Frequency-dependent cannabinoid receptor-independent modulation of glycine receptors by endocannabinoid 2-AG

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INTRODUCTION

Cannabinoids have widespread actions in the brain: they influence learning and memory, modulate locomotor activity and reward pathways. Majority of its effects suggested to be mediated by specific G-protein-coupled cannabinoid receptors (CBRs), their endogenous ligands (endocannabinoids) and the cellular machinery for their synthesis and degradation. Endocannabinoids are thought to act as retrograde messengers, being released from the postsynaptic neuron and acting on presynaptic CBRs to decrease neurotransmitter release. Also, at physiologically relevant concentrations cannabinoids can directly modulate the function of voltage-gated and receptor-operated ion channels. Using patch-clamp recording we analyzed the consequences of the direct action of an endocannabinoid, 2-arachidonoylglycerol (2-AG), on the functional properties of glycine receptor channels (GlyRs) and ionic currents in glycineergic synapses. At physiologically relevant concentrations (0.1–1 μM), 2-AG directly affected the functions of recombinant homomeric α1H GlyR: it inhibited peak amplitude and dramatically enhanced desensitization. The action of 2-AG on GlyR-mediated currents developed rapidly, within ~300 ms. Addition of 1 μM 2-AG strongly facilitated the depression of glycine-induced currents during repetitive (4–10 Hz) application of short (2 ms duration) pulses of glycine to outside-out patches. In brainstem slices from CB1 receptor knockout mice, 2-AG significantly decreased the extent of facilitation of synaptic currents in hypoglossal motoneurons during repetitive (10–20 Hz) stimulation. These observations suggest that endocannabinoids can modulate postsynaptic metaplasticity of glycineergic synaptic currents in a CB1 receptor-independent manner.

Keywords: glycine receptors, endocannabinoids, desensitization, patch-clamp, metaplasticity

Abbreviations: 2-AG, 2-arachidonoylglycerol; ACSF, artificial cerebrospinal fluid; CB1R−/− mice, CB1 receptor knockout mice; CBRs, cannabinoid receptors; CHO cells, Chinese hamster ovary cells; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinazoline; CNS, central nervous system; GABA, gamma-aminobutyric acid; GlyR, glycine receptor; HM, hypoglossal motoneurons; IGLIC, glycine-induced current.

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In hypoglossal nucleus glycinergic synapses provide the main inhibitory drive regulating respiratory activity and other physiological functions (Peever et al., 2001). Glycinergic synaptic currents in hypoglossal motoneurons (HM) are partially modulated presynaptically through the retrograde cannabinoid signaling pathway (Mukhtarov et al., 2005), while the existence of additional postsynaptic modulation of glycinergic synapses in these motoneurons has also been suggested.

Indeed, recent data indicate that cannabinoids directly modulate GlyR. The nature of this modulation depends on concentration of glycine. At low doses of the glycine (EC50; Lozovaya et al., 2005; Hejazi et al., 2006; Iatsenko et al., 2007; Yang et al., 2008; Zhang and Xiong, 2009). Thus cannabinoids exert dual concentration-dependent effects on IGly.

In this study, firstly we analyzed the basic properties of direct modulation by 2-AG of homeric α1 H GlyRs expressed in chine nese hamster ovary (CHO) cells that do not contain endogenous CBs. Secondly, we showed the functional significance of direct endocannabinoid-induced modulation for the glycinergic synaptic transmission in HMs in brainstem slices from CB1R-knockout mice.

MATERIALS AND METHODS

The experiments were carried out on cultured CHO cell lines and on brainstem slices from postnatal (P15–P20) CB1R-knockout (CB1R–/−) mice (Ledent et al., 1999).

CELL CULTURE AND TRANSFECTIONS

The CHO cells were cultivated as described earlier (Fucile et al., 1999; Medina et al., 2000; Markova et al., 2008). Cells were transfected with cDNA encoding the human GlyRα1 subunit using the lipofectamine reagent method (Life Technology, USA). To facilitate identification of transfected cells, cells were cotransfected with GFP.

ELECTROPHYSIOLOGICAL RECORDINGS FROM CHO CELLS

Glycine-activated currents were studied in the whole-cell and outside-out patch-clamp configurations using a EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany) under visualization with a Nikon microscope. The experiments were performed at room temperature (20–22˚) at a holding potential of −30 mV.

The cells were incubated in solution of the following composition (in mM): 140 NaCl, 2.8 KCl, 2 CaCl2, 4 MgCl2, 20 HEPES/NaOH, 10 glucose, pH 7.4. The patch pipettes were filled with a solution containing (in mM): 140 CsCl, 2 MgCl2, 2 MgATP, 0.4 Na2GTP, 10 HEPES/CsOH, 20 BAPTA/KOH, 6 CaCl2, pH 7.2, 290–300 mOsm. For rapid replacement of solutions, a system of three parallel rectangular tubes, 100 μm in diameter, located at a distance of 40–50 μm from the tested cell, was used. The position of tubes was controlled by a computer regulating the fast perfusion system (SF 77A Perfusion Fast-Step; Warner, USA). As measured by open tip electrode controls (1/10 NaCl), in this system a 20–80% solution exchange time was within 3 ms.

For experiments mimicking synaptic release concentration, steps of glycine lasting 1–2 ms were applied to outside-out patches at various frequencies depending on the purpose of the experiment. Solution exchange across the tip of the patch pipette was performed using a piezoelectric fast application system (P 245.70, Physik Instrumente, Waldbronn, Germany). With an open tip patch electrode (resistance >5 MΩ and a tip diameter <1 μm) the estimated 20–80% exchange time was 0.1–0.2 ms. In these experiments standard outside-out recordings were made under direct visualization (Leica microscope) of the CHO cells transfected by cDNA encoding the human GlyRα1 subunit. The resistance of the pipettes was 7–8 MΩ for outside-out recordings. Only outside-out patches with an input resistance exceeding 1 GΩ for were selected for analysis.

SLICE PREPARATION

CB1R-knockout (CB1R–/−) mice (P15–P20) were anaesthetized with either and killed by decapitation in agreement with the European Directive 86/609/EEC requirements. The brainstem was rapidly removed and placed in an oxygenated ice-cold saline buffer. Transverse 200 μm-thick brainstem slices were cut using an HM 650V (Microm, Germany) vibrating microslicer in ice-cold protecting solution containing (in mM): 130 K-gluconate, 14.6 KCl, 2 EGTA, 20 HEPES, 25 glucose, and 0.05 d-APV, equilibrated at pH 7.3 with KOH. Prior to recording, slices were incubated in an ACSF solution containing (in mM): 125 NaCl, 3.5 KCl, 2 CaCl2, 1.3 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose, equilibrated at pH 7.3 with 95% O2 and 5% CO2 at room temperature (22–25˚C) for at least 1 h to allow recovery.

ELECTROPHYSIOLOGICAL RECORDINGS IN BRAIN SLICES

For patch-clamp recordings brainstem acute slices were visualized through a ×40 water-immersion objective using an upright microscope (Axioskop, Zeiss, Germany). HM were identified by their location in the hypoglossal nucleus (n.XII), their large somata (25–40 μm) and their dendritic arborization.

During measurements, slices were superfused with oxygenated saline (1.0–1.5 ml min−1) with the same ionic composition as that used for slice incubation. Borosilicate glass patch pipettes (Harvard Apparatus) with a tip resistance of 3–5 MΩ were filled with an intracellular solution containing (in mM): 140 CsCl, 2 MgCl2, 0.03 CaCl2, 0.1 BaCl2, 10 HEPES, 2 MgATP, 0.4 Na2GTP, and 10 Na2-phosphocreatine, equilibrated at pH 7.3 with CaOH, 290 mOsm. QX 314 (1 mM) was routinely added into the intracellular solution to block voltage-gated sodium conductances.

Membrane currents were recorded with an EPC-9 (HEKA Elektronik, Germany) amplifier and filtered at 3–10 kHz. Stored data were analyzed using pClamp software (Axon Instruments) or the PulseFit program (HEKA Elektronik). The series resistances, ranging between 5 and 15 MΩ as estimated from slow transient cancelation, were compensated by 30–80% depending on the cell. The holding potential (V h) was −70 mV. The postsynaptic currents were elicited by stimulating pulses (1–90 V,100 μs) with glass bipolar electrodes. The stimulus intensity was adjusted to obtain the evoked responses without failures. Stimulating electrodes were obtained by pulling theta-glass tubes to a final tip diameter of 1–2 μm and filling with external solution. Trains of 10 stimuli at 10 or 20 Hz were applied.

To isolate strychnine-sensitive glycinergic synaptic currents all experiments were performed in the presence of blockers of glutamate (CNQX, 10 μM; d-APV, 40 μM) and GABA.
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(bicuculline, 10 μM) receptors. Antagonists were bath-applied via a gravity-driven perfusion system. For external application, 2-AG (5–10 μM) was applied via a puff pipette close to the cell soma.

d-APV, bicuculline and 2-AG were from Tocris (UK). All the other chemicals were from Sigma (USA).

Statistical analysis was done in Origin (OriginLab Corporation, USA) using Student’s paired t-test. Results are given as mean ± SEM.

RESULTS

DIRECT MODULATION OF GlyRα1H-MEDIATED CURRENTS BY THE ENDOCANNABINOID 2-AG

Application of glycine (10 μM–1 mM) to CHO cells transfected with α1H GlyR cDNA elicited whole-cell chloride currents (I\textsubscript{GlyR}), which exhibited weak desensitization even at a saturating concentration of agonist. The half-effective concentration of glycine (EC\textsubscript{50}) estimated from the dose–response curve was in the range

![FIGURE 1 | Modulation of human GlyRα1H-mediated currents by 2-AG in outside-out patches. (A) Representative traces of I\textsubscript{Gly}, induced by 50 μM Gly, recorded in outside-out patch-clamp configuration in control (blue) and in the presence of 1 μM 2-AG (red). Here and below representative traces correspond to the saturation of the effects of 2-AG. (B) Superimposed and normalized traces shown in (A) at expanded time scale to illustrate 2-AG-induced changes in the rise time of I\textsubscript{Gly}. (C) Deactivation of I\textsubscript{Gly}. Traces shown in (A) superimposed and normalized by the current amplitude at the end of glycine application before (blue) and after addition of 2-AG (gray; the same trace smoothed by Savitzky–Golay method is shown in red). (D–G) Time courses of the changes in I\textsubscript{Gly} peak amplitude (D), charge transfer of I\textsubscript{Gly} normalized by peak amplitude (E), 10–90% rise time (F), and 10–90% deactivation time (G) following application of 1 μM 2-AG.](www.frontiersin.org)
40–70 μM (data not shown), which corresponds closely to previously reported values in cell lines expressing α1 GlyR (Fucile et al., 1999, 2000; Buldakova et al., 2007; Markova et al., 2008). These values are also similar to those reported for acutely isolated rat hippocampal neurons (Kondratskaya et al., 2002) and for other preparations (Chesnok-Marchais, 2003; Ge et al., 2007).

Effects of the endogenous cannabinoid 2-AG on I_{GlyR} were studied in outside-out patches pulled from CHO cells transfected with α1H GlyR cDNA. GlyR-mediated chloride currents (I_{GlyR}) were evoked by application of 10 s-long pulses of 50 μM glycine (concentration close to EC_{50}). After control recordings, cells were exposed to the external solution with 2-AG (1 μM) and 2 min later I_{GlyR} were recorded every 2 min in the continuous presence of 2-AG. **Figure 1** shows that continuous application of 2-AG caused dramatic effects on glycine-induced currents. It inhibited the peak, decreased the rise time, strongly accelerated the rate of desensitization and slowed down the deactivation of I_{GlyR}. Effects reached saturation within 3–6 min. A particularly dramatic effect was on the kinetics of desensitization, which accelerated by hundreds of times. For instance, in the patch presented in **Figure 1**, the half time of current decay is less than 50 ms after 6 min of 2-AG application. All the effects of 2-AG were partially reversible (**Figures 2A–C**). To quantify the effects of 1 μM 2-AG we calculated averaged I_{GlyR} peak amplitudes, charge transfer of I_{GlyR} normalized by peak amplitude (reflecting changes in the speed of desensitization) and 10–90% rise time and deactivation time values in the presence of 2-AG, and compared these with control values.

After 4–6 min of the presence of 2-AG, mean peak amplitude values, calculated as I_{2-AG}/I_{CTRL}, decreased to 0.35 ± 0.04 (p = 0.0001; n = 9). Charge transfer of I_{GlyR} normalized by peak amplitude, used as an index of desensitization of I_{GlyR}, decreased to 0.066 ± 0.01 (p < 0.0001; n = 9). The rise time strongly decreased while deactivation time increased, with mean values of, respectively, 0.18 ± 0.02 (p < 0.004; n = 9), and 3.64 ± 0.87 (p = 0.03, n = 4) relative to control (**Figure 2C**).

The effects of 2-AG on I_{GlyR} depended on the concentration of endocannabinoid and were significant starting from 100 nM of 2-AG (**Figures 2D,E**). After 6 min of 100 nM 2-AG application, the mean peak amplitude of glycine-induced currents decreased to 0.68 ± 0.04 (p < 0.02; n = 5), normalized charge transfer by I_{GlyR} decreased to 0.55 ± 0.12 (p < 0.002; n = 5) of control and I_{GlyR} rise time decreased to 0.61 ± 0.05 (p < 0.005; n = 5) of control.

To estimate the rate of interaction of 2-AG with GlyR in outside-out patches we tested the modulation of I_{GlyR} by 2-AG (1 μM) applied simultaneously with glycine. Under these conditions the peak amplitude and the rise time of I_{GlyR} were unaltered; however, the normalized charge transfer decreased to 0.59 ± 0.02 (p < 0.02; n = 3; **Figure 3**). Changes in I_{GlyR} desensitization kinetics were first detected at 264 ± 65 ms from the beginning of co-application of glycine and 2-AG (n = 5; **Figure 3**).

These observations demonstrate that the endocannabinoid 2-AG modulates GlyR function by a CBR-independent mechanism, as CHO cells do not endogenously express any CBR. The effects developed in the time scale of hundreds milliseconds.

**EFFECTS OF 2-AG ON CURRENTS MEDIATED BY GlyRα1H DEPENDED ON AGONIST CONCENTRATION**

Several studies have shown that the modulatory effects of cannabinoids on GlyRs depend on the glycine concentration used for receptor activation (Hejazi et al., 2006; Yang et al., 2008). Therefore, we tested whether modulation of I_{GlyR} by 2-AG (1 μM) was dependent on the concentration of the agonist. Three concentrations of glycine were tested: low (20 μM), half-effective (50 μM), and saturating (1 mM). **Figure 4** shows that the maximal effects of 2-AG on I_{GlyR} peak and charge transfer were reached at 50 μM of glycine. I_{GlyR} induced by low and saturating glycine concentrations was significantly less affected by 2-AG, resulting in a bell-shaped glycine concentration dependence (**Figure 4D**). I_{GlyR} induced by 50 μM of glycine in the presence of 2-AG was inhibited to 0.35 ± 0.04 (p = 0.0001, n = 9), while I_{Gly} evoked by 20 μM, and 1 mM in the presence of 2-AG were either not affected or affected to a much lesser extent (1.04 ± 0.06, p = 0.52, n = 4 and 0.8 ± 0.08, p = 0.03, n = 4, respectively). Normalized charge transfer of I_{GlyR} induced by 50 μM glycine in the presence of 2-AG decreased to 0.07 ± 0.02, p = 0.0001, while normalized charges of I_{GlyR} induced by 20 μM and 1 mM were less affected (0.31 ± 0.10, p = 0.001, n = 4 and 0.16 ± 0.05, p = 0.001, n = 4, respectively). It should be specifically noted that when measured in the whole-cell configuration 2-AG induced potent augmentation of I_{GlyR} induced by a low concentration of glycine 20 μM (data not shown). This is in agreement with previous papers on the effects of cannabinoids on GlyRs (Hejazi et al., 2006; Iatsenko et al., 2007; Yang et al., 2008; Zhang and Xiong, 2009), while in outside-out configuration (as indicated above) this facilitation was not apparent. This difference could be a result of the distortion of the cytoskeleton and/or more efficient wash–out of intracellular factors in the outside-out patches that might be involved in up-regulation of GlyR by 2-AG. However, in the present paper we did not focus on this issue.

**FREQUENCY-DEPENDENT MODULATION OF I_{GlyR} BY ENDOCANNABINOIDS IN OUTSIDE-OUT PATCHES**

Recent study on neurons of zebrafish hindbrain suggest that the slow GlyR desensitization process mainly accounts for the frequency-dependent decrease in GlyR-mediated responses (Rigo and Legendre, 2006). We hypothesized, therefore, that the strong acceleration of GlyR desensitization induced by endocannabinoids would contribute to the reinforcement of the frequency-dependent decrease in synaptic glycineric current amplitudes.

We tested this hypothesis in outside-out patches from CHO cells transfected with GlyRs. Since in synapses neurotransmitter release is evoked by saturating glycine concentration, we first tested the effects of 2-AG on I_{GlyR} activated by 1 mM of glycine. **Figures 5A,B** shows that with long (10s) application of 1mM glycine 2-AG (1 μM) also significantly inhibited the peak and accelerated the rate of desensitization of I_{GlyR} (although the effects were less strong as with 50 μM glycine). The same effect of 2-AG on the peak current was observed with short (2 ms) ultra fast application of 1mM glycine mimicking synaptic transmission (**Figures 5C,D**). Whereas, the decay kinetics of the current representing deactivation was slightly, but not significantly increased (**Figure 5C**).
Next we tested effects of 2-AG on \(I_{\text{GlyR}}\) evoked by a train of short glycine pulses using ultra fast agonist application techniques. The amplitude of the outside-out currents evoked by repetitive applications (10 Hz) of short (2 ms) pulses of a saturating glycine concentration (1 mM) progressively declined (Figure 6A). The extent of this decline increased with increasing application frequencies (not shown).

This depression of \(I_{\text{GlyR}111}\) during repetitive application of glycine was strongly enhanced in the presence of 2-AG (1 μM; Figures 6A–C). The ratios of amplitudes of the 10th and first...
FIGURE 3 | Effect of 2-AG (1 μM) on $I_{Gly}$ occurs within hundreds of milliseconds. (A) Representative traces of $I_{Gly}$ obtained in control (blue) and in the presence 2-AG (1 μM, red) applied simultaneously with glycine (50 μM).

(B) Corresponding traces are normalized and superimposed. Note that changes in $I_{Gly}$ kinetics can be detected from 200 ms after the beginning of glycine and 2-AG co-application.

FIGURE 4 | Modulation of $I_{Gly}$ by 2-AG depends on the concentration of glycine. (A–C) Left, representative outside-out recordings of $I_{Gly}$ induced by 50 μM (A), 1 mM (B), and 20 μM (C) of glycine obtained in control and in the presence 2-AG (1 μM). (A–C) Right, corresponding traces are normalized and superimposed. (D) Summary plots showing the effects of 1 μM of 2-AG on $I_{Gly}$, the amplitude (left), normalized charge (middle), and 10–90% rise time (right), of $I_{Gly}$ at various concentrations of Gly. Data are plotted as a fraction of control values.
FIGURE 5 | Modulation by 2-AG of $I_{\text{GlyR}}$ evoked by saturated concentration of glycine. (A) Left, representative traces of $I_{\text{GlyR}}$ induced by long (10 s) pulse of 1 mM Gly, obtained in control (blue), in the presence of 1 μM 2-AG (red). Right, corresponding traces are normalized and superimposed. (B) Summary plots showing the effects of 1 μM 2-AG on $I_{\text{GlyR}}$ amplitude and charge transfer of $I_{\text{GlyR}}$ normalized by peak amplitude. Data are plotted as a fraction of control values. (C,D) The same as in (A,B), but for (2 ms) pulse of 1 mM glycine.

FIGURE 6 | Frequency-dependent modulation of $I_{\text{Gly}}$ by endocannabinoids in outside-out patches. (A) Representative traces of $I_{\text{Gly}}$, induced by the train (10 Hz, 10 pulses) of 10 short (2 ms) applications of Gly (1 mM) in control (left), in the presence 2-AG (1 μM; Middle), and after wash-out of 2-AG (right). Arrows indicate the glycine applications. (B) Summary plot showing decline in $I_{\text{Gly}}$ in response to a train of stimuli, in control (blue circles) and in the presence of 1 μM 2-AG (red circles). (C) Averaged total charge of $I_{\text{Gly}}$ in response to a train of stimuli normalized by first peak amplitude, in the presence of 2-AG as a fraction of control value. Data from four outside-out patches.
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Correspondingly, charge transfer in the train normalized to the first peak was also significantly decreased, to 0.50 ± 0.11 (p = 0.006, n = 4; Figure 6C), while the absolute charge of $I_{\text{GlyR}}$ induced by a train of short pulses of Gly, applied at 10 Hz, decreased to 0.28 ± 0.06; $p = 0.004$. The same phenomenon, but to a lesser extent, was observed at 4 Hz stimulation (data not shown).

These observations indicate that 2-AG can be a powerful modulator of glycine responses amplitude at high-frequency activation.

**SHORT-TERM PLASTICITY OF GLYCINERGIC TRANSMISSION INDUCED BY DIRECT ACTION OF 2-AG ON RAT HYPOGLOSSAL MOTONEURONS**

It has been suggested previously that in HMs, in addition to the CB1R-dependent retrograde signaling pathway, there exist additional mechanisms of modulation of glycine responses synaptic transmission by endocannabinoids (Mukhtarov et al., 2005). One of these mechanisms would be direct interaction of GlyRs and endocannabinoids. To test whether the direct modulation of the GlyR desensitization by endocannabinoids contributes to the activity–dependent changes in glycine transmission we performed experiments in HMs using the CB1R−/− mouse brainstem slice preparation. This model eliminates the possible impact of presynaptic retrograde modulation by endocannabinoids.

Short-term changes in glycine responses synaptic currents induced by repetitive stimulation of presynaptic axons in the presence of 2-AG were investigated using whole-cell voltage-clamp recordings. In control conditions a train of repetitive stimuli applied at 20 Hz induced significant short-term facilitation of the glycine response synaptic currents. Figure 7A shows representative averaged traces of the increased IPSCs evoked by trains. In the presence of 2-AG the extent of synaptic facilitation during the train was significantly less developed (Figures 7A,B). The ratios of amplitudes of the 10th and first currents in trains ($I_{\text{IPSC10}}/I_{\text{IPSC1}}$) were $1.79 ± 0.44$ in control and $0.94 ± 0.12$ in the presence of 2-AG; $p < 0.02, n = 5$. Correspondingly, normalized charge transfer in the train also significantly decreased to $0.74 ± 0.07; p < 0.02, n = 5$ (Figure 7C). The peak amplitude of the first IPSC in the train in the presence of 2-AG was $0.64 ± 0.15 (p < 0.05, n = 5)$ of the control value. The same phenomena, but to a smaller extent, were observed at 10 Hz stimulation (data not shown).

These data directly demonstrate the physiological implications for the CB1-independent modulation of GlyR by endocannabinoids.

**DISCUSSION**

The key findings of the present paper are the following. First, the endocannabinoid 2-AG directly affected functions of recombinant homomeric α1H GlyR: it inhibited peak amplitude, dramatically enhanced desensitization, decreased the rise time and slowed down the deactivation of $I_{\text{Gly}}$ induced by a long pulse of agonist. Second,
Moreover, it has been shown that desensitization shifts the 2-AG affinity for CB1Rs (60–472 nM; Mechoulam et al., 1995; Howlett et al., 2002). We had already observed significant modulation of the amplitude, activation and decay kinetics of $I_{Gly}$ at a 2-AG concentration of 100 nM. Furthermore, since 2-AG, highly lipophilic compound, is believed to be synthetized and accumulated within lipid bilayer (Di Marzo et al., 1999) its concentration in the vicinity of GlyR might be much higher.

It is unlikely that the observations described here can be attributed to the activation of any known CBR. First of all, cannabinoids affected currents mediated by recombinant GlyRs expressed in CHO cells. Secondly, in our experiments were used CB1 knockout mice, excluding interaction with this subtype. Thirdly, the pharmacological profile of cannabinoid-induced effects on GlyRs was remarkably different from that of classical CB1R. While CB1R mediated effects can be inhibited by the antagonist SR141716A (Thomas et al., 2006), in our earlier experiments SR141716A failed to block cannabinoid induced reduction of native GlyR-mediated current amplitude in isolated hippocampal neurons. In contrast SR141716A itself modulated GlyRs in a similar way as CB1 agonist. (Lozovaya et al., 2005).

Another striking difference in pharmacological profile of the two mechanisms is that the order of potency for different agonists differs from that observed for CB1R activation. WIN55,212-2 is much more potent at activating the CB1R compared to endogenous cannabinoids such as 2-AG (Breivogel et al., 2001). However, in our experiments, 2-AG was much more potent at modulating of both recombinant alpha 1 (see Figure S1 in Supplementary Material) and native GlyR-mediated currents (Lozovaya et al., 2005).

The current decay after removal of glycine represents transitions from active ligand-bound receptor states to inactive unbound states, including channel closure and glycine dissociation. In addition, desensitized receptors may be reopened before agonist dissociation, prolonging the deactivation time course (Jones and Westbrook, 1995). The fact that endocannabinoids accelerated the $I_{Gly}$ desensitization suggested the possibility of coupling between $I_{Gly}$ desensitization and deactivation. Indeed, acceleration of $I_{Gly}$ desensitization by 2-AG was accompanied by a slowdown of $I_{Gly}$ deactivation. This phenomenon of desensitization–deactivation coupling has been observed in a number of experimental conditions (Jones and Westbrook, 1995; Largrane et al., 2007). Moreover, it has been shown that desensitization shifts receptor-operated channels to a high-affinity state (Chang et al., 2002).

Our experimental data and model simulations (see Appendix) using simplified model of GlyR proposed by (Grewer, 1999 and the ChannelLab 2.0 software (developed by S. Traynelis for Synaptosoft, Decatur, GA, USA, unpublished data) indicate that modulation of GlyR gating by endocannabinoids considered here appears to be most compatible with enhancement of binding and desensitization. However, we cannot exclude that 2-AG additionally affected conformational transitions other than binding and desensitization but their impact is minor.

Thus 2-AG strongly affects parameters of $I_{Gly}$ induced by long application of glycine. However, macroscopic desensitization does not seem to contribute to GlyR function during brief agonist exposure. The question arises as to whether endocannabinoid-induced modulation of GlyR gating has implications for synaptic transmission. It has recently been suggested that the GlyR desensitization process may contribute to a frequency-dependent decrease in GlyR-mediated response amplitude (Rigo and Legendre, 2006). Indeed, our data show that modulation of GlyR by 2-AG during repetitive stimulation occurs in a strongly frequency-dependent manner and becomes prominent at 10 Hz. Receptor activity can be down-regulated as a result of progressive accumulation of the number of postsynaptic receptors being in a long-lasting desensitization state. It has been shown that desensitization of postsynaptic AMPA and GABA receptors plays a role in short-term plasticity (STP) of synaptic transmission (for review see Zucker and Regehr, 2002). This phenomenon can be developed on elevation of the low concentration of neurotransmitter in the synaptic cleft during prolonged synaptic activation (Larkman et al., 1997; Overstreet et al., 2000) or at accumulation of a small fraction of postsynaptic receptors in a desensitized state when activation frequency is close to the recovery rate constant from the desensitized state (Rigo and Legendre, 2006). In our experiments 2-AG induced a dramatic increase in the fraction of desensitized GlyRs; correspondingly, this leads to an enhancement of short-term depression (STD) in a condition of repetitive stimulation.

Short-term plasticity plays an essential role in filtering sensory information (O’Donovan and Rinzel, 1997; Zucker and Regehr, 2002; Thomson, 2003; Abbott and Regehr, 2004). At the synaptic level, the efficacy of fast inhibitory synaptic transmission depends on the number of presynaptic release sites, the presynaptic release probability and the parameters of postsynaptic responses. Prominent postsynaptic alterations include modulation of desensitization. STP in turn can be modulated by previous synaptic activity or by neuromodulatory inputs. This second degree of plasticity, plasticity of plasticity, has been termed metaplasticity (Abraham and Tate, 1997; Fischer et al., 1997) and is regarded to be essential for information processing in neuronal networks (for review see Abraham and Tate, 1997; Abbott and Regehr, 2004). While persistent changes in synaptic efficacy, such as long-term potentiation and long-term depression, are essential for information storage in neural networks (Malenka and Bear, 2004), a complementary mechanism, metaplasticity, maintains network stability and ensures that synapses operate within a functional dynamic range by modifying the expression of synaptic plasticity as a function of prior activity (Abraham, 2008). Endocannabinoids are widely regarded as negative modulators of presynaptic release, acting retrogradely via presynaptic G-protein-coupled CB1 receptors, and have emerged as key activity-dependent signals that can mediate short-term and long-term
We suggest that this is a result of an increased contribution from Betz, H., and Laube, B. (2006). Glycine.

Breivogel, C. S., Griffin, G., Di Marzo, Becker, C. M. (1990). Disorders of

and Regehr, 2004). Dysfunctioning of STD modulation could

control to increase their sensitivity to sudden changes (Abbott

networks with functional capabilities such as filtering and gain

interaction of the two types of synapses (excitatory and inhibitory)

postsynaptically induced STD in the presence of 2-AG. The inter-

play of various STP components at each synapse and the dynamic interaction of the two types of synapses (excitatory and inhibitory)

are thought to determine the types of computations performed

by neural circuits (Tsodyks and Markram, 1997; Dobrunz and Stevens, 1999; Dittman, et al., 2000; Pouille and Scanziani, 2004).

STD has been postulated to be essential in the regulation of many cognitive functions including habituation (Chung et al.,

2002; Nicollels, 2002) and coordination of motor control (Nadim

and Manor, 2000). STD has been shown to bestow neuronal networks with functional capabilities such as filtering and gain control to increase their sensitivity to sudden changes (Abbott and Regehr, 2004). Dysfunctioning of STD modulation could

be of particular relevance in pathophysiology, such as Parkinson’s disease (Tecuapetla et al., 2007) and epilepsy (Doherty and Dingleline, 2001).

Thus endocannabinoids enhance the low-pass filtering ability of GlyR synapses for inhibitory signals and consequently increase the probability of transfer of the high-frequency excitatory signals conveyed to hypoglossal neurons. Therefore, endocannabinoids may induce striking changes in the temporal information processing in glycineric synapses by direct action on postsynaptic GlyRs.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/molecular_neuroscience/10.3389/ fnmol.2011.00013/abstract

Figure S1 | Summary of the saturated effects of the modulation of human GlyRα1δ1δ2-mediated currents recorded from outside-out patches by WIN 55,212-2. (A) Superimposed representative traces of I_Gly, induced by 50 μM Gly, obtained in control, in the presence of 1 μM WIN 55,212-2, and after wash-out of cannabinoids (left). (Middle and right) corresponding traces are normalized. (B) The averaged numbers for changes of I_Gly, peak (left), normalized charge (middle), and 10–90% rise time (right) induced by WIN 55,212-2 plotted as a fraction of the control values.

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APPENDIX

SIMPLIFIED MODEL OF GLYCINE RECEPTOR MODULATION

The kinetic modeling was performed using the ChannelLab 2.0 software (developed by S. Traynelis for Synaptosoft, Decatur, GA, USA).

Our experimental data indicate that modulation of desensitization may offer a potent mechanism of an amplitude regulation of responses evoked by saturating concentration of glycine. The main goal of model simulation presented here was to consider the impact of desensitization on the amplitude regulation of $I_{\text{Gly}}$ induced by saturating concentration of glycine under condition of high-frequency stimulation.

Taking into account the available experimental evidence, our strategy was to propose a minimum requirement for 2-AG effects by adapting a previously published model (Grewer, 1999; **Figure A1**). In the model the $k_4$ in control conditions was increased, because in the present study the currents elicited by 10 s pulse of glycine yielded currents with slower decay kinetics, than that predicted by model. The major simplifying assumption of this model is that it postulates only one set of fully bound states (open, closed, desensitized). In particular, it is known that besides unique desensitized state included in the model, there could be several other slower desensitized components which might additionally shape the current responses, especially those elicited by long glycine pulses (Harty and Manis, 1998; Legendre, 1998; Beato et al., 2004).

The major effect of 2-AG was the dramatic acceleration of $I_{\text{Gly}}$ desensitization. At saturating (1 mM) and at 50 μM of glycine, 2-AG reduced the current amplitude indicating that, as mentioned above, 2-AG favors the occupancy of the desensitized state. As an attempt to model this possibility, we assumed that 2-AG increases the rate constant of entrance into the desensitized state (DL3). Indeed this assumption was sufficient to qualitatively reproduce our experimental finding on acceleration of decay, inhibition of peak amplitude of $I_{\text{Gly}}$ by 2-AG and our main finding: enhancement of frequency-dependent decrease of peak amplitude of $I_{\text{Gly}}$. In addition, 2-AG induces acceleration of $I_{\text{Gly}}$ onset. Such a 2-AG effect can be easily reproduced by increasing the binding rate $k_2$ (**Tables A1** and A2).

However, our model data are quantitatively slightly contradicted with experimental data: the extent of inhibition of the peak amplitude of $I_{\text{Gly}}$ in train has been more pronounced in model simulation. To adapt our experimental data we had to introduce changes in rate of conformational transition open-fully occupied closed state ($k_{-4}$). As a matter of fact, the rate of decline of $I_{\text{Gly}}$ reflects both the intrinsic molecular rate constants for desensitization and the probability of occupying states from which desensitization can occur. A perturbation that accelerates the decline of current may do so by increasing either or both of these processes. The results of simulation are presented in **Figures A1B,D**.

Nevertheless, to confirm the assumption that 2-AG affect conformational transition open-fully occupied closed state additional single-channel recordings should be performed. On the other hand quantitative contradiction with experimental data can reflect the oversimplification of this model assumptions.

Thus, our model simulations indicate that modulation of GlyR gating by endocannabinoids considered here appears to be most compatible with enhancement of binding and desensitization. Furthermore, these processes mainly account for the strong enhancement of frequency-dependent decrease in GlyR-mediated responses in the presence of 2-AG.
Table A1 | The rate constant values in control conditions and in the presence of 2-AG.

| [2-AG] [Gly] | k₁ (μM⁻¹ s⁻¹) | k₋₁ (s⁻¹) | k₂ (μM⁻¹ s⁻¹) | k₋₂ (s⁻¹) | k₃ (μM⁻¹ s⁻¹) | k₋₃ (s⁻¹) | k₄ (s⁻¹) | k₋₄ (s⁻¹) | k₅ (s⁻¹) | k₋₅ (s⁻¹) |
|-------------|----------------|----------|----------------|----------|----------------|----------|----------|----------|----------|----------|
| Control     | 27             | 1900     | 18             | 3800     | 9              | 5700     | 2.2*10⁴  | 38       | 23.5     | 0.02     |
| 20μM        | 27             | 1900     | 18             | 350      | 9              | 5700     | 2.2*10⁴  | 300      | 90       | 0.02     |
| 50μM        | 27             | 1900     | 18             | 350      | 9              | 5700     | 2.2*10⁴  | 2000     | 90       | 0.02     |
| 1 mM        | 27             | 1900     | 18             | 350      | 9              | 5700     | 2.2*10⁴  | 100      | 800      | 0.02     |

Table A2 | The effects of 2-AG (1 μM) on I_Gly at different concentration of glycine. ED, experimental data; MS, model simulation.

| Glycine concentration | Amplitude | Rise time | Charge | Amplitude | Rise time | Charge |
|-----------------------|-----------|-----------|--------|-----------|-----------|--------|
| 20μM                  | 104       | 23        | 32.24  | 105.00    | 13.91     | 37     |
| 50μM                  | 35        | 10        | 2.45   | 37        | 11        | 2.16   |
| 1 mM                  | 80        | –         | 12.8   | 95        | 93        | 4      |

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