ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Electrophysiological Characterization of Methyleugenol: A Novel Agonist of GABA(A) Receptors

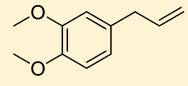
Jing Ding,[†,‡,§] Chen Huang,[§] Zhong Peng,[§] Yuxuan Xie,[†] Shining Deng,[†] Yan-Zhen Nie,[†] Tian-Le Xu,[§] Wei-Hong Ge,[‡] Wei-Guang Li,*[,†,§] and Fei Li*[,†]

[†]Department of Developmental and Behavioral Pediatrics, Shanghai Institute of Pediatric Translational Medicine, Shanghai Children's Medical Center, Ministry of Education-Shanghai Key Laboratory of Children's Environmental Health, Shanghai Jiao Tong University School of Medicine, Shanghai 200129, China

[‡]Department of Chinese Materia Medica, College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou 310053, China

[§]Neuroscience Division, Departments of Anatomy, Histology and Embryology, Biochemistry, and Molecular Cell Biology, Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

ABSTRACT: Methyleugenol (ME) is a natural constituent isolated from many plant essential oils having multiple biological effects including anticonvulsant and anesthetic activities, although the underlying mechanisms remain unclear. Here, we identify ME as a novel agonist of ionotropic γ-aminobutyric acid (GABA) receptors. At lower concentrations (~30 μM), ME significantly sensitized GABA-induced, but not glutamate- or glycine-induced, currents in cultured hippocampal neurons, indicative of a preferentially modulatory role of this compound for A type GABA receptors ($GABA_A$Rs). In addition, ME at higher concentrations (≥100 μM) induced a concentration-dependent, Cl^--permeable current in hippocampal neurons, which was inhibited by a $GABA_A$R channel blocker, picrotoxin, and a competitive $GABA_A$R antagonist, bicuculline, but not a specific glycine receptor inhibitor, strychnine. Moreover, ME activated a similar current mediated by recombinant α1-β2-γ2 or α5-β2-γ2 $GABA_A$Rs in human embryonic kidney (HEK) cells. Consequently, ME produced a strong inhibition of synaptically driven neuronal excitation in hippocampal neurons. Together, these results suggest that ME represents a novel agonist of $GABA_A$Rs, shedding additional light on future development of new therapeutics targeting $GABA_A$Rs. The present study also adds $GABA_A$R activation to the list of molecular targets of ME that probably account for its biological activities.



1,2-dimethoxy-4-prop-2-en-1-ylbenzene (Methyleugenol, ME)

KEYWORDS: *Methyleugenol, GABA, $GABA_A$ receptor, neuronal excitability*

Methyleugenol (1,2-dimethoxy-4-prop-2-en-1-ylbenzene, ME; Figure 1A) is a type of phenylpropanoid compound isolated from various essential oils of plants[1−3] such as *Myristica fragrans*, *Ocimum basilicum*, *Pimenta officinalis*, *Cinnamomum oliveri*, *Thapsia villosa*, *Doryphora sassafras*, and *Croton nepetaefolius*. ME possesses a substantial place in daily life and biomedical applications. Because of its flavoring nature, ME is widely used as a supplemental agent in food and a fragrance in products such as cosmetics, soaps, and shampoos.[4] In addition, ME has been used as an olfactory stimulant to attract insects[5] and kill them by combination with insecticides. Moreover, many biological actions of ME in mammals have been proposed, including anesthetic,[6,7] antianaphylaxis,[8] antidepressive,[9] anticonvulsant,[10,11] antinociceptive,[12] neuroprotective,[13] and vasodilatory[3,14] effects, although underlying mechanisms remain elusive. In consideration of the growing interest in the pharmacological activities of ME targeting the central nervous system,[6,7,9−13] probing its molecular targets, such as membrane receptors or ion channels in the brain, represents a promising way to further characterize additional pharmacological activities and therapeutic uses of this traditional agent.

Type A γ-aminobutyric acid receptors ($GABA_A$Rs) are pentameric proteins that form Cl^- permeable ion channels and are widely distributed in the central nervous system.[15] $GABA_A$Rs confer primary inhibitory control of neural activity physiologically and pathophysiologically.[16−19] To date, as many as 19 $GABA_A$R subunits have been identified,[20−22] which associate into functional heteromeric receptors with various physiological and pharmacological properties. The main subunit combination is α1β2γ2, at a ratio of 2:2:1, which constitutes up to 43% of the $GABA_A$Rs in the adult brain.[22] Based on the key roles of $GABA_A$Rs, this type of receptor is no doubt an important molecular target for the treatment of numerous neurological disorders.[17,18,23−27] Up to now, progress has been made in the development of $GABA_A$R-targeted drugs[28] for preclinical and clinical use, including benzodiazepines, barbiturates, steroids, and anesthetics. These $GABA_A$R modulators have made a significant contribution in the treatment of a variety of neurological diseases or disorders[17,18,23−27] including insomnia, epilepsy, anxiety, and depression. Given the complexity of $GABA_A$R physiology[20] and the increasing need for improved

Received: February 9, 2014
Revised: June 30, 2014
Published: July 1, 2014

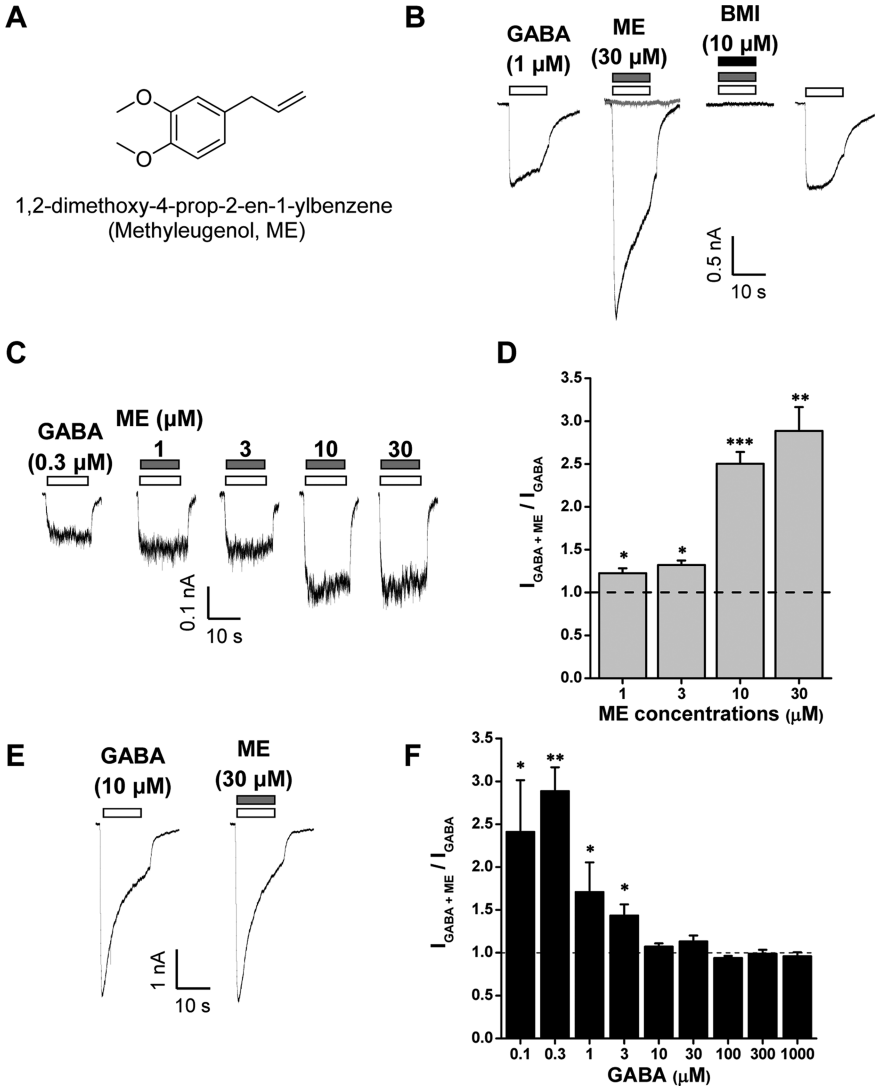


Figure 1. Methyleugenol (ME) facilitation of GABA response in cultured hippocampal neurons. (A) Chemical structure of ME. (B) Representative traces showing the currents evoked by GABA (1 μM) alone, ME (30 μM) alone (gray), GABA (1 μM) plus ME (30 μM) (black), or GABA (1 μM) plus ME (30 μM) in the presence of BMI (10 μM) as indicated. (C, D) Representative traces (C) and pooled data (D) showing the currents evoked by GABA (0.3 μM) in the absence or presence of ME with various concentrations (n = 4–6; *P < 0.05, **P < 0.01, and ***P < 0.01, compared with I_{GABA}, paired Student's t test). (E, F) Representative traces (E) and pooled data (F) showing the effects of ME (30 μM) on the current induced by various concentrations of GABA (n = 4–5; *P < 0.05 and **P < 0.01, compared with I_{GABA}, paired Student's t test).

treatment of neuropsychiatric diseases, continued efforts are required to discover or develop novel $GABA_AR$ modulators including agonists[29−31] and antagonists.[32]

In the present study, we electrophysiologically identified ME as a novel agonist of $GABA_ARs$. Using cell culture and electrophysiological recordings, we characterized the effects of ME on neurotransmitter-gated ion channels and found that ME preferentially sensitized $GABA_AR$ activation at lower concentrations (~30 μM) in cultured hippocampal neurons. ME (~30 μM) alone induced negligible currents, but it significantly potentiated lower concentrations (~3 μM) of GABA-induced currents. Strikingly, at higher concentrations (≥100 μM), ME directly activated endogenous and recombinant $GABA_ARs$. Considering the neuropharmacological effects of ME, these findings raise a potential involvement of $GABA_AR$ activation that probably accounts for its biological activities.

RESULTS AND DISCUSSION

Facilitation of GABA Response in Hippocampal Neurons by ME. Previous studies have suggested that ME possesses inhibitory effects on the activity of the central nervous system, including anesthetic,[10,11] anticonvulsant,[6,7] and antinociceptive[12] effects. To examine whether ME (Figure 1A) exerts its central action by enhancing GABAergic inhibition, GABA-induced currents (1 μM) in the absence and presence of ME (30 μM) were electrophysiologically measured in cultured hippocampal neurons. ME (30 μM) alone induced negligible currents in hippocampal neurons (Figure 1B). However, simultaneous application of ME (30 μM) dramatically enhanced GABA-induced currents (Figure 1B). The ME-facilitated current was fully inhibited by the $GABA_AR$ antagonist bicuculline (BMI, 10 μM; Figure 1B), suggesting that activation of $GABA_ARs$ is sufficient to underlie the enhanced current by ME. The present observation of ME facilitation of GABA response in hippocampal

dx.doi.org/10.1021/cn500022e | ACS Chem. Neurosci. 2014, 5, 803−811

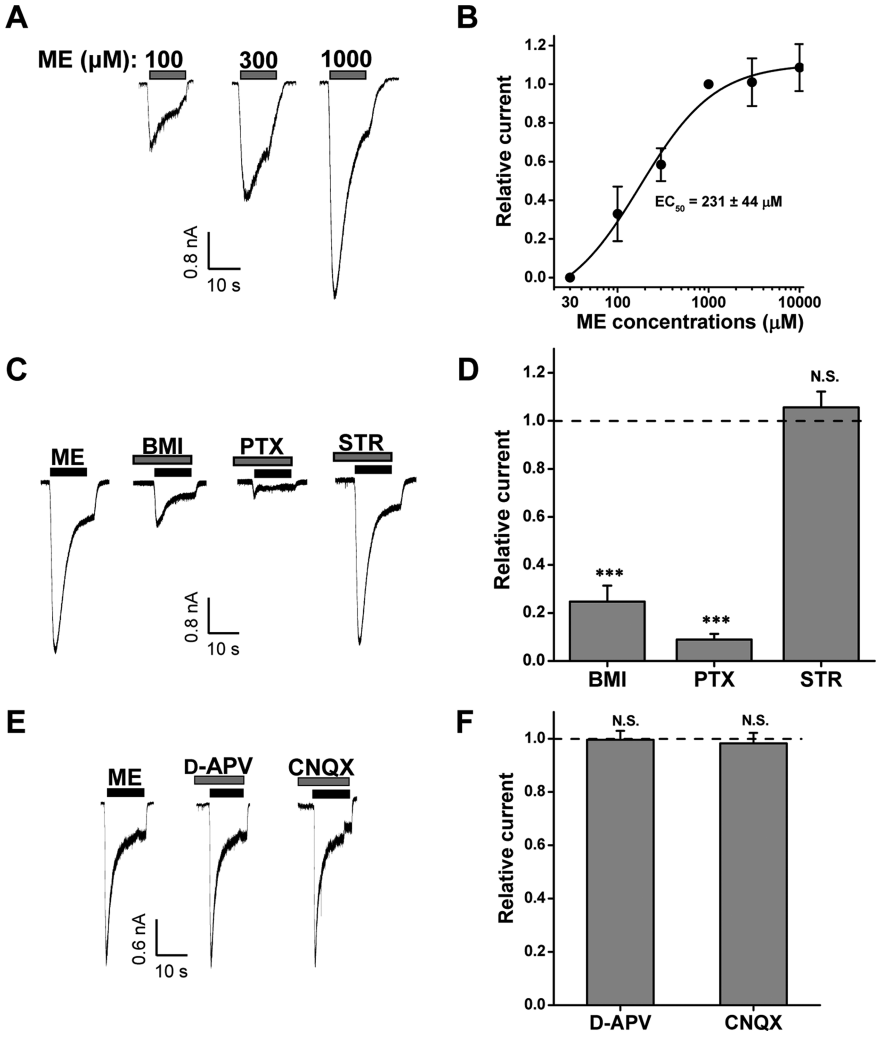


Figure 2. ME-activated currents (I_{ME}) in cultured hippocampal neurons. (A) Representative traces showing the inward currents induced by various concentrations of ME in cultured hippocampal neurons. (B) Concentration−response relationship of I_{ME}. All currents were normalized to the peak current evoked by ME (1 mM). Data points represented means ± SEM of four or five neurons. (C, D) Representative traces (C) and summary (D) of the normalized data of I_{ME} evoked by ME (1 mM) alone or in the presence of 10 μM BMI (n = 4), 100 μM PTX (n = 4), or 1 μM STR (n = 4). Dashed line in part D indicates the control values of ME-induced currents without antagonist treatment. ***P < 0.001 and NS = no significant difference, compared with I_{ME}, paired Student's t test. (E, F) Representative traces (E) and summary (F) of the normalized data of I_{ME} evoked by ME (1 mM) alone or in the presence of 20 μM D-APV (n = 4) or 10 μM CNQX (n = 5). Dashed line in part F indicates the control values of ME-induced currents without antagonist treatment. NS = no significant difference, compared with I_{ME}, paired Student's t test.

neurons raised a potential participation of $GABA_AR$ activation to mediate its pharmacological effects.

Next, we further characterized the effects of ME on the GABA response by measuring currents over a wide range of concentrations of ME and GABA. When various concentrations of ME were applied together with GABA (0.3 μM), the GABA-induced currents were potentiated in a concentration-dependent manner (Figure 1C,D). Likewise, the effect of ME (30 μM) on currents induced by a wide range of GABA concentrations from 0.1 to 1000 μM were measured in hippocampal neurons. As shown in Figure 1E,F, ME selectively increased peak current amplitudes at GABA concentrations of ≤3 μM. The prominent modulation by ME at lower concentrations of GABA (0.1−3 μM) and the resistance of GABA response at higher concentrations (≥10 μM) of ME were reminiscent of the fact that synaptic and extrasynaptic concentrations of GABA differ widely and probably activate different types of $GABA_ARs$. While the concentration of GABA in the synaptic cleft can reach the millimolar range,[33,34] the ambient level of GABA in the cerebral spinal fluid[35] is estimated to vary between 0.8 and 2.9 μM, which is sufficient to induce tonic inhibition by activating slowly desensitizing extrasynaptic high-affinity $GABA_ARs$.[36] ME enhanced GABA responses in hippocampal neurons, which potentially regulate neuronal excitability in the central nervous system.

Activation of $GABA_ARs$ in Hippocampal Neurons by ME. As shown in Figure 2A, ME alone at higher concentrations (≥100 μM) induced large inward currents (I_{ME}) in hippocampal neurons. Quantification of the ME-induced currents yielded an EC_{50} of 367 ± 58 μM (Figure 2B). ME-activated currents in hippocampal neurons were inhibited by the $GABA_AR$ antagonist BMI (10 μM) or the channel blocker picrotoxin (PTX, 100 μM) but not a specific glycine receptor inhibitor strychnine (STR, 1 μM) (Figure 2C,D) nor the N-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptor antagonists D(−)-2-amino-5-phosphonopentanoic acid (D-APV, 20 μM) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) (Figure 2E,F),

dx.doi.org/10.1021/cn500022e | ACS Chem. Neurosci. 2014, 5, 803−811

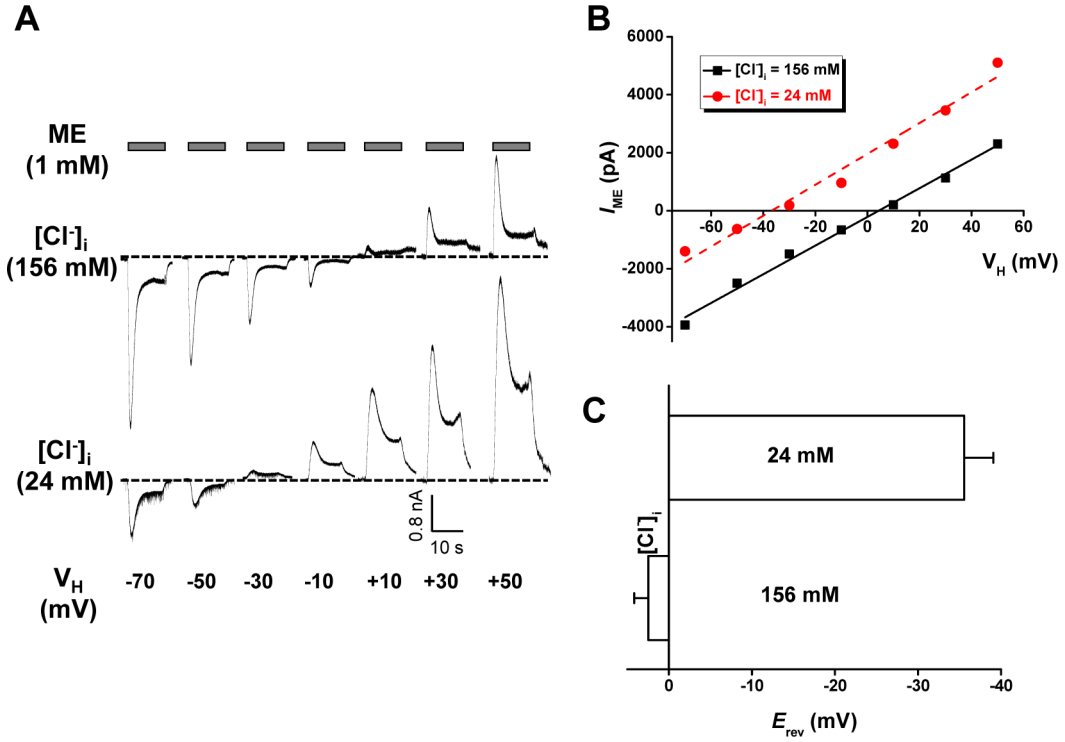


Figure 3. Ion selectivity of ME-activated currents (I_{ME}) in cultured hippocampal neurons. (A) Representative current traces showing I_{ME} evoked by ME (1 mM) at various holding potentials (V_H) with $[Cl^-]_i$ of 156 mM (upper traces) and 20 mM (lower traces). (B) Representative current−voltage relationship of I_{ME} with $[Cl^-]_i$ at 156 and 20 mM, respectively. The reversal potential (E_{rev}) of I_{ME} moved toward hyperpolarizing direction upon lowering of $[Cl^-]_i$. (C) Summary results (n = 4 for each group) exemplified as in part B.

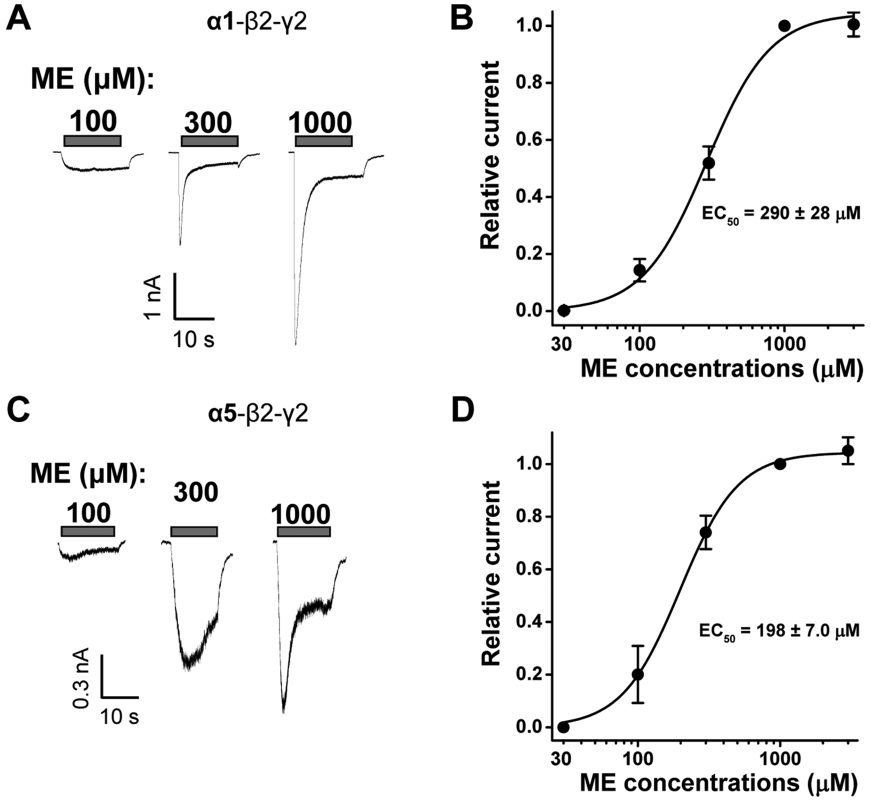


Figure 4. Activation of the recombinant $GABA_A$Rs by ME. (A) Representative current traces induced by various concentrations of ME as shown in HEK-293T cells that expressed α1, β2, and γ2 $GABA_A$R subunits. (B) Statistical data showing the relative current normalized to I_{ME} evoked by ME (1 mM), n = 5. (C) Representative current traces induced by various concentrations of ME as shown in HEK-293T cells that expressed α5, β2, and γ2 $GABA_A$R subunits. (D) Statistical data showing the relative current normalized to I_{ME} evoked by ME (1 mM), n = 5.

dx.doi.org/10.1021/cn500022e | ACS Chem. Neurosci. 2014, 5, 803−811

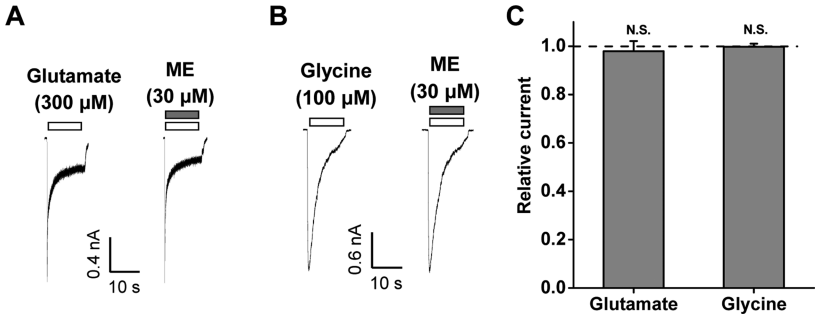


Figure 5. Effects of ME on ionic glutamate or glycine receptors in hippocampal neurons. (A, B) Representative traces showing ionic currents elicited by glutamate (300 μM, plus 1 μM glycine to activate NMDA subtype glutamate receptors), or glycine (100 μM) as indicated. Note: The currents mediated by ionic glutamate receptors were evoked under the Mg^{2+}-free external solution to relieve the Mg^{2+} blockade in the NMDA subtype glutamate receptors. (C) Bar graph summary showing the relative current amplitudes in the presence of 30 μM ME normalized to that induced by the agonist alone; n = 5 for each group; NS, no significant difference.

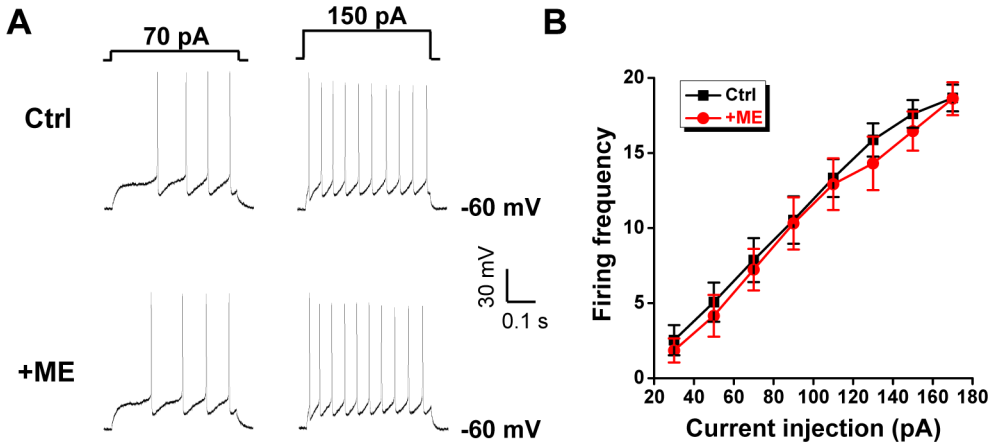


Figure 6. Effects of ME on intrinsic membrane excitability in hippocampal neurons. (A) Representative traces showing voltage responses in hippocampal neurons to 500 ms current injection of +70 and +150 pA in the absence (Ctrl, upper panel) and presence of 30 μM ME (lower panel). CNQX (10 μM), D-APV (20 μM), BMI (10 μM), and STR (1 μM) were added to the bath to block synaptic transmission. (B) Firing frequency at different levels of currents injected in the absence (Ctrl) or presence of ME (30 μM); n = 13−15 for each group.

respectively, suggesting that ME-induced currents occurred through the specific activation of $GABA_A$Rs.

To confirm further whether ME-induced currents were mediated by the permeation of Cl^- ions, the reversal potentials (E_{rev}) of ME currents at two different intracellular Cl^- concentrations ($[Cl^-]_i$ = 156 or 20 mM, respectively) were calculated based on the measurement of currents under different membrane potentials (from −70 to +50 mV at 20 mV steps). As shown in Figure 3, the E_{rev} shifted from −2.5 ± 1.7 (n = 4) to −35.6 ± 3.5 mV (n = 4) when the Cl^- concentration in the pipet solution was changed from 156 to 24 mM. This change in E_{rev} was consistent with the theoretical value for Cl^- calculated using the Nernst equation. Collectively, these findings demonstrated that ME-induced currents in hippocampal neurons were mediated by Cl^- flux through $GABA_A$R channels.

Activation of Recombinant $GABA_A$Rs by ME. To characterize more definitively the molecular identity of $GABA_A$R activation by ME in hippocampal neurons, the effects of ME were examined on recombinant $GABA_A$Rs expressed in human embryonic kidney (HEK)-293T cells. As stated previously,[22] the $\alpha1\beta2\gamma2$ composition is the most common form of $GABA_A$Rs in the adult brain including the hippocampus. In addition, both $\alpha5$ and δ $GABA_A$R subunits are required to mediate tonic inhibition in hippocampal neurons. More specifically, whereas CA1 pyramidal cells predominantly express $\alpha5$ subunits, dentate gyrus granule cells primarily express δ subunits.[37−39] Mechanistically, the effects of ME on HEK-293T cells expressing $\alpha1$-$\beta2$-$\gamma2$ (Figure 4A,B) or $\alpha5$-$\beta2$-$\gamma2$ (Figure 4C,D) $GABA_A$R subunits were investigated to reveal the potential contribution of phasic over tonic $GABA_A$Rs. As shown in Figure 4, ME induced significant inward currents (I_{ME}) in HEK-293T cells that expressed $\alpha1$-$\beta2$-$\gamma2$ or $\alpha5$-$\beta2$-$\gamma2$ $GABA_A$R subunits with an EC_{50} of 290 ± 28 and 198 ± 7.0 μM, respectively. These results were comparable with observations in hippocampal neurons (Figure 2A,B), suggesting that activation of $\alpha1\beta2\gamma2$- or $\alpha5\beta2\gamma2$-$GABA_A$Rs by ME mediated at least part of the effects of ME in hippocampal neurons described previously.

Negligible Effects of ME on Ionic Glutamate or Glycine Receptors in Hippocampal Neurons. The finding of direct activation of $GABA_A$Rs (Figure 2) together with the significant facilitation of GABA responses (Figure 1) prompted us to investigate the membrane receptor/ion channel selectivity affected by ME in hippocampal neurons. The effects of ME at a concentration of 30 μM, which sensitized $GABA_A$Rs (Figure 1), on the channel activity of excitatory transmitter (i.e., glutamate) receptors was examined in hippocampal neurons. Glutamate (plus glycine)-induced currents were recorded under Mg^{2+}-free conditions; therefore, these are probably the result of collective activation of AMPA, NMDA, and kainate receptors. As shown in Figure 5A,C, there were no significant differences in the amplitudes of the peak currents evoked by glutamate (300 μM) plus glycine (1 μM) in the absence or presence of ME (30

dx.doi.org/10.1021/cn500022e | ACS Chem. Neurosci. 2014, 5, 803−811

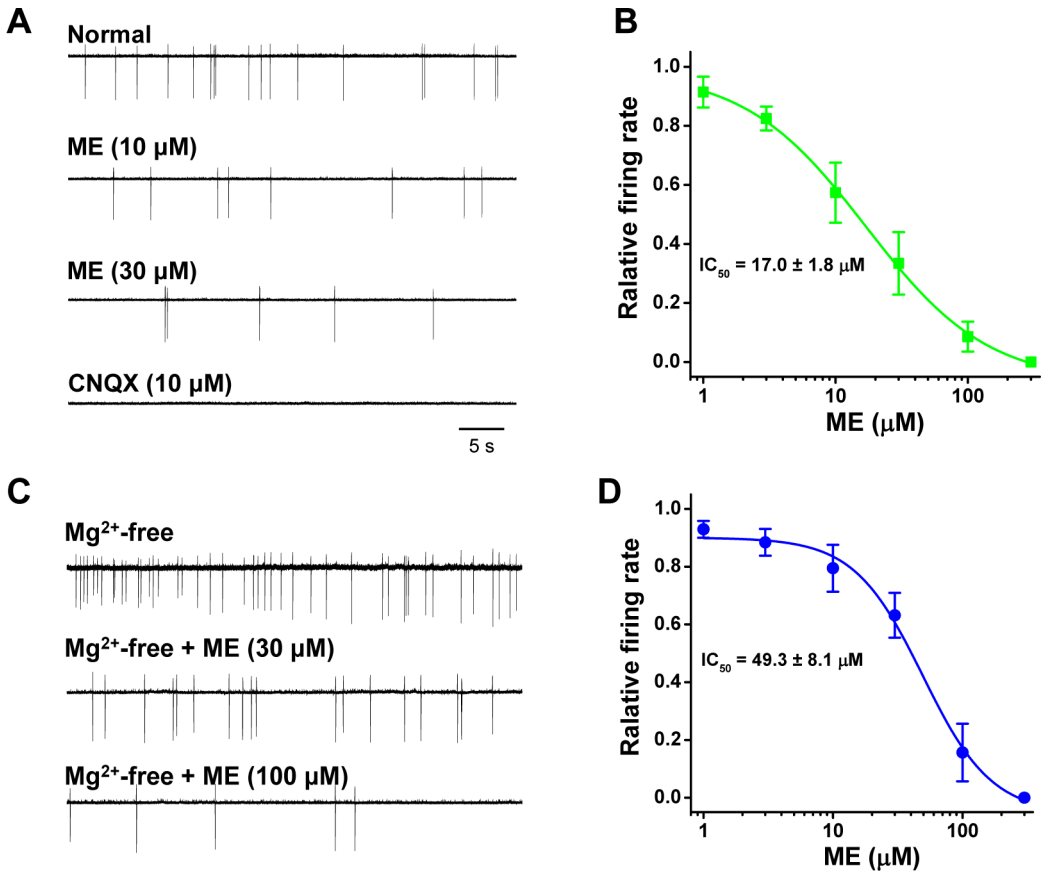


Figure 7. Inhibition of synaptically driven spiking by ME in hippocampal neurons. (A) Representative traces of cell-attached recordings of spontaneous spiking from a single hippocampal neuron bathed in the standard external solution in the presence or absence of ME and CNQX as indicated. (B) Concentration-dependent inhibition of ME on the spontaneous firing rate under normal external solution, n = 7. (C) Representative traces of spontaneous spiking from a single hippocampal neuron bathed in the Mg^{2+}-free external solution in the absence or presence of ME. (D) Concentration-dependent inhibition of ME on neuronal firing rate under Mg^{2+}-free external solution, n = 6.

μM) (97.9% ± 4.2% of the Ctrl current, n = 4, P > 0.05), suggestive of a negligible involvement of glutamate receptors in the ME effects. Analogously, the effects of ME (30 μM) on glycine receptors, another type of ionic inhibitory receptor in the central nervous system, were also examined. As shown in Figure 5B,C, there was no significant effect of ME (30 μM) on glycine (100 μM)-induced currents (99.8% ± 1.3% of the Ctrl current, n = 5, P > 0.05). The negligible impacts of ME on the activation of ionic glutamate or glycine receptors were in agreement with the resistance of ME-activated currents to inhibitors of glutamate (i.e., D-APV and CNQX) or glycine receptors (i.e., STR) in hippocampal neurons described previously (Figure 2C−F). Hence, neither ionic glutamate nor glycine receptors were involved in ME effects in neurons.

Negligible Effects of ME on Intrinsic Membrane Excitability in Hippocampal Neurons. To determine whether ME affects intrinsic membrane excitability, the impact of ME on the evoked spike rates of hippocampal neurons was examined in the presence of a cocktail of transmitter receptor antagonists, including CNQX (10 μM), D-APV (20 μM), BMI (10 μM), or STR (1 μM) for the selective inhibition of AMPA, NMDA, $GABA_A$, and glycine receptors, respectively. As shown in Figure 6, ME (30 μM) had no significant effects on the firing rates induced by step-depolarization current injections. Collectively, these negative results strengthened the notion that selective enhancement of $GABA_A$R activity but not involvement of ionic glutamate or glycine receptors nor intrinsic membrane excitability dominates ME actions in neurons.

Inhibition of Synaptically-Driven Spiking by ME in Hippocampal Neurons. Finally, to obtain the functional output of ME facilitation and activation of $GABA_A$Rs, the acute effects of ME on synaptically driven excitation in hippocampal neurons were examined. A cell-attached voltage-clamp configuration was used to evaluate neuronal spiking.[40,41] Mean spontaneous firing rates were 1.2 ± 0.2 Hz (n = 6) in cultured hippocampal neurons 12−16 days *in vitro* (DIV), which were fully eliminated by the AMPA receptor antagonist, CNQX (10 μM) (Figure 7A), confirming the synaptically driven nature. Strikingly, neuronal firing rates were significantly decreased after application of ME in a dose-dependent manner (IC_{50} = 17.0 ± 1.8 μM, Figure 7B). Moreover, in a cellular model of hyperexcitation[31,32]caused by removal of external Mg^{2+}, ME still gave rise to a concentration-dependent suppression of spontaneous spiking rates (IC_{50} = 49.3 ± 8.1 μM, Figure 7C,D). Of note, at lower concentrations up to 3 μM, ME was able to yield a significant reduction of neuronal excitability under normal or even hyperexcitation conditions (Figure 7B,D). The above results show that ME exerted more noticeable effects on the currents induced by lower concentrations of GABA (0.1−3 μM) and failed to affect the GABA responses at higher concentrations (≥10 μM) (Figure 1); thus, we speculated that the synergy between ME and (more likely extrasynaptic) GABA on $GABA_A$Rs probably conferred the ME regulation of neuronal

dx.doi.org/10.1021/cn500022e | *ACS Chem. Neurosci.* 2014, 5, 803−811

firing, a notion that was strengthened by the fact that ME was capable of activating phasic and tonic types of recombinant $GABA_A$Rs as well (Figure 4). Collectively, these results indicated that ME effectively inhibited neuronal firing probably by promoting the activation of $GABA_A$Rs.

In summary, we electrophysiologically demonstrated in the present study that ME acts as a novel agonist for the $GABA_A$Rs. At lower concentrations (~30 μM), ME significantly sensitized GABA (~3 μM)-induced currents in hippocampal neurons (Figure 1); at higher concentrations (≥100 μM), ME induced a concentration-dependent activation of endogenous (Figures 2 and 3) and recombinant (Figure 4) $GABA_A$Rs. By contrast, ME exerted negligible effects on the activation of ionic glutamate or glycine receptors (Figure 5). In addition, intrinsic membrane excitability was not affected by ME (Figure 6). As a result of enhanced $GABA_A$R activation, ME produced a strong inhibitory effect on synaptically driven neuronal excitation in hippocampal neurons (Figure 7). Thus, the present identification of ME as a $GABA_A$R agonist provided the first mechanistic link between the natural compound ME and inhibitory $GABA_A$Rs in the central nervous system, which adds $GABA_A$R activation to the list of molecular targets of ME that probably accounts for its central inhibition activities.

As stated above, ME has been increasingly recognized to possess diverse therapeutic efficacies including anesthetic,[6,7] antidepressive,[9] anticonvulsant,[10,11] antinociceptive,[12] and neuroprotection[13] effects by acting in the central nervous system, although the underlying mechanisms remain to be established. All of these central effects can be accounted for by ME-induced activation of $GABA_A$Rs widely distributed in different regions of the brain. It is well-known that the hippocampus is a pivotal region for the genesis of epileptic seizure activity;[42−47] hence the finding of ME-induced activation of $GABA_A$Rs and reduction of network excitability in the hippocampus constitutes a probable mechanism of its anticonvulsant properties, although other possibilities cannot be excluded.

The $GABA_A$R is the primary receptor responsible for inhibitory control over neural activity and information processing in the central nervous system, thereby enabling it to affect various processes such as sensorimotor action, emotion, cognition, and memory. Based on the present identification of ME as a $GABA_A$R agonist, we speculate that ME might exert its effects more widely. As one kind of insect attractant, ME was found in much earlier studies[48−50] to attract male oriental fruit flies and to cause a compulsive feeding behavior that continues until flies die from engorgement. Up to now, the neurochemical basis underlying the characteristic feeding response to ME was not fully understood. Whether ME activates a $GABA_A$R-analogous receptor in flies remains an open question awaiting additional studies in the future. Coincidentally, in mammals, ME as a flavoring agent is commonly used in food supplementation.[4] We anticipate that ME likely promotes feeding behavior in mammals in part due to the agonistic action on $GABA_A$Rs in addition to acting as an olfactory stimulus. The well-established $GABA_A$R modulator, allopregnanolone, has also been reported to produce strong orexigenic effects.[51−53] On the contrary, long-term administration of $GABA_A$R ligands such as benzodiazepines usually produce dependence and associated withdrawal reactions.[54] Here identification of ME as a $GABA_A$R agonist thus raises a caution of substance dependence associated with this compound due to its wide use during daily exposure or oral administration.

The present study characterizing ME as a novel agonist of $GABA_A$Rs sheds more light on the future development of new therapeutics associated with GABAergic inhibition. On the one hand, based on the present observations and previous studies,[6,7,9−13] ME itself can be a potential drug candidate to treat neuropsychiatric diseases by augmentation of $GABA_A$R function or through other unidentified mechanisms. On the other hand, ME probably represents a novel chemical scaffold for developing $GABA_A$R agonists by generation of additional ME analogs. To attain this aim, it is undoubtedly a prerequisite to perform additional structure−activity relationship studies on this compound as well as on the receptors with which it interacts.[55] The present electrophysiological identification of ME as a $GABA_A$R activator represents a first step in revealing the molecular mechanisms of ME action in the central nervous system.

METHODS

Animals. Pregnant C57BL/6J mice (15-day-old embryonic) were obtained from Shanghai Slac Laboratory Animal Company Limited, Shanghai, China. All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental protocols were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine, China.

Cell Culture. Primary cultures of mouse hippocampal neurons were prepared according to previously described techniques.[56] In brief, 15-day-old embryonic C57BL/6J mice were isolated by a standard enzyme treatment protocol. Brains were removed rapidly and placed in ice-cold Ca^{2+}- and Mg^{2+}-free phosphate buffered solution. Tissues were dissected and incubated with 0.05% trypsin−EDTA for 10 min at 37 °C, followed by trituration with fire-polished glass pipettes, and plated on poly(D-lysine)-coated 35 mm culture dishes at a density of 1×10^6 cells per dish. Neurons were cultured with Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 atmosphere incubator. Cultures were fed twice a week and used for electrophysiological recording 10−20 days after plating. Glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine (20 μg/mL, Sigma-Aldrich) and uridine (20 μg/mL, Sigma-Aldrich).

The HEK-293T cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin (all from Invitrogen).

Chemicals. All drugs were purchased from Sigma-Aldrich (St. Louis, MO). In electrophysiological experiments, the final concentration of dimethyl sulfoxide (DMSO) was lower than 0.1% and was verified as ineffective alone at the same concentration in control experiment.[31] Other drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use or dissolved directly in the standard external solution.

Expression of Recombinant $GABA_A$Rs. The cDNAs of rat α1, β2, and γ2 subunits of $GABA_A$Rs were obtained from Dr. Yu Tian Wang (University of British Columbia, Vancouver, BC, Canada). The cDNA of rat α5 $GABA_A$R subunit was kindly provided by Dr. David H. Farb (Boston University School of Medicine, Boston, Massachusetts, USA). Transient transfection of HEK293T cells was carried out using HilyMax liposome transfection reagent (Dojindo Laboratories, Japan). Cotransfection with a green fluorescent protein expression vector, pEGFP-C3, was used to enable identification of transfected cells for patch clamp recording by monitoring the fluorescence of green fluorescent protein. Electrophysiological measurements were performed 24−48 h after transfection.

Electrophysiological Recording. Whole-cell or cell-attached recordings were made using an Axon 200B patch-clamp amplifier (Axon Instruments). Membrane currents were sampled and analyzed using a Digidata 1440 interface and a personal computer running Clampex and Clampfit software (Version 10, Axon Instruments). In

voltage clamp mode, the membrane potential was held at −60 mV for whole-cell current recording, and the patch potential was held at the potential that gives a holding current of 0 pA in the cell-attached recording for evaluation of firing activity.[41] In current clamp mode, action potentials were elicited by applying 500 ms current (ranging from −10 to +170 pA at 20 pA steps and 5 s intervals).

The standard extracellular solution contained as follows (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), and 10 glucose, pH 7.4, adjusted with Tris-base. Except where otherwise indicated, most of the whole-cell recordings were performed using the patch pipet solution with high Cl^- concentration, which was composed of (mM): 150 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 ethylene glycol tetraacetic acid (EGTA), 2 Mg-ATP, 10 HEPES, pH 7.2, adjusted with Tris-base. The pipet solution with the low Cl^- concentration contained (mM): 130 Cs-gluconate, 20 CsCl, 2 $MgCl_2$, 0.2 EGTA, 2 Mg-ATP, 10 HEPES, and pH was adjusted to 7.2 with gluconic acid. When the current−voltage ($I-V$) relationships were constructed under voltage clamp configuration, tetrodotoxin (TTX, 300 nM) and $CdCl_2$ (100 μM) were added to the standard extracellular solution to exclude the potential activation of voltage-gated ion channels.

Statistics. Except where otherwise indicated, the data were presented as means ± SEM. Statistical comparisons were made with Student's *t* test: NS, no significant difference; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant differences. The smooth concentration−response curve of ME response was drawn according to a modified Michaelis−Menten equation by the method of least-squares (the Newton−Raphson method) after normalizing to the ME (1 mM)-induced current: $I = I_{max} \times C^h/(C^h + EC_{50}{}^h)$, where I is the normalized value of the current, I_{max} is the maximal response, C is the drug concentration, EC_{50} is the concentration that induces the half-maximal response, and h is the apparent Hill coefficient. The curve for the effect of ME on the neuronal firing was fitted to the equation: $I = I_{max} \times (IC_{50})^h/(C^h + IC_{50}{}^h)$, where IC_{50} represents the concentration of ME producing a half-maximal inhibitory effect, and the others are the same as described above.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: feili@shsmu.edu.cn (F.L.).
*E-mail: wgli@shsmu.edu.cn (W.G.L.).

Author Contributions

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

Funding

This study was supported by grants from the National Basic Research Program of China (Grants 2013CB835100 and 2014CB910300), the National Natural Science Foundation of China (Grants 31230028, 81222012, 91132303, 91213306, and 91232706), Shanghai Committee of Science and Technology (Grants 11DZ2260200 and 14JC1404600), Shanghai Municipal Education Commission (Leading Academic Discipline Projects J50201; Dawning Plan 13SG18), and Project HOPE.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank all groups that provided us with $GABA_AR$ cDNAs. We thank Ms. Ying Li and Mr. Hai-Long Zhang for technique assistance.

ABBREVIATIONS

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BMI, bicuculline; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Ctrl, control; D-APV, D(−)-2-amino-5-phosphonopentanoic acid; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GABA, γ-aminobutyric acid; $GABA_AR$, A type γ-aminobutyric acid receptor; GlyR, glycine receptor; HEK, human embryonic kidney; HEPES, *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; ME, methyleugenol; NMDA, *N*-methyl-D-aspartate; STR, strychnine; TTX, tetrodotoxin

REFERENCES

(1) Leal-Cardoso, J. H., and Fonteles, M. C. (1999) Pharmacological effects of essential oils of plants of the northeast of Brazil. *An. Acad. Bras. Cienc. 71*, 207−213.

(2) De Vincenzi, M., Silano, M., Stacchini, P., and Scazzocchio, B. (2000) Constituents of aromatic plants: I. Methyleugenol. *Fitoterapia 71*, 216−221.

(3) Lahlou, S., Figueiredo, A. F., Magalhaes, P. J., Leal-Cardoso, J. H., and Gloria, P. D. (2004) Cardiovascular effects of methyleugenol, a natural constituent of many plant essential oils, in normotensive rats. *Life Sci. 74*, 2401−2412.

(4) Council of Europe-Committee of Experts on Flavouring Substances (2001) Opinion of the scientific committee on food on methyleugenol (4-Allyl-1,2-dimethoxybenzene), Document SCF/CS/Flavour/4 ADD1 Final, pp 1−10

(5) Tan, K. H., and Nishida, R. (2012) Methyl eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. *J. Insect Sci. 12*, 56.

(6) Sell, A. B., and Carlini, E. A. (1976) Anesthetic action of methyleugenol and other eugenol derivatives. *Pharmacology 14*, 367−377.

(7) Carlini, E. A., Dallmeier, K., and Zelger, J. L. (1981) Methyleugenol as a surgical anesthetic in rodents. *Experientia 37*, 588−589.

(8) Shin, B. K., Lee, E. H., and Kim, H. M. (1997) Suppression of L-histidine decarboxylase mRNA expression by methyleugenol. *Biochem. Biophys. Res. Commun. 232*, 188−191.

(9) Norte, M. C., Cosentino, R. M., and Lazarini, C. A. (2005) Effects of methyl-eugenol administration on behavioral models related to depression and anxiety, in rats. *Phytomedicine 12*, 294−298.

(10) Dallmeier Zelger, K. R., Zelger, J. L., and Carlini, E. A. (1983) New anticonvulsants derived from 4-allyl-2-methoxyphenol (Eugenol): comparison with common antiepileptics in mice. *Pharmacology 27*, 40−49.

(11) Sayyah, M., Valizadeh, J., and Kamalinejad, M. (2002) Anticonvulsant activity of the leaf essential oil of Laurus nobilis against pentylenetetrazole- and maximal electroshock-induced seizures. *Phytomedicine 9*, 212−216.

(12) Yano, S., Suzuki, Y., Yuzurihara, M., Kase, Y., Takeda, S., Watanabe, S., Aburada, M., and Miyamoto, K. (2006) Antinociceptive effect of methyleugenol on formalin-induced hyperalgesia in mice. *Eur. J. Pharmacol. 553*, 99−103.

(13) Choi, Y. K., Cho, G. S., Hwang, S., Kim, B. W., Lim, J. H., Lee, J. C., Kim, H. C., Kim, W. K., and Kim, Y. S. (2010) Methyleugenol reduces cerebral ischemic injury by suppression of oxidative injury and inflammation. *Free Radical Res. 44*, 925−935.

(14) Magalhaes, P. J., Lahlou, S., Juca, D. M., Coelho-de-Souza, L. N., da Frota, P. T., da Costa, A. M., and Leal-Cardoso, J. H. (2008) Vasorelaxation induced by the essential oil of Croton nepetaefolius and its constituents in rat aorta are partially mediated by the endothelium. *Fundam. Clin. Pharmacol. 22*, 169−177.

(15) Hevers, W., and Luddens, H. (1998) The diversity of GABAA receptors. Pharmacological and electrophysiological properties of GABAA channel subtypes. *Mol. Neurobiol. 18*, 35−86.

(16) Brickley, S. G., and Mody, I. (2012) Extrasynaptic GABA(A) receptors: Their function in the CNS and implications for disease. *Neuron 73*, 23−34.
(17) Pavlov, I., and Walker, M. C. (2013) Tonic GABA(A) receptor-mediated signalling in temporal lobe epilepsy. *Neuropharmacology 69*, 55−61.
(18) Rudolph, U., and Mohler, H. (2014) GABAA Receptor Subtypes: Therapeutic Potential in Down Syndrome, Affective Disorders, Schizophrenia, and Autism. *Annu. Rev. Pharmacol. Toxicol. 54*, 483−507.
(19) Fritschy, J. M., and Brunig, I. (2003) Formation and plasticity of GABAergic synapses: Physiological mechanisms and pathophysiological implications. *Pharmacol. Ther. 98*, 299−323.
(20) Enna, S. J., and Mohler, H. (2007) *The GABA Receptor*, 3rd ed., Humana Press Inc., Totowa, NJ.
(21) Olsen, R. W., and Sieghart, W. (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: Classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol. Rev. 60*, 243−260.
(22) McKernan, R. M., and Whiting, P. J. (1996) Which GABAA-receptor subtypes really occur in the brain. *Trends Neurosci. 19*, 139−143.
(23) Foster, A. C., and Kemp, J. A. (2006) Glutamate- and GABA-based CNS therapeutics. *Curr. Opin. Pharmacol. 6*, 7−17.
(24) D'Hulst, C., and Kooy, R. F. (2007) The GABAA receptor: A novel target for treatment of fragile X. *Trends Neurosci. 30*, 425−431.
(25) Rudolph, U., and Knoflach, F. (2011) Beyond classical benzodiazepines: Novel therapeutic potential of GABAA receptor subtypes. *Nat. Rev. Drug Discovery 10*, 685−697.
(26) Fernandez, F., Morishita, W., Zuniga, E., Nguyen, J., Blank, M., Malenka, R. C., and Garner, C. C. (2007) Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome. *Nat. Neurosci. 10*, 411−413.
(27) Yu, Z., Fang, Q., Xiao, X., Wang, Y. Z., Cai, Y. Q., Cao, H., Hu, G., Chen, Z., Fei, J., Gong, N., and Xu, T. L. (2013) GABA transporter-1 deficiency confers schizophrenia-like behavioral phenotypes. *PLoS One 8*, No. e69883.
(28) D'Hulst, C., Atack, J. R., and Kooy, R. F. (2009) The complexity of the GABAA receptor shapes unique pharmacological profiles. *Drug Discovery Today 14*, 866−875.
(29) Zhang, X. B., Jiang, P., Gong, N., Hu, X. L., Fei, D., Xiong, Z. Q., Xu, L., and Xu, T. L. (2008) A-type GABA receptor as a central target of TRPM8 agonist menthol. *PLoS One 3*, No. e3386.
(30) Huang, C., Li, W. G., Zhang, X. B., Wang, L., Xu, T. L., Wu, D., and Li, Y. (2013) α-Asarone from *Acorus gramineus* alleviates epilepsy by modulating A-type GABA receptors. *Neuropharmacology 65*, 1−11.
(31) Ding, J., Wang, J. J., Huang, C., Wang, L., Deng, S., Xu, T. L., Ge, W. H., Li, W. G., and Li, F. (2014) Curcumol from *Rhizoma curcumae* suppresses epileptic seizure by facilitation of GABA(A) receptors. *Neuropharmacology 81*, 244−255.
(32) Xiao, X., Zhu, M. X., and Xu, T. L. (2013) 2-Guanidine-4-methylquinazoline acts as a novel competitive antagonist of A type γ-aminobutyric acid receptors. *Neuropharmacology 75*, 126−137.
(33) Mozrzymas, J. W., Zarnowska, E. D., Pytel, M., and Mercik, K. (2003) Modulation of GABA(A) receptors by hydrogen ions reveals synaptic GABA transient and a crucial role of the desensitization process. *J. Neurosci. 23*, 7981−7992.
(34) Nusser, Z., and Mody, I. (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J. Neurophysiol. 87*, 2624−2628.
(35) Lerma, J., Herranz, A. S., Herreras, O., Abraira, V., and Martin del Rio, R. (1986) In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Res. 384*, 145−155.
(36) Mtchedlishvili, Z., and Kapur, J. (2006) High-affinity, slowly desensitizing GABAA receptors mediate tonic inhibition in hippocampal dentate granule cells. *Mol. Pharmacol. 69*, 564−575.
(37) Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., Jackson, M. F., Lambert, J. J., Rosahl, T. W., Wafford, K. A., MacDonald, J. F., and Orser, B. A. (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U. S. A. 101*, 3662−3667.
(38) Glykys, J., Mann, E. O., and Mody, I. (2008) Which GABA(A) receptor subunits are necessary for tonic inhibition in the hippocampus? *J. Neurosci. 28*, 1421−1426.
(39) Serwanski, D. R., Miralles, C. P., Christie, S. B., Mehta, A. K., Li, X., and De Blas, A. L. (2006) Synaptic and nonsynaptic localization of GABAA receptors containing the alpha5 subunit in the rat brain. *J. Comp. Neurol. 499*, 458−470.
(40) Sipila, S. T., Huttu, K., Soltesz, I., Voipio, J., and Kaila, K. (2005) Depolarizing GABA acts on intrinsically bursting pyramidal neurons to drive giant depolarizing potentials in the immature hippocampus. *J. Neurosci. 25*, 5280−5289.
(41) Perkins, K. L. (2006) Cell-attached voltage-clamp and current-clamp recording and stimulation techniques in brain slices. *J. Neurosci. Methods 154*, 1−18.
(42) Scheibel, A. B. (1980) Morphological correlates of epilepsy: Cells in the hippocampus. *Adv. Neurol. 27*, 49−61.
(43) Schwartzkroin, P. A. (1994) Role of the hippocampus in epilepsy. *Hippocampus 4*, 239−242.
(44) Sutula, T. P., Cavazos, J. E., and Woodard, A. R. (1994) Long-term structural and functional alterations induced in the hippocampus by kindling: Implications for memory dysfunction and the development of epilepsy. *Hippocampus 4*, 254−258.
(45) Lopes da Silva, F. H., Kamphuis, W., Titulaer, M., Vreugdenhil, M., and Wadman, W. J. (1995) An experimental model of progressive epilepsy: the development of kindling of the hippocampus of the rat. *Ital. J. Neurol. Sci. 16*, 45−57.
(46) Thom, M., Zhou, J., Martinian, L., and Sisodiya, S. (2005) Quantitative post-mortem study of the hippocampus in chronic epilepsy: seizures do not inevitably cause neuronal loss. *Brain 128*, 1344−1357.
(47) Joels, M. (2009) Stress, the hippocampus, and epilepsy. *Epilepsia 50*, 586−597.
(48) Steiner, L. F. (1952) Methyl eugenol as an attractant for oriental fruit fly. *J. Econ. Entomol. 45*, 241−248.
(49) Metcalf, R. L., Mitchell, W. C., Fukuto, T. R., and Metcalf, E. R. (1975) Attraction of the oriental fruit fly, Dacus dorsalis, to methyl eugenol and related olfactory stimulants. *Proc. Natl. Acad. Sci. U. S. A. 72*, 2501−2505.
(50) Boeckh, J., Kaissling, K. E., and Schneider, D. (1965) Insect olfactory receptors. *Cold Spring Harb. Symp. Quant. Biol. 30*, 263−280.
(51) Fudge, M. A., Kavaliers, M., and Ossenkopp, K. P. (2006) Allopregnanolone produces hyperphagia by reducing neophobia without altering food palatability. *Eur. Neuropsychopharmacol. 16*, 272−280.
(52) Holmberg, E., Backstrom, T., Johansson, M., Lofgren, M., and Haage, D. (2013) Allopregnanolone induces a diurnally dependent hyperphagic effect and alters feeding latency and duration in male Wistar rats. *Acta Physiol. 208*, 400−409.
(53) Nakhate, K. T., Subhedar, N. K., Bharne, A. P., Singru, P. S., and Kokare, D. M. (2013) Involvement of cocaine- and amphetamine-regulated transcript peptide in the hyperphagic and body weight promoting effects of allopregnanolone in rats. *Brain Res. 1532*, 44−55.
(54) Schoch, P., Moreau, J. L., Martin, J. R., and Haefely, W. E. (1993) Aspects of benzodiazepine receptor structure and function with relevance to drug tolerance and dependence. *Biochem. Soc. Symp. 59*, 121−134.
(55) Li, W. G., and Xu, T. L. (2012) Emerging approaches to probing ion channel structure and function. *Neurosci. Bull. 28*, 351−374.
(56) Duan, B., Wang, Y. Z., Yang, T., Chu, X. P., Yu, Y., Huang, Y., Cao, H., Hansen, J., Simon, R. P., Zhu, M. X., Xiong, Z. G., and Xu, T. L. (2011) Extracellular spermine exacerbates ischemic neuronal injury through sensitization of ASIC1a channels to extracellular acidosis. *J. Neurosci. 31*, 2101−2112.