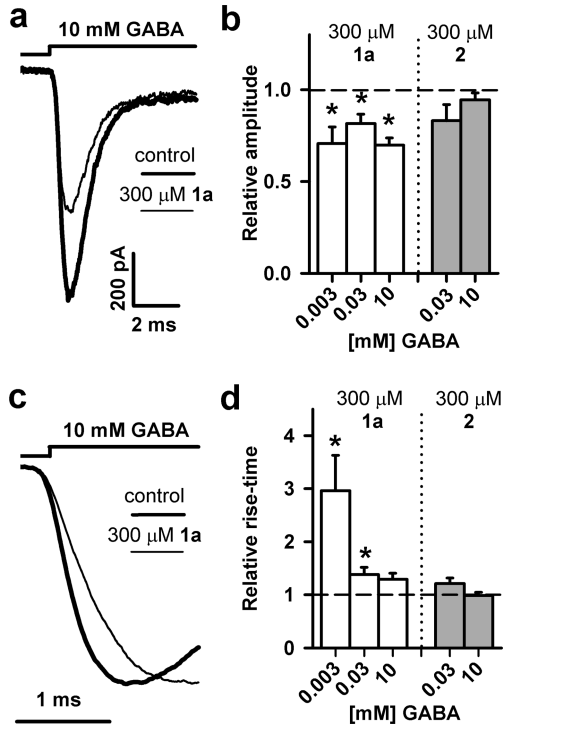

Figure 4. α-Thujone (**1a**) significantly reduces the amplitude of currents evoked by a saturating GABA concentration (10 mM). (a) Superimposed current traces recorded in control conditions (10 mM GABA, thick line) and in the presence of 300 μM **1a** (thin line). (b) Statistics of the action of **1a** and **2** on current responses evoked by different GABA concentrations. Note that **1a** exerted a similar effect on amplitudes of current responses elicited by 3 μM to 10 mM GABA concentrations. (c) Normalized and overlapped initial phases of current traces (current onset) in control conditions (10 mM GABA, thick line) and in the presence of 300 μM **1a** (thin line). Note a slower onset in the presence of **1a**. (d) Statistics of the action of **1a** and **2** on the rise time of responses evoked by different GABA concentrations. Insets above the current traces indicate GABA application. Asterisks indicate statistically significant differences.

the current fading could be described by a sum of one exponential and a constant value representing the steady-state value. In control conditions, the mean value of the desensitization time constant was τ_{des} = 1.36 ± 0.06 ms (n = 7), and the steady-state component (ss/peak) was 0.17 ± 0.06 (n = 7). Interestingly, **1a** at 300 μM affected the time course of desensitization onset by significantly increasing the value of the time constant τ_{des} (relative τ_{des} 1.38 ± 0.12, n = 7, p < 0.05, Figure 5f), while the effect on the ss/peak was at the limit of statistical significance (relative ss/peak −1.38 ± 0.12, n = 6, 0.1 > p > 0.05; Figure 5d, e).

Since hippocampal neurons in the adult brain[19] and in the long-term culture[20,21] express predominantly the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, it seemed interesting to examine in addition the effect of α-thujone (**1a**) on these receptors in experimental conditions similar to those used for neurons. For this purpose, recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in HEK293 cells, and whole-cell currents, elicited by 3 μM GABA, were recorded. Amplitudes of currents mediated by $\alpha_1\beta_2\gamma_2$ receptors were reduced by 300 μM **1a** to 0.71 ± 0.07 (n = 5, p < 0.05) of control values. This extent of **1a**-mediated blockade is comparable to that reported by Hall et al.[9] for the same $GABA_A$ receptor type expressed in *Xenopus laevis* oocytes. Moreover, the fact that **1a** reduced current responses in neurons and in the considered recombinant

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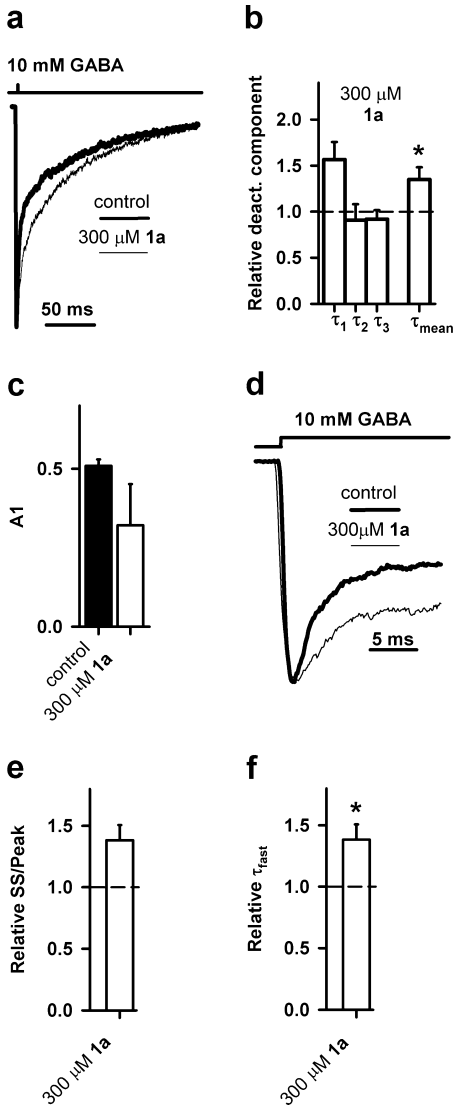

Figure 5. α-Thujone (**1a**) slows deactivation of currents evoked by brief (3 ms) applications of a saturating (10 mM) GABA concentration significantly. (a) Normalized and superimposed current traces recorded in control conditions (10 mM GABA, black line) and in the presence of 300 μM **1a** (thin line). (b) Statistics of relative values of time constants (τ_1, τ_2, τ_3) and mean time constant (τ_{mean}) for currents recorded in the presence of 300 μM **1a** (relative to those recorded in control conditions). (c) Compound **1a** reduces the percentage (A_1) of the fastest deactivation time constant (for control: A_1 = 0.50 ± 0.02, A_2 = 0.26 ± 0.06, A_3 = 0.22 ± 0.04, n = 4; for 300 μM α-thujone: A_1 = 0.32 ± 0.13, A_2 = 0.35 ± 0.07, A_3 = 0.31 ± 0.06, n = 4). (d) Normalized and superimposed current traces evoked by a prolonged (50 ms) application of a saturating GABA concentration (control, thick line) and in the presence of 300 μM **1a** (thin line). Note that **1a** reduces the rate and extent of desensitization. (e) Statistics of the relative value of the ss/peak, calculated as a ratio of the steady-state (at 50 ms) and the peak signal. (f) Statistics for the relative value of desensitization time constant, τ_{Des}, determined for currents recorded in the presence of 300 μM **1a**. Insets above the current traces indicate GABA application. Asterisks indicate statistically significant differences.

model to a similar extent suggests that the effect of **1a** in hippocampal neurons may be largely due to its action on the $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs, which are the major receptor type in these cells.

Taken together, the present results show that **1a** inhibits GABAergic currents and affects their time course. Within the concentration range evaluated (up to 300 μM), the strongest effect was observed for the frequency of mIPSCs (Figure 1a, c). The combination of a moderate effect on mIPSC amplitudes and kinetics and a marked action on the frequency of these currents gave rise to a particularly strong reduction of the total charge transfer mediated by mIPSCs (approximately 85% at 300 μM **1a**; Figure 1d). These results thus point to a strong inhibitory effect of **1a** on the phasic inhibition. To some extent, reduction of mIPSC amplitudes could contribute to a decrease in the frequency of the currents, which could fall to a nondetectable level. However, it seems unlikely that reduction of mIPSC amplitude by less than 50% could give rise to a frequency reduction of nearly 74%, therefore suggesting an additional, presynaptic mechanism. The strong effect of **1a** on the mIPSC frequency is reminiscent of the presynaptic suppression of inhibition by activation of CB1 receptors. However, **1a** could bind to these receptors but do not induce their activation,[6] therefore arguing against this interpretation. A strong decrease in the mIPSCs frequency seems therefore to indicate a presynaptic action of **1a**, although its mechanism is unknown. Interestingly, **2**, did not affect mIPSC frequency significantly, suggesting that the effect is not simply related to the hydrophobicity of thujones and a nonspecific alteration of membrane fluidity, but rather due to a specific interaction with the agonist releasing machinery.[22]

Besides a phasic (synaptic) form of inhibition, GABA also induces a tonic inhibition[23,24] that relies on regulation of the cell's input resistance by ambient GABA present in the extracellular fluids at concentrations close to 1 μM.[24] The present data on current responses to comparable GABA concentrations (3 μM, Figure 3) indicate a roughly 40% reduction of current amplitude, suggesting a potentially prominent reduction of tonic inhibition. Remarkably, amplitude reductions of mIPSC and of responses to 3 μM GABA were similar (Figures 1 and 3), implying that the extent of inhibition of synaptic and extrasynaptic $GABA_A$Rs by **1a** is similar. Thus, since mIPSCs were down-regulated additionally by a strong reduction of their frequency, these data indicate that **1a** affects the phasic inhibition to a larger extent than the tonic one. These actions combine to weaken GABAergic inhibition, potentially leading to a pro-epileptic action.

Regarding the mechanism by which **1a** affects the functioning of $GABA_A$Rs and modulates its binding and gating properties, it is worth noting that a hallmark of agents exerting a strong effect on the binding step is a markedly larger sensitivity of synaptic currents compared to the responses elicited by exogenous GABA applications.[12,25] The major reason for this difference is a very short duration of synaptic agonist transient that renders the synaptic signal extremely sensitive to modulation of the binding process.[12,25] The fact that the amplitudes of mIPSCs and of current responses to a wide range of GABA concentrations were affected by **1a** to a similar extent provides an indirect indication that the major mechanism by which **1a** affects $GABA_A$Rs is to alter gating rather than binding. The existence of only a weak, if any, effect of **1a** on the binding step is further suggested by the observation that the responses elicited by concentrations of GABA both close to and lower than its Ec_{50} value (30 and 3 μM, Figure 3) showed a similar sensitivity to **1a**, despite the higher sensitivity to binding modulation expected at concentrations close to the Ec_{50} value. Additional evidence indicating a

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major effect of **1a** on the mechanisms of gating came from the analysis of current responses to saturating GABA concentrations, which indicated a marked effect on the receptor desensitization (Figure 5). Moreover, the slowdown of the onset kinetics for responses to 10 mM GABA (Figure 4) and of mIPSCs (Figure 2) possibly reflects a reduction in the transition rate from closed to open bound conformations. The present data are, however, insufficient to quantify the effect of **1a** on each specific rate constant governing the $GABA_AR$ gating. In addition, the observation that **1a** reduced to a similar extent the currents evoked by low or high GABA concentrations indicates a noncompetitive blocking mechanism, in agreement with a previous proposal.[8] It could be considered that, besides modulating $GABA_AR$ properties, **1a** can also reduce GABAergic currents by directly occluding the channel pore (open channel block). However, in this case, no marked effect on the rising phase and increased current fading would be expected, since the receptor is "undisturbed" by the open channel blocker prior opening, but gets occluded after opening, giving rise to a faster and deeper current fading. Notably, our recordings show the opposite, in that rise time is slowed and fading is reduced. The lack of use-dependence further argues against a prominent role of the use-dependent blocking by **1a**, even though it cannot be ruled out that this compound exerts a mixed action, combining allosteric modulation of $GABA_AR$ properties (gating), responsible for observed alterations in current kinetics, and the blocking of the channel pore, contributing to down-regulation of current amplitudes. In this context, it is interesting to note that **1a** affected in a similar way the rising phase of mIPSC and of current responses to short applications of saturating [GABA], while their decays were affected differently (Figures 2 and 5). The reason for this discrepancy is unclear. It could result from the involvement of different $GABA_A$ receptor types in synapses and in excised patches, which could contain an unknown mixture of synaptic and extrasynaptic receptors.[24] However, the extent of **1a**-mediated blocking of the whole-cell current responses (to 3 μM GABA) in neurons and in the HEK293 cells expressing $\alpha1\beta2\gamma2$ receptors was similar, indicating that this $GABA_AR$ type is predominant in our model or that other types of $GABA_ARs$ present in these neurons show a similar **1a**-sensitivity. On, the other hand, it needs to be also considered that the time course of neurotransmitter in the two situations is markedly different, with an exponential-like decay being expected in synapses and a step-like agonist waveform being applied when using a rapid agonist application.[12,25]

α-Thujone (**1a**) has been reported to block $GABA_ARs$ with an IC_{50} of 21 μM,[8] while in the present study a weaker effect was observed, since at 100−300 μM **1a** the extent of blocking was below 50%. This discrepancy might well be related to the different preparations used (dorsal root ganglia[8] and hippocampal neurons), since in dorsal root ganglia the predominant form of $GABA_A$ receptors is the $\alpha_2\beta_3\gamma_2$,[26,27] while in hippocampal neurons the dominant somatic subunit arrangement does not contain the α_2 subunit.[19] Interestingly, **1a** has been reported to inhibit the $5\text{-}HT_3$ receptor, another member of the cys-loop family, by increasing agonist-induced receptor blockade.[10] Remarkably, $GABA_A$ receptors also show a similar property of self-blocking,[28] but this effect was observed using much higher agonist concentrations than those administred here. Also the observation that the extent of $GABA_AR$ blocking by **1a** apparently does not depend on GABA concentration does not support this view. Further experiments on other cys-loop receptors are, however, needed to assess if α-thujone acts indeed as a broadly tuned modulator for the cys-loop receptor family.

In conclusion, the present study provides the first direct evidence that α-thujone (**1a**) affects $GABA_A$ receptor functioning, down-regulating the phasic GABAergic transmission by modulating mainly $GABA_A$ receptor gating and by decreasing the mIPSC frequency via unknown presynaptic mechanisms. A moderate reduction of the tonic inhibition was observed with **1a**, while its closely related structural analogue **2** was either ineffective or exerted a much smaller effect. This observation suggests that the action of **1a** on GABAergic activity is not simply the result of a perturbation of membrane fluidity, but involves specific interactions amenable to structure−activity studies. While providing solid evidence of GABA-inhibiting properties for α-thujone, our results, nevertheless, add to the growing evidence that neurotoxicity by α-thujone could not be involved in absinthism,[1,4] since the GABA-inhibiting action of this compound is observed only at dosages unlikely to be achieved in a dietary context. Thus, the plasma concentration of α-thujone corresponding to the EC_{50} value reported for the inhibition of $GABA_ARs$ (21 μM)[8] is 3.19 μg/L. Despite the heated debate on the epileptogenic and psychotropic properties of thujones, their bioavailability in humans is unknown from both oral and nasal administration, and very little information is, in general, available for the oral absorption of monoterpenes in humans. Data are available for menthol.[29] Administration of 180 mg of peppermint oil (ca. 40% menthol) led to plasma concentrations in the range 1.2 −1.5 μg/mL. Assuming a similar oral biovailability for thujones, the intake of over 2 L of absinthe at the highest allowed thujone titer (35 mg/L) would be necessary to reach these concentration. While thujones, just like other monoterpene ketones, might induce seizures when assumed in concentrated form like an essential oil, the GABA-potentiating properties of alcohol make the epileptogenicity of absinthe a veritable pharmacological oxymoron.[1] Despite these considerations, the specific action of α-thujone (**1a**) on GABAergic activity is interesting, since a monoterpene-recognition site on $GABA_ARs$ seems definitely to exist, confirming that volatile compounds can show a host of specific actions on various druggable pharmacological end-points.[30] Given the rapid brain penetration on nasal absorption of many of these,[31] the pharmacological potential of these compounds seems worthy of systematic investigation.

EXPERIMENTAL SECTION

Chemicals. α-Thujone (**1a**, >96% by GC) was purchased from Fluka, while **2** (>95% purity by GC) was prepared by catalytic hydrogenation of umbellulone.[32]

Neuronal Primary Cell Culture. Primary cell cultures of hippocampal neurons were prepared from P2−P3-day-old Wistar rats. All experiments were performed under conditions in agreement with the Polish Animal Welfare Act and were approved by the local Ethical Committee. Animals were killed by decapitation, brains were removed, and hippocampi were isolated in ice-cold dissociation medium (DM; composed in mM: 81.8 Na_2SO_4; 30 K_2SO_4; 15.8 $MgCl_2$; 0.25 $CaCl_2$; 1.5 HEPES; 20 glucose; 1 kynurenic acid; 0.001% phenol red). Hippocampi were then rinsed three times in DM and incubated twice for 15 min at 37 °C in 100 U papain (Worthington, NY, USA). After digestion, hippocampi were again rinsed three times in DM and three times in MEM+FBS solution (MEM; 10% FBS; 1% MEM nonessential amino acids; 1% GlutaMAX; 1 mM pyruvate; 0.18 mM glucose; 1% pencillin/streptomycin). Hippocampi were

dissociated, diluted in OptiMEM (Gibco, Billings, MT, USA), and centrifuged at room temperature at 1000 rpm. Following centrifugation, cells were resuspended in MEM+FBS and plated at 120 000 cells per well on 18 mm diameter coverslips covered with 1 mg/mL poly-L-lysine and 2.5 μg/mL laminin (Roche). Two to four hours after plating, the medium was changed to a growth medium (neurobasal-A without phenol red; 2% B-27 supplement; 1% penicilin/streptomycin; 0.5 mM glutamine; 12.5 μM glutamate; 25 μM β-mercaptoethanol). Neurons were kept in an incubator at 37 °C in 5% CO_2. For electrophysiological experiments, cells cultured for 9−18 days were used. Chemicals were from Sigma except where otherwise specified.

Expression of Recombinant $GABA_A$ Receptors. HEK 293 cells were cotransfected with pCMV-based plasmids encoding rat α_1, β_1, and γ_2 $GABA_AR$ (1 μg/mL each), using a calcium phosphate precipitation technique.[33] For a reporter gene the pCMVCD4 plasmid encoding human CD4 receptor was added to each transfection. Cells expressing recombinant proteins were identified by CD4 binding magnetic beads (Dynabeads M-450 CD4, Dynal Biotech ASA, Oslo, Norway). Following transfection, cells were shocked with 15% glycerol and replated the following day. Cells were used for measurements 48−72 h after transfection.

Electrophysiological Recordings and Agonist Application. Currents were recorded in the whole-cell configuration (mIPSCs and responses to low GABA concentrations) or in the outside-out mode (for responses to medium and saturating GABA concentrations) of the patch-clamp technique, using the Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA), at a holding voltage of −40 mV (for current responses) or −70 mV (for mIPSC recordings). Signals were sampled and filtered at 10 kHz (except for ultrafast perfusion recordings, for which sampling was increased to 100 kHz), using a Digidata 1440 acquisition card (Molecular Devices Corporation) equipped with pClamp 10.2 software (Molecular Devices Corporation). mIPSCs and responses to 3 or 30 μM GABA were low-pass filtered at 3 kHz, whereas the responses to 10 mM GABA were at 10 kHz (Butterworth filter). Intracellular solution contained (in mM) 137 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 BAPTA (tetra cesium salt), 2 ATP, and 10 HEPES with pH adjusted to 7.2 with CsOH. The extracellular solution used was standard Ringer solution composed of (in mM) 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose, and 10 HEPES with pH adjusted to 7.2 with NaOH. To isolate the GABAergic mIPSCs, 1 mM kynurenic acid was used to block glutamatergic currents and 1 μM tetrodotoxin (LaToxan, Valence, France) was also added to extracellular solution to block the network excitability. Chemicals were purchased from Sigma except for tetrodotoxin. During measurements of current responses to low GABA concentrations in the whole-cell mode, 1 μM tetrodotoxin was added to the extracellular solution to suppress the spontaneous activity. For application of a low GABA concentration (3 μM), the multibarrel system was used (Bio-Logic RSC-200, Bio-Logic, Grenoble, France; exchange time approximately 20−30 ms). To accurately establish the kinetics of current responses to medium (30 μM) and saturating (10 mM) GABA concentrations, the piezoelectric (Physik Instrumente, Karlsruhe, Germany)-driven ultrafast perfusion system supplying solutions through theta glass capillaries (Hilgenberg, Malsfeld, Germany) was used. Open tip junction potentials showed that 10−90% solution exchange occurred in 100−150 μs. In experiments in which the effects of **1a** (or **2**) were assessed, patches were always pretreated (for at least 1 min) with external solution containing the same concentration of the compound as in GABA-containing saline (applied to evoke the current response). Access resistance was monitored, and appropriate compensation was applied if needed. Cells exhibiting access resistance greater than 15 MΩ after compensation or rundown of recorded currents larger than 20% during the course of measurements were excluded from the analysis. To avoid accumulation of toxin in the measurement dish, it was constantly superfused with Ringer solution at the rate of ca. 1 mL/min.

Data Analysis. Data were analyzed using pClamp 10.2 software. Current onset was quantified as 10−90% rise time. mIPSC current decay and deactivation of current responses were fitted with a sum of exponents (two for mIPSC decay and three in the case of deactivation of current response to saturating GABA concentration): $I(t) = \sum_{n=1}^{f} A_n \exp(-t/\tau_n)$, where f is the number of exponents, A_n is amplitude of the component, and τ_n is its decay constant. The time course of desensitization was fitted with a sum of one exponent and a constant value representing the steady-state current. In the case of normalized currents, $\sum_{n=1}^{f} A_n = 1$, and the mean time constant was calculated as $\tau_{mean} = \sum_{n=1}^{f} A_n\tau_n$. Current fading was quantified as the difference between the current at its peak and at the end of application divided by peak current: fading = $(I_{peak} - I_{end})/I_{peak}$. Total charge transfer per time unit was calculated as charge transferred by an averaged mIPSC multiplied by overall mIPSC frequency. Steady state to peak ratio was calculated as a ratio of the steady-state current after 50 ms of GABA application to the peak value. Since recordings in the presence of treated and control measurements were conducted on the same cell, results are expressed as relative values normalized to the respective control obtained for the same cell. Statistical significance was assessed with the Student t-test, with a confidence level of 0.05. All experiments were performed at room temperature, 22−24 °C.

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Notes

The authors declare no competing financial interest.

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