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Δ^9-Tetrahydrocannabinol and Endogenous Cannabinoid Anandamide Directly Potentiate the Function of Glycine Receptors

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Running Title: Cannabinoids Directly Potentiate Glycine Currents

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ABBREVIATIONS: ANOVA: analysis of variance; GABA: γ-aminobutyric acid; I_{Gly}: glycine-activated current; THC: Δ^9-tetrahydrocannabinol; TM: transmembrane domain; VTA: ventral tegmental area.

FOOTNOTES

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ABSTRACT

Anandamide (AEA) and Δ^9-tetrahydrocannabinol (THC) are endogenous and exogenous ligands, respectively, for cannabinoid receptors. While most of the pharmacological actions of cannabinoids are mediated by CB1 receptors, there is also evidence that these compounds can produce effects that are not mediated by the activation of identified cannabinoid receptors. Here, we report that THC and AEA, in a CB1 receptor-independent manner, cause a significant potentiation of the amplitudes of glycine-activated currents (I_{Gly}) in acutely isolated neurons from rat ventral tegmental area (VTA) and in *Xenopus* oocytes expressing human homomeric (α1) and heteromeric (α1β1) subunits of glycine receptors (GlyRs). The potentiation of I_{Gly} by THC and AEA is concentration-dependent with respective EC_{50} values of 86 ± 9 nM and 319 ± 31 nM for α1 homomeric receptors, 73 ± 8 nM and 318 ± 24 nM for α1β1 heteromeric receptors, and 115 ± 13 nM and 230 ± 29 nM for native GlyRs in VTA neurons. The effects of THC and AEA are selective for I_{Gly}, since GABA-activated current in VTA neurons or in *Xenopus* oocytes expressing α2β3γ2 $GABA_A$ receptor subunits were unaffected by these compounds. The maximal potentiation by THC and AEA was observed at the lowest concentration of glycine; with increasing concentrations of glycine, the potentiation significantly decreased. The site for THC and AEA seems to be distinct from that of the alcohol and volatile anesthetics. The results indicate that THC and AEA, in pharmacologically relevant concentrations, directly potentiate the function of GlyRs through an allosteric mechanism.

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Cannabis is the most commonly used illicit psychotropic substance in the world. The psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC) and endogenously produced cannabinoid anandamide (AEA) have widespread actions in the brain (Howlett et al., 2002): in the hippocampus, it influences learning and memory (Gerdeman et al., 2002; Howlett et al., 2002); in the basal ganglia and hypothalamus, it modulates locomotor activity, food intake and reward pathways (Gerdeman et al., 2002; Tanda and Goldberg, 2003); and in the spinal cord and supra spinal sites, it has antinociceptive effects (Cravatt and Lichtman, 2004; Walker and Huang, 2002). In the nervous system, these effects of cannabinoids are mediated primarily by the activation of the G-protein coupled cannabinoid CB1 receptors (Gerdeman et al., 2002; Howlett et al., 2002). However, several recent studies indicate that these compounds can produce effects that are not mediated by the activation of identified cannabinoid receptors (Barann et al., 2002; Di Marzo et al., 2002; Lester et al., 2004; Maingret et al., 2001; Oliver et al., 2004; Oz et al., 2003; Oz et al., 2000; Oz et al., 2002; Poling et al., 1996; Venance et al., 1995). For example, it has been demonstrated that THC inhibits the function of serotonin type 3 ($5\text{-}HT_3$) receptor in a cannabinoid receptor-independent manner (Barann et al., 2002). Similarly, AEA can inhibit the function of gap junctions (Venance et al., 1995), voltage-dependent-Ca^{2+} channels (Oz et al., 2000), various types of K^+ channels (Munro et al., 1993; Oliver et al., 2004; Poling et al., 1996), $5\text{-}HT_3$ receptor (Fan et al., 1995), and nicotinic acetylcholine (nACh) receptors (Oz et al., 2003). This suggests that additional molecular targets for cannabinoids exist in the CNS, and that these targets may represent important sites for cannabinoids to alter neuronal function.

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The Glycine receptors (GlyRs) belong to the cys-loop superfamily, which comprises both *cationic* receptors such as nACh and 5-HT_3 receptors, and *anionic* receptors such as GABA (γ -aminobutyric acid) type A ($GABA_A$) and GlyRs (Lynch, 2004). The GlyR consists of α and β subunits, which combine to form a pentameric receptor complex. While the α1 subunit is expressed mainly in the spinal cord, the α2 subunit was found in several brain regions including layer VI of the cerebral cortex, hippocampus, and ventral tegmental areas (VTA) (Betz et al., 1999). The anatomical locations of GlyRs and the potential involvement of GlyRs in pain transmission (Betz et al., 1999; Lynch, 2004) and dopamine release from VTA neurons (Molander and Soderpalm, 2005a; Molander and Soderpalm, 2005b) suggest that these receptors can play a role in analgesia and drug addiction.

To date, although direct effects of THC and AEA have been demonstrated on *cationic* (nACh and 5-HT_3) receptors (Barann et al., 2002; Fan, 1995; Oz et al., 2003; Oz et al., 2002), such an interaction between these cannabinoids and *anionic* ($GABA_A$ and Gly) receptors has not been described. Here we report that THC and AEA in pharmacologically relevant concentrations directly potentiate the function of native GlyRs in rat VTA neurons and recombinant human GlyRs expressed in *Xenopus* oocytes.

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Material and Methods

Site-Directed Mutagenesis—Point-mutations of a cloned human glycine α1 subunit were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The authenticity of the DNA sequence through the mutation sites was confirmed by double strand DNA sequencing using an ABI Prism 377 Automatic DNA Sequencer (Applied Biosystems).

Preparation of cRNAs and Expression of Receptors - Complementary RNAs (cRNAs) were synthesized in vitro from linearized template cDNAs with a mMACHINE RNA transcription kit (Ambion Inc., Austin, TX). The quality and sizes of synthesized cRNAs were confirmed by denatured RNA agarose gels. Mature female *Xenopus* laevis frogs were anesthetized by submersion in 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St Louis MO), and oocytes was surgically excised. Oocytes were separated and the follicular cell layer was removed by treatment with type I collagenase (Boehringer Mannheim, Indianapolis, IN) for 2 hr at room temperature. Each oocyte was injected with 20 ng of cRNA in 20 nl of diethylpyrocarbonate-treated water and was incubated at 19 °C in modified Barth's solution (MBS): 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM HEPES, pH7.4.

Recording from Xenopus Oocytes—After incubation for 2-5 days, oocytes were studied at room temperature (20-22C°) in a 90 µl chamber. The oocytes were superfused with MBS at a rate of 6 ml/min. Agonists and chemical agents were diluted in the bath solution and applied to the oocytes for a specified time, using a solenoid valve controlled superfusion

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system. Agonists and other chemical agents were diluted either directly in the bath solution or dissolved in ethanol before dilution. The final ethanol concentration was less than 0.7 mM. Membrane currents were recorded by two-electrode voltage-clamp at a holding potential of –70 mV, using a Gene Clamp 500 amplifier (Axon Instruments Inc., Burlingame, CA). The recording microelectrodes were filled with 3 M KCl and had electrical resistances of 0.5-3.0 MΩ. Data were routinely recorded on a chart recorder (Gould 2300S; Gould Inc., Cleveland, OH). Average values are expressed as means ± S.E.

Neuron dissociation - Neurons were freshly isolated from the ventral tegmental area (VTA) of Sprague Dawley rats between 4 to 15 postnatal days. The VTA neurons were prepared as described previously (Ye et al., 2005). In brief, rats were decapitated and the midbrain was isolated and transversely sliced (400μm) with a Vibroslice (Campden Instruments, Leicester, UK) or a VF-100 Slicer (Precisionary Instruments, Greenville, NC) while kept in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95%O_2/ 5%CO_2 containing: 126 mM NaCl, 1.6 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose. Slices were transferred to the standard external solution (see below for components) saturated with O_2, containing 1 mg pronase/6 ml and incubated (31^o C) for 20 min. After additional 20 min incubation in 1 mg thermolysin/6 ml, the VTA was identified under a dissecting microscope as the region medial to the accessory optic tract and lateral to the fasciculus retroflexus. Micro-punches of the VTA were isolated and transferred to a 35 mm culture dish. Mild trituration of these tissue punches through heat-polished pipettes of progressively smaller tip diameters

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dissociated single neurons. Within 20 min of trituration, isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments.

Electrophysiological Recording - Whole cell patch-clamp techniques were used to record electrical activity with an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA), a Digidata 1320A analog-to-digital converter (Axon Instruments), and Clampex 9 (Axon Instruments). Data were sampled at 5 kHz and filtered at 1 kHz. All currents were recorded with standard external solution containing: NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 2.0 mM, HEPES 10 mM, glucose 10 mM. The pH was adjusted to 7.4 with 1 N NaOH. The junction potential between the pipette and the bath solutions was nullified just before giga-seal formation. The patch electrodes had a resistance of 3-5 MΩ, when filled with the following: 140 mM CsCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.1 mM GTP, the pH was adjusted to 7.2 with KOH, and the osmolarity to 280-300 mOsm with sucrose. All recordings were performed at room temperature (22-24°C).

Data analysis - Statistical analysis of concentration-response data was performed with the use of the nonlinear curve-fitting program ALLFIT. Data were fitted to the logistic equation

$$Y = \{(E_{max} - E_{min})/[1 + (X/EC_{50})^{n}]\} + E_{min}$$

where X and Y are concentration and response, respectively; E_{max} and E_{min} are the maximal and minimal responses, respectively, EC_{50} is the half-maximal concentration; and n is the slope (apparent Hill coefficient). Data were statistically compared by the

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paired *t*-test, or analysis of variance (ANOVA), as noted. Average values are expressed as mean ± SE.

Chemicals and application - Most of the chemicals including glycine and GABA were from Sigma (St. Louis, MO). Solutions were prepared on the day of experiment and applied to neurons using a 'Y- tube' perfusion system (Ye et al., 2005). A perfusion pipette with a diameter of 50 μm was placed 50–100 μm away from the neuron. With this perfusion system, solutions in the vicinity of a neuron can be completely exchanged within 20 ms without loss of mechanical stability.

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Results

Although α subunit alone is sufficient to form a homomeric functional channel, native GlyRs are thought to consist of both α and β subunits (Lynch, 2004). In this regard, we expressed both homomeric (α1 subunit alone) and heteromeric (α1 with β1 subunits) GlyRs in *Xenopus* oocytes. The EC_{50} and slope values for glycine were 85 ± 8 μM and 1.2 ± 0.3 for the α1 homomeric receptors and 141 ± 18 μM and 1.1 ± 0.2 for the α1β1 heteromeric receptors, respectively. Consistent with previous observations (Betz et al., 1999; Lynch, 2004), the heteromeric GlyRs were less sensitive to picrotoxin, a chloride channel blocker. Picrotoxin at 100 μM inhibited 75 ± 3% of I_{Gly} activated by Gly at EC_{50} in cells expressing the α1 homomers and 25 ± 2% of I_{Gly} activated by Gly at EC_{50} Gly in cells expressing the α1β1 heteromers.

Application of AEA (300 nM) or THC (300 nM) for 15 sec. did not alter baseline holding currents in oocytes expressing homomeric or heteromeric GlyRs, suggesting that, under control conditions, these compounds do not alter endogenously expressed ionic conductances in oocytes and they do not have an agonistic activity on GlyRs. In the first series of experiments, we have investigated the effects of THC (300 nM) or AEA (300 nM) on glycine-activated currents (I_{Gly}). The peak amplitudes of currents activated by 3 μM glycine were markedly increased when glycine was coapplied with THC or AEA in oocytes expressing α1 homomers (Fig. 1A) and α1β1 heteromers (Fig. 1B). In the continued presence of glycine, I_{Gly} reached steady-state levels after the peak amplitude (Fig. 1C). During this plateau phase, addition of THC or AEA increased the amplitudes of I_{Gly} in oocytes expressing homomeric and heteromeric GlyRs (Fig. 1, C and D). Both THC and AEA increased the amplitude of I_{Gly} in a reversible and concentration-

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dependent manner. The EC_{50} values for THC and AEA were 86 ± 9 nM and 319 ± 31 nM, respectively, for the α1 homomers (Fig. 2A); and 73 ± 8 nM and 318 ± 24 nM, respectively, for the α1β1 heteromers (Fig. 2B).

To exclude the possibility that the THC and AEA-induced potentiation of GlyRs was mediated by CB1 receptors, we tested the effects of the CB1 receptor antagonist SR141716A on the THC and AEA potentiation of GlyR-mediated currents (Fig. 3). In cells expressing α1 homomeric GlyRs, applications of SR 141716A alone at 1 µM did not significantly alter the amplitude of I_{Gly} induced by 3 µM glycine (5 ± 7% of control; P>0.1, unpaired t-test, n=5). Similarly, the pretreatment with either SR141716A at 1 µM did not significantly alter the AEA and THC-induced potentiation of I_{Gly} in cells expressing α1 homomeric GlyRs (Fig. 3; P>0.1, unpaired t- test, n=5). In addition, we tested the effect of AM404, anadamide membrane transport inhibitor, on the THC and AEA potentiation of I_{Gly}. Application of 1 µM AM404 did not significantly affect the magnitude of THC and AEA-induced potentiating effects on I_{Gly}.

We next examined whether THC and AEA could potentiate I_{Gly} in acutely dissociated rat VTA neurons. As demonstrated in figure 4A, SR141716A at 1 µM alone did not change holding current of VTA neurons nor did it alter the amplitude of currents activated by 5 µM glycine. After 15 sec. preincubation with SR141716A, 300 nM THC continued to potentiate I_{Gly} induced by 5 µM glycine (Fig. 4B). THC-induced potentiation was fully reversible upon cessation of THC application and remained unaltered in the absence (60 ± 15%) and presence of 1 µM SR141716A (53 ± 14%; n=5, P=0.38). These results indicate that THC-induced potentiation of I_{Gly} is independent of CB1 receptors.

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AEA at 300 nM also increased I_{Gly} of a VTA neuron pretreated with SR141716A (Fig. 4C). The magnitude of the AEA-induced potentiation of I_{Gly} was similar in the absence and presence of SR141716A (17 ± 8% and 20 ± 4% potentiation in the absence and presence of SR141716A, n=4-8, *P*=0.13). Both THC and AEA potentiated I_{Gly} in a concentration-dependent manner over a concentration range of 10 to 3000 nM (Fig. 4D). However, THC was more potent than AEA to potentiate I_{Gly}. The EC_{50} values for THC and AEA potentiation were 115 ± 13 nM, and 230 ± 29 nM, respectively. The maximal potentiation by THC and AEA were 55 ± 5% and 20 ± 4%, respectively. These values are significantly different (n=5-6, *P*<0.001).

$GABA_A$ receptors are the major inhibitory neurotransmitter-gated ion channels in the brain and they are functionally and structurally similar to GlyRs (Lynch, 2004). For this reason, we next investigated whether THC could also modulate $GABA_A$ receptor function. Neither THC nor AEA at 0.3 μM significantly altered the amplitude of currents activated by 3 μM GABA in *Xenopus* oocytes expressing α2β3γ2 subunits of $GABA_A$ receptor (Fig. 5A). We also observed that THC at 0.3 μM did not affect currents activated by various sub maximal concentrations of GABA I_{GABA} in isolated VTA neurons (Fig. 5B). In the presence of THC, the magnitudes of GABA-activated currents were 97 ± 2% of control at 1 μM GABA (n=3), 99 ± 2% of control at 3 μM GABA (n=5), 97 ± 2% of control at 10 μM GABA (n=4), and 98 ± 2% of control at 30 μM GABA (n=4). These values are not significantly different (*P*>0.1).

Next, we examined the effects of increasing glycine concentration on the THC and AEA potentiation of I_{Gly}. In *Xenopus* oocytes expressing the α1 GlyR homomers, the magnitude of THC and AEA-induced potentiation of I_{Gly} decreased significantly with

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increasing concentrations of glycine (Fig 6A). The magnitudes of potentiation by 300 nM THC and AEA were 76 ± 9 and 48 ± 6 at 3 µM glycine, respectively and 2.8 ± 1and 7 ± 8 at 30 µM glycine, respectively in *Xenopus* oocytes expressing the α1 subunits (Fig. 6B; *P*<0.01, unpaired *t*-test, n=5). The dependence on agonist concentration was also evidenced by THC potentiation of I_{Gly} in VTA neurons. A fixed concentration of THC (300 nM) was applied with increasing glycine concentrations (from 5 µM to 30 µM). As illustrated in Fig. 6C, while the currents induced by 5 µM glycine were markedly enhanced by 0.3 µM THC, the I_{Gly} induced by 30 µM glycine remained unchanged in the presence of THC (Fig. 6C). On average, 300 nM THC potentiated I_{Gly} induced by 5, 10, 30, and 100 µM glycine by 51 ± 5% (n=8, *P*=0.001), 42 ± 13% (n=5, *P*=0.005), and 11 ± 10% (n=7, *P*=0.2), respectively (Fig. 6D).

The above observations have suggested that ANA and THC are likely to directly interact with glycine receptors. Previous studies have identified single amino acid residue at 267 in the TM2 domain of glycine receptor α subunits as a distinct site for a variety of volatile anesthetics, n-alcohols and commonly abused inhalants (Mihic et al., 1997; Wick et al., 1998). Given that both THC and AEA are hydrophobic substance with massive volume, we proposed that the potentiation of I_{Gly} induced by ANA and THC may share a molecular basis similar to that of alcohol/anesthetics. If so, a point-mutation of S267 should reduce AEA and THC-induced poentiating effects of I_{Gly}. To test this hypothesis, we substituted a serine at 267 with an asparagine (Q) and injected cRNA into the oocytes. The S267Q mutation did not significantly alter the receptor’s sensitivity to glycine (72 ± 9 µM vs 85 ± 8 µM; n=5, *P*=0.5). Consistent with previous reports (Mihic et al., 1997), while 100 mM

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EtOH significantly potentiated I_{gGy} by 91% (Fig. 7A), the S267Q mutation completely abolished the potentiating effect induced by 100 mM ethanol (Fig. 7, B and C), respectively (Mihic et al., 1997). However, the S267Q mutation did not significantly alter the potentiation induced by THC and AEA (Fig. 7, B and C; n=5, *P*=0.2).

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Discussion

In this study, we have shown that THC and AEA induced a concentration-dependent potentiation of currents mediated by α1 homomeric and α1β1 heteromeric GlyRs expressed in *Xenopus* oocytes or by native GlyRs in acutely isolated VTA neurons. THC appears to be more potent than AEA on both recombinant and native GlyRs. The EC_{50} values for the effect of THC on heteromeric GlyRs (73 nM) and native GlyRs (115 nM) are in the pharmacological ranges that induce psychotropic and antinociceptive effects in humans. For example, the plasma THC concentration was found to be 162 nM after 1 h of low-dose cannabis smoking (Huestis and Cone, 2004). Similarly, the EC_{50} values for the AEA potentiation of recombinant and native GlyRs are in the range of 230 to 318 nM, comparable to AEA's affinity (89-300 nM) for CB1 receptor (Howlett et al., 2002).

AEA and THC, at the concentration range used in this study, have been shown to activate native cannabinoid receptors (Howlett et al., 2002). However earlier studies indicate that the cloned cannabinoid receptors CB1 and CB2 are not expressed in *Xenopus* oocytes (Henry and Chavkin, 1995; Ho et al., 1999; Jin et al., 1999). Furthermore, SR141716A, CB1 receptor antagonist, did not prevent the AEA or THC potentiation of I_{Gly} in both *Xenopus* oocytes and VTA neurons. This suggests that the potentiating effects induced by both THC and AEA is not mediated by CB1 receptors since the IC_{50s} of SR141716A to block endogenous CB1 receptors is found to be around nanomolar concentrations (Di Marzo et al., 2004). We did not directly examine the ability of CB2 antagonists to block THC and AEA potentiation of I_{Gly} in VTA neurons, since CB2 receptors are not found in the brain (Howlett et al., 2002; Walker and Huang, 2002).

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Based on our data, we conclude that ability of AEA and THC to potentiate GlyR-mediated currents does not result from activation of identified cannabinoid receptors.

Our findings in VTA neurons is consistent with a recent study, which has reported that GlyR-mediated synaptic currents in hypoglossal motoneurons were potentiated directly by conditions that promote Ca^{2+}-dependent endocannabinoid production (Diana and Bregestovski, 2005). This result indicates that in native neurons production of endocannabinoids potentiates the function of GlyRs in a CB1 receptor-independent mechanism. Similarly, another recent study has reported that endogenous cannabinoids modulated I_{Gly} in hippocampal neurons through a CB1-independent mechanism (Lozovaya et al., 2005). However, the authors in this paper observed that AEA directly produced inhibition rather than potentiation of I_{Gly} in the presence of CB1/CB3 receptor antagonists. There are a number of reasons to explain the discrepancy between our and their observations such as different compositions of glycine receptor subunits and different signal transduction pathways in presynaptic and postsynaptic neurons. In addition, the inhibition of I_{Gly} appeared to occurr at high concentrations of glycine (100 μM) (Lozovaya et al., 2005), whereas THC and AEA potentiation reported in our study only occurred at low concentrations of IGly less than 5 μM. Collectively, these findings suggest that, under physiological conditions where the activation of cannabinoid receptors cause a strong presynaptic inhibition, endocannabinoids can induce dual modulatory effects on neuronal excitability, i.e. presynaptic inhibition of the glycinergic synaptic transmission and the potentiation of postsynaptic (both synaptically and extrasynaptically located) GlyRs.

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It is worth mentioning that neurons in the VTA area contain vanilloid receptors, which are another possible non-cannabinoid site of action for AEA (Di Marzo et al., 2002; Kim et al., 2005). However, it is unlikely that modulation of GlyR by THC and AEA observed in this study is mediated by endogenous vanilloid receptors. First, the EC_{50} of THC and AEA-induced potentiating effects on GlyR are found to be at nanomolar concentrations, whereas AEA action on the vanilloid receptor is observed at submicromolar concentrations. Second, application of THC and AEA alone did not induce any detectable inward currents in cells voltage-clamped at -60 mV. Third, *Xenopus* oocytes have been widely used to express and study the function of recombinant vanilloid receptors since these cells do not have endogenous vanilloid receptors (Premkumar and Ahern, 2000; Schumacher et al., 2000).

Recent studies using exogenously supplied glycine and specific inhibitors of glycine transporters have revealed that the glycine-binding site associated with NMDA receptors (which has 100-fold higher glycine affinity than GlyRs) is not saturated at the synapse (Bradaia et al., 2004; Eulenburg et al., 2005; Gomeza et al., 2003). Extracellular glycine concentrations in rat spinal cord were determined to be in the range of 2 to 3 μM, and the glycine content of cerebrospinal fluid was determined to be 6 μM (Whitehead et al., 2001). These findings indicate that synaptic and extrasynaptic glycine is often present at concentrations that produce only low occupancy of GlyRs. Therefore, cannabinoid-induced potentiation of GlyRs at low occupancy is likely to have physiological roles in neuronal excitability.

Interestingly, both AEA and THC did not affect $GABA_A$ receptor-mediated responses even though these receptors share structural and functional similarities with

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GlyRs (Lynch, 2004). Given that the $GABA_A$ α2β3γ2 receptors are abundantly expressed in the central nervous system (Sigel and Kannenberg, 1996), the observation reported in this study suggests that direct actions of AEA and THC are relatively specific for GlyR. However, other cannabinoid receptor ligands, such as AM251 (1 μM), a CB1 receptor antagonist, caused significant (31%) inhibition of exogenous GABA-activated currents in basolateral amygdala neurons (Zhu and Lovinger, 2005). Thus, the effects of other cannabinoid receptor ligands on different $GABA_A$ receptor subunit combinations expressed in other brain regions cannot be excluded.

The role of GlyRs in pain transmission (Lynch, 2004) suggests another potential role for cannabinoid and endocannabinoid effects on the receptor. For instance, while THC-induced analgesic effects are completely abolished in the hotplate and formalin tests in the CB1 knockout mouse, THC-induced analgesia in the tail-flick test remains intact (Zimmer et al., 1999). On the other hand, AEA still exerts a cannabimimetic activity in all three analgesic tests in CB1 knockout mice, suggesting that not all cannabinoid and endocannabinoid-induced analgesic effects are mediated by CB1 receptors (Di Marzo et al., 2000). Future studies should address if enhancement of GlyR function by THC and AEA could contribute to their non-CB1-mediated antinociceptive effects.

The VTA is thought to be a potential target region for the direct actions of cannabinoids on the GlyR, and it is the origin of mesolimbic dopamine system, which mediates the reinforcing properties of cannabinoids (Gerdeman et al., 2002). Increased dopamine release by exogenous glycine application has also been shown (Molander and Soderpalm, 2005a; Molander and Soderpalm, 2005b). In this regard, we tentatively

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predict that the potentiation of the GlyR function by THC and ANA may have a modulatory role in CB1 receptor-dependent release of dopamine in the mesolimbic system.

The precise molecular mechanisms underlying THC and AEA potentiation of GlyR-mediated responses are currently unknown. The potentiation by THC and AEA exhibited fast onset and offset times (see Fig. 2). Similar to ethanol potentiation of 5-HT_3 receptor-meditated currents (Lovinger and Zhou, 1998; Zhang et al., 2002), the cannabinoid potentiation of GlyRs decreased significantly with increasing agonist concentrations. Our earlier studies have shown that the agonist-concentration dependency of ethanol actions resulted from direct modulatory actions of ethanol on the gating properties of 5-HT_3 receptor-channel complex (Zhang et al., 2002). It is important to note that while cannabinoids, endocannanoids and ethanol can potentiate GlyR, these effects seem to be mediated by different molecular determinants. The amino acid residue at 267 or the equivalent position of GlyR has been shown to be critical for alcohol and volatile anesthetics-induced modulation of GlyR function (Mihic et al., 1997; Tao and Ye, 2002; Ye et al., 2001). The similar case was also found to be true for $GABA_A$ receptor (Ueno et al., 2000). However, point-mutation of S267 did not affect THC and AEA-induced potentiation of GlyR. This finding also excludes the possibility that the potentiation of GlyR by THC and AEA is due to a contamination from ethanol-induced effects since we dissolved both THC and AEA in ethanol in stock solutions. It is likely that THC and AEA may still exert their effects by modulating the gating properties of GlyRs through allosteric mechanisms. Further studies are needed to identify molecular determinants of the actions of cannabinoids on GlyRs. It should be also interesting to determine if low

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concentrations of ethanol and THC or AEA may impose a synergetic potentiating effects on GlyR since such synergisms by alcohol and THC have been reported in animal and human studies (Chait and Perry, 1994; Doty et al., 1992; Liguori et al., 2002).

In conclusion, we have shown that THC and AEA potentiate the function of GlyRs in a CB1 receptor-independent manner. The effects of THC and AEA are mediated by a direct interaction between cannabinoids and GlyRs. Collectively, the results suggest that GlyRs represent a novel target in mediating the pharmacological actions of endogenous and exogenous cannabinoids.

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Figure Legends

Fig. 1. THC and AEA potentiation of human recombinant GlyRs. A, trace records showing THC and AEA (300 nM) enhancement of amplitude of currents activated by 3 μM glycine in oocytes expressing the α1 subunits. B, trace records showing THC and AEA (300 nM) enhancement of amplitude of currents activated by 5 μM glycine in oocytes expressing the α1β1 subunits. C, trace records showing THC and AEA (300 nM) enhancement of amplitude of steady state activated by 3 μM glycine in oocytes expressing the α1 subunits. D, trace records showing THC and AEA (300 nM) enhancement of steady-state of currents activated by 5 μM glycine in oocytes expressing the α1β1 subunits.

Fig. 2. The concentration-response curves of THC and AEA potentiation of the α1 and α1β1 subunits. A and B, graph plotting average percentage potentiation by THC and AEA as a function of THC and AEA concentration. Each data point represents the average of 5-10 oocytes. The curves shown are the best fits of the data using the Hill equation described in 'Material and Methods'. Error bars not visible are smaller than the size of symbols.

Fig. 3. Effects of SR141716A and AM404 on THC and AEA potentiation of I_{Gly}. The solid bar graphs plotting average percentage potentiation by 300 nM AEA and THC of I_{Gly} activated by 3 μM glycine in the presence and absence of 1 μM SR141716A. The oocytes expressing the α1 subunits were preincubated with SR141716A or AM404 for 15

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sec. prior to exposure to a mixture of THC plus glycine or AEA plus glycine. Each data point represents mean ± s.e. of 5-9 oocytes.

Fig. 4. THC and AEA potentiation of glycine-activated current in isolated VTA neurons. A, membrane currents recorded after 1 µM SR141716A (SR) application (left), after 5 µM glycine was applied alone and after coapplication of SR and glycine. B, I_{Gly} (induced by 5µM glycine) recorded before (left), during (middle) and after (right) the application of 300 nM THC in the absence and presence of 1µM SR. The SR was applied 15 seconds before the application of the mixture of glycine + THC + SR. C, I_{Gly} (induced by 5 µM glycine) recorded before (left), during (middle) and after (right) the application of 300 nM AEA in the presence of 1µM SR. The SR was applied 15 seconds before the application of the mixture of glycine + AEA + SR. D, concentration-response curves of the THC and AEA potentiation of I_{Gly} (activated by 5 µM glycine) in the presence of 1µM SR. The error bars not visible are smaller than the size of symbols.

Fig. 5. THC and AEA do not affect I_{GABA}. A, trace records of currents activated by 3 µM GABA in the absence and presence of 300 nM THC and AEA in *Xenopus* oocytes expressing the α1 subunits. The solid bar on top of each record indicates the time of drug application. B, traces of currents activated by 3 µM GABA in the absence and presence of 300 nM THC in a VTA neuron. The cell was preincubated with 1 µM SR 141716A for 15 sec.

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Fig. 6. Glycine-concentration dependence of THC and AEA potentiation. A, trace records showing THC (300 nM) and AEA (300 nM) enhancement of currents activated by 3 and 30 μM glycine in oocytes expressing the α1 subunits. B, average potentiation by THC and AEA of I_{Gly} activated by various concentrations of glycine in oocytes expressing the α1 subunits. Each bar represents the average of 4-5 oocytes. C, trace records showing THC enhancement of amplitude of currents activated by 5 and 30 μM glycine in VTA neurons. D, potentiation by THC of I_{Gly} by various concentrations of glycine in VTA neurons. Each bar represents the average of 3-5 cells.

Fig. 7. The S267Q mutation abolished the potentiation by EtOH but not AEA and THC. A, trace records of current activated by 1 μM glycine in the presence of AEA (300 nM), THC (300 nM) and EtOH (300 mM) in oocytes expressing the wild type α1 subunits. B, trace records of current activated by 1 μM glycine in the presence of THC, AEA and EtOH in oocytes expressing the mutant S267Q subunits. C, average potentiation by AEA, THC and EtOH of I_{Gly} in oocytes expressing the wild type and mutant α1 subunits. Each bar represents the average of 4-5 oocytes.

Figure 1

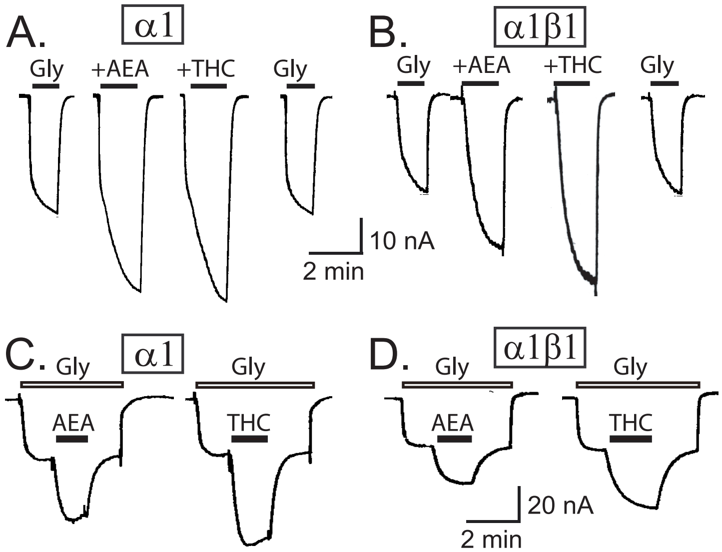
A.
α1
Gly
+AEA
+THC
Gly
10 nA
2 min
B.
α1β1
Gly
+AEA
+THC
Gly
C.
α1
Gly
AEA
Gly
THC
D.
α1β1
Gly
AEA
Gly
THC
20 nA
2 min

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Figure 2

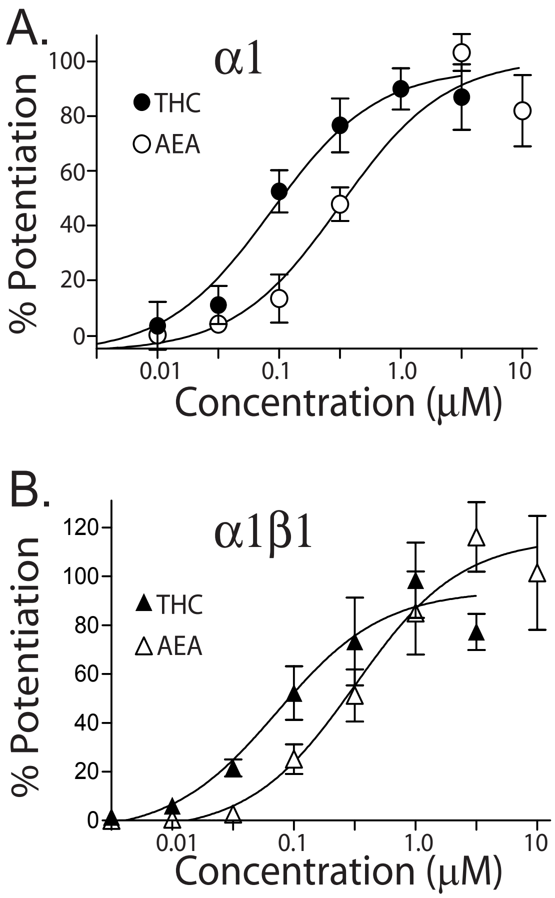

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Figure 3

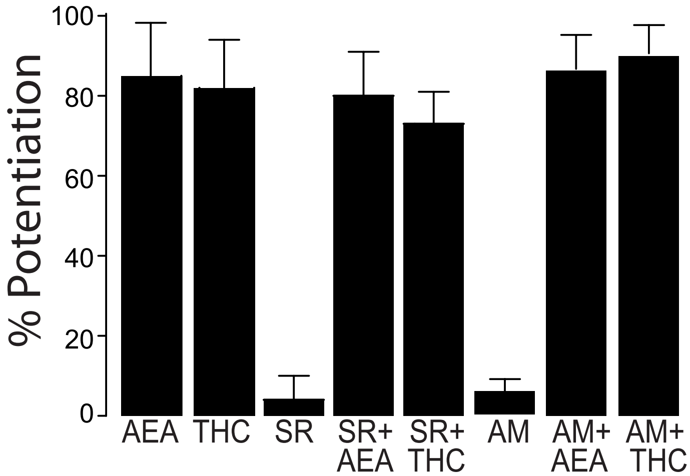

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Figure 4

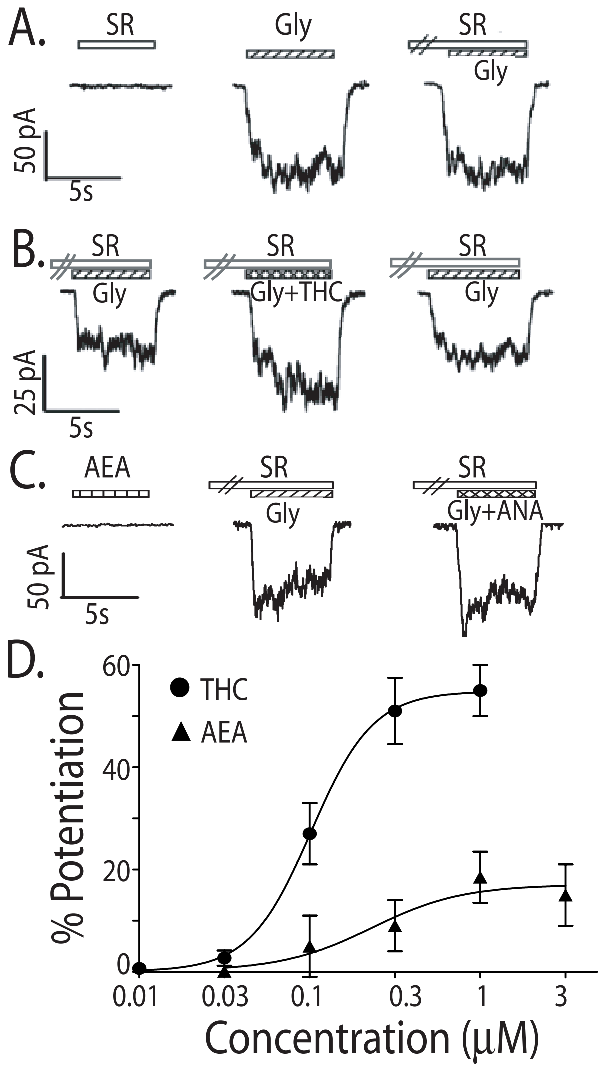

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Figure 5

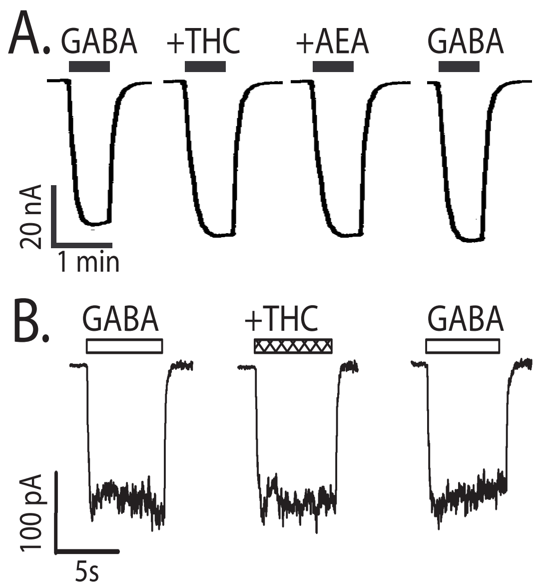

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Figure 6

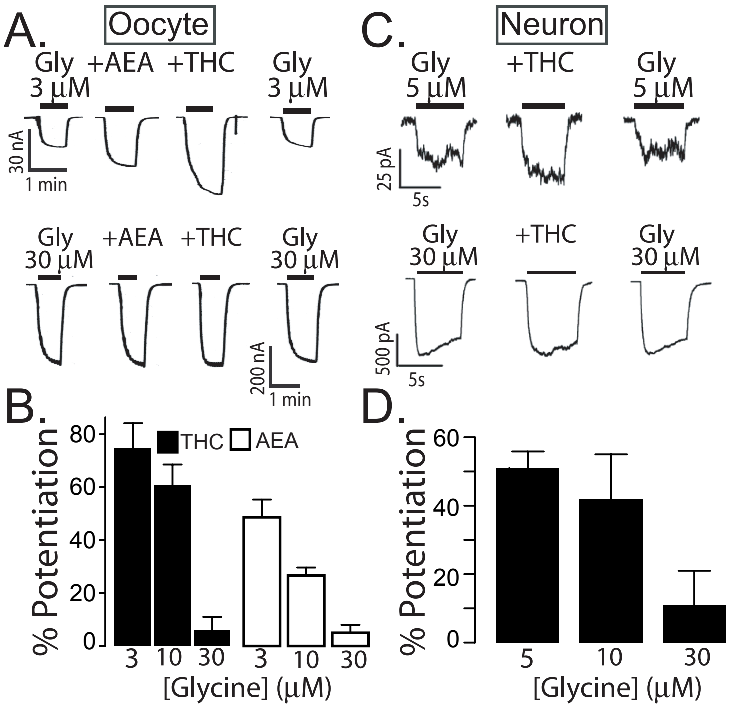

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Figure 7