available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Subunit-specific modulation of glycine receptors by cannabinoids and N-arachidonyl-glycine

Zhe Yang [a], *Karin R. Aubrey* [b], *Iris Alroy* [c], *Robert J. Harvey* [d], *Robert J. Vandenberg* [b], *Joseph W. Lynch* [a,*]

[a] *Queensland Brain Institute and School of Biomedical Sciences, University of Queensland, Brisbane, QLD 4072, Australia*
[b] *Discipline of Pharmacology, School of Medical Sciences, Bosch Institute, University of Sydney, Sydney, NSW 2006, Australia*
[c] *Pharmos Ltd., Kiryat Weizmann, Rehovot 76326, Israel*
[d] *Department of Pharmacology, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK*

ARTICLE INFO

Article history:
Received 24 June 2008
Accepted 30 July 2008

Keywords:
Cys-loop receptor
Ligand-gated ion channel
Nociception
Analgesia
Neurotransmission
Endocannabinoid
HU-210
HU-308
WIN55,212-2
Anandamide
N-arachidonyl-glycine

ABSTRACT

Glycine receptors (GlyRs) mediate inhibitory neurotransmission in spinal cord motor and pain sensory neurons. Recent studies demonstrated apparently contradictory (potentiating versus inhibitory) effects of the endocannabinoid anandamide on these receptors. The present study characterised the effects of cannabinoid agonists on α1, α1β, α2 and α3 GlyRs recombinantly expressed in HEK293 cells with the aims of reconciling effects of cannabinoids on these receptor subtypes and to establish the potential of different GlyR isoforms as novel physiological or analgesic targets for cannabinoids. The compounds investigated were anandamide, HU-210, HU-308, WIN55,212-2 and the endogenous non-cannabinoid, *N*-arachidonyl-glycine. The latter compound was chosen due to the structural similarity with anandamide and known analgesic actions in the spinal cord. Recombinant α1 and α1β GlyRs were potentiated by anandamide and HU-210 at submicromolar concentrations, whereas WIN55,212-2 had no effect and HU-308 produced only weak inhibition. By contrast, *N*-arachidonyl-glycine exerted complex effects including both potentiation and inhibition. Anandamide had no effect at α2 or α3 GlyRs although the other cannabinoids produced potent inhibition. On α2 GlyRs, the inhibitory potency sequence was HU-210 = WIN55,212-2 > HU-308 > *N*-arachidonyl-glycine but on α3 GlyRs it was HU-210 = WIN55212 = HU-308 > *N*-arachidonyl-glycine. These results suggest that α1, α2 and α3 containing GlyRs exhibit distinct pharmacological profiles for cannabinoids. We conclude that cannabinoid agonists may be useful as pharmacological tools for selectively inhibiting α2 and α3 GlyRs. Our results also establish GlyRs as potential novel targets for endogenous and exogenous cannabinoids.

1. Introduction

Pharmacological and gene knockout experiments have shown that the analgesic effects of cannabinoids are mediated primarily through CB_1 and CB_2 G protein-coupled receptors [1,2]. However, cannabinoids also produce actions that are not mediated by these receptors [3]. Because cannabinoid agonists exert effects on a wide variety of membrane proteins at

* *Corresponding author*. Tel.: +61 7 3365 3157; fax: +61 7 3365 1766.
E-mail address: j.lynch@uq.edu.au (J.W. Lynch).

Abbreviations: AEA, anandamide; $GABA_AR$, γ-aminobutyric acid type-A receptor chloride channel; GlyR, glycine receptor chloride channel; IPSC, inhibitory postsynaptic current; NA-Gly, *N*-arachidonyl-glycine; nAChR, nicotinic acetylcholine receptor cation channel; THC, Δ^9-tetrahydrocannabinol; WT, wild type.

0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.07.037

physiologically relevant concentrations, there are many potential candidate receptors for these actions [4].

Potent direct potentiating effects of the cannabinoids anandamide (AEA) and Δ^9-tetrahydrocannabinol (THC) have recently been identified at the glycine receptor (GlyR) chloride channel [5]. Because GlyRs mediate inhibitory neurotransmission onto nociceptive sensory neurons in peripheral laminae of the spinal cord dorsal horn [6–10], agents that specifically potentiate glycinergic inhibitory postsynaptic currents (IPSCs) should dampen the transmission of nociceptive impulses to the brain and thereby exert analgesic activity. It is thus feasible that GlyRs could mediate some of the analgesic effects of cannabinoids in the spinal cord.

AEA is closely related in structure to *N*-arachidonyl-glycine (NA-Gly), with the two molecules differing only by an OH group. NA-Gly is also found endogenously in the nervous system where it is distributed at highest levels in the spinal cord [11,12]. Spinal administration of NA-Gly also produces analgesia via an unknown mechanism [12–14]. Whereas AEA potently activates both CB_1 and CB_2 receptors, NA-Gly has no effects on either receptor [15].

GlyRs are members of the Cys-loop ligand-gated ion channel superfamily and can be formed either as pentameric homomers of α1, α2 or α3 subunits or as αβ subunit heteromers [16].

Several recent publications have investigated the effects of cannabinoids on GlyRs [5,17,18]. One study showed that the endocannabinoid agonists AEA and 2-arachidonylglycerol inhibited glycine-gated currents in isolated hippocampal and cerebellar neurons [17]. Since the effect was not abolished by CB_1 receptor antagonists or G protein inhibitors, a direct interaction between endocannabinoids and GlyRs was proposed. In apparent contradiction, AEA and THC increased the amplitude of subsaturating glycine-induced currents in *Xenopus* oocytes expressing homomeric α1 or heteromeric α1β GlyRs [5], with similar effects being observed in native GlyRs in rat ventral tegmental area neurons. This study also showed that GlyR modulation occurred independently of the CB_1 receptor. The third study identified no direct effect of the synthetic cannabinoid agonist, WIN55,212-2, on glycinergic IPSCs in hypoglossal motor neurons [18]. The inconsistency between these reports is possibly the result of the lower glycine concentrations used in the study reporting potentiation. Alternatively, it may have resulted from differences in GlyR subunit composition.

The present study systematically investigated the pharmacological profiles of several cannabinoids on recombinantly expressed α1, α1β, α2 and α3 GlyRs with the aims of reconciling the apparently contradictory results obtained to date, and to establish the potential of different GlyR isoforms as possible non-CB receptor analgesic targets for cannabinoids. At each of the four GlyR isoforms, we compared the actions of the following five compounds: AEA which is a non-specific agonist of CB_1 and CB_2 receptors [19], HU-308 which is highly specific for CB_2 over CB_1 receptors [20], HU-210 which is a highly potent CB_1 and CB_2 receptor agonist [19,21], WIN55,212-2 which is selective for CB_2 over CB_1 receptors [19,21] and NA-Gly which exhibits no activity at either CB_1 or CB_2 receptors, but is structurally related to AEA and exerts potent analgesic effects in the spinal cord. The synthetic cannabinoid compounds used in this study belong to three different structural classes: HU-210 is a classical cannabinoid, HU-308 is a non-classical bicyclic cannabinoid, and WIN55,212-2 belongs to the aminoalkylindoles which bear no obvious structural similarities to either classical or non-classical cannabinoids.

2. Materials and methods

2.1. Mutagenesis and expression of GlyR cDNAs

The human GlyR α1 subunit cDNA was cloned into the pCIS2 plasmid vector. The human α2 and rat α3L subunits were cloned into the pcDNA3.1 plasmid vector (Invitrogen, Carlsbad, CA). The human β subunit was cloned into the pIRES2-EGFP plasmid vector (Clontech, Mountain View, CA). HEK293 cells, cultured in Dulbecco's Modified Eagles Medium, were transfected using a calcium phosphate precipitation protocol. When co-transfecting plasmids encoding the GlyR α and β subunits, cDNAs were combined in a ratio of 1:20. After exposure to transfection solution for 24 h, cells were washed twice in calcium-free phosphate buffered saline and used for recording over the following 24–72 h.

2.2. Electrophysiology

Cells were visualised using an inverted fluorescent microscope and currents were measured by whole cell patch-clamp recording. Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had a tip resistance of 1–2 MΩ when filled with the standard pipette solution which contained (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configuration, cells were voltage-clamped at −40 mV and membrane currents were recorded using an Axon Instruments MultiClamp 700B amplifier and pClamp9 software (Molecular Devices, Union City, CA). The cells were perfused by a parallel array of microtubular barrels through which solutions were gravity-induced. All experiments were conducted at room temperature (19–22 °C).

Because α subunits can form functional GlyRs, it was necessary to confirm the incorporation of β subunits into functional αβ heteromers. As the GlyR β subunit cDNA was cloned into the pIRES2-EGFP plasmid vector, we used EGFP fluorescence to identify cells expressing the GlyR β subunit. The successful incorporation of β subunits into functional heteromeric GlyRs was inferred by their characteristic insensitivity to 5 μM picrotoxin [22]. Picrotoxin (PTX), from Sigma (St Louis, MO), was dissolved in DMSO at a concentration of 100 mM.

HU-210 and HU-308 (both gifts from Pharmos Ltd., Rehovot, Israel) were dissolved in ethanol at a concentration of 100 mM. WIN55,212-2 (Biomol, Plymouth Meeting, PA, USA) was dissolved in DMSO at a concentration of 50 mM. NA-Gly and AEA (both from Cayman Chemical, Ann Arbor, MI, USA) were

dissolved in DMSO at a concentration of 100 mM. Stock solutions were kept frozen for up to 2 weeks in small aliquots. Once thawed, aliquots were used or discarded. Substances were applied to cells within 2 h of being dissolved into room temperature bathing solution at their final concentration. The different solvents were employed in line with supplier's instructions. Solvent application alone had no effect at the concentrations used here. The molecular weights of the compounds used in this study were as follows: HU-210, 386.6 g; HU-308, 414.6 g; WIN55,212-2, 522.6 g; AEA, 347.5 g; NA-Gly, 361.5 g.

2.3. Data analysis

Results are expressed as mean ± S.E.M. of three or more independent experiments. The Hill equation was used to calculate the half-maximal concentration (EC_{50}) and Hill coefficient (n_H) values for activation. A similar equation was also used to calculate the half maximal concentrations for inhibition (IC_{50}) and n_H values for inhibition. All curves were fitted using a non-linear least squares algorithm (Sigmaplot 9.0, Jandel Scientific). Statistical significance was determined by unpaired Student's *t*-test with $p < 0.05$ representing significance.

3. Results

3.1. Effects on α1 GlyRs

Because glycine concentrations >EC_{30} have previously been shown to dramatically reduce the sensitivity of GlyRs to AEA [5], all drug dose–response relationships were measured at the EC_{10} glycine concentration. The EC_{10} concentrations, determined empirically for each cell, were in the range of 10–15, 50–70, and 100–150 μM for α1, α2 and α3 GlyRs, respectively. Examples of the effects of increasing concentrations of AEA, HU-308 and WIN55,212-2 on the amplitude of EC_{10} glycine

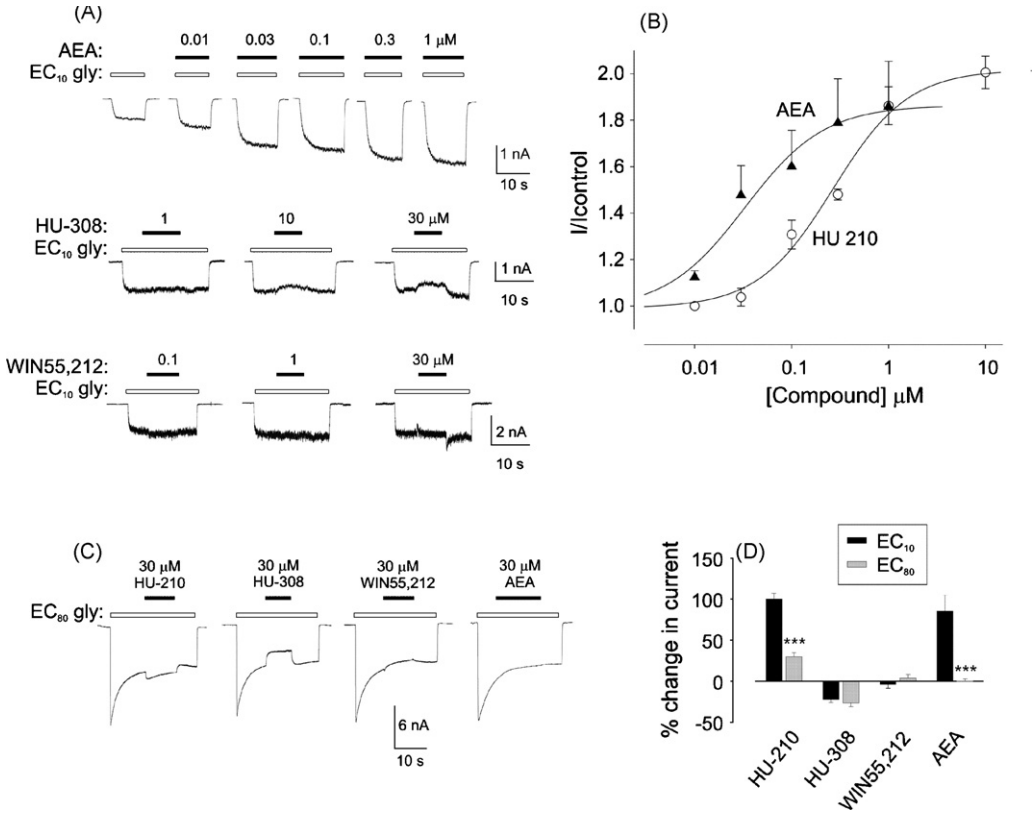

Fig. 1 – Effects of cannabinoids on α1 GlyRs. (A) Examples of the effects of indicated concentrations of AEA, HU-308 and WIN55,212-2 on currents activated by EC_{10} (10–15 μM) glycine. In this and subsequent figures, glycine and cannabinoid applications are indicated by unfilled and filled bars, respectively. (B) Averaged potentiating dose–responses for AEA and HU-210 are shown together with curves of best fit to the Hill equation. Averaged parameters of best fit to individual dose–responses are presented in Table 1. (C) Examples of the effects of 30 μM concentrations of HU-210, HU-308, WIN55,212-2 and AEA on currents activated by EC_{80} (100 μM) glycine. The first three traces were recorded from the same cell. (D) Averaged effects on current magnitude of 30 μM concentrations of each compound at EC_{10} and EC_{80} glycine concentrations. A 30 μM concentration of each compound corresponds to the following quantities in g l^{-1}: HU-210: 0.0116 g l^{-1}; HU-308: 0.0124 g l^{-1}; WIN55,212-2: 0.0157 g l^{-1}; AEA: 0.0104 g l^{-1}; NA-Gly: 0.0108 g l^{-1}. Potentiation by HU-210 and AEA was significantly reduced in magnitude at the higher glycine concentration (*$p < 0.001$ using unpaired *t*-test). Only HU-210 produced a statistically significant potentiation at EC_{80} glycine. The inhibitory potency of HU-308 was not significantly affected by the increased glycine concentration.**

Table 1 – Cannabinoid sensitivity of recombinantly expressed GlyRs

Compound	α1		α2		α3	
	EC_{50} (μM)	n_H	IC_{50} (μM)	n_H	IC_{50} (μM)	n_H
AEA	0.038 ± 0.011*	1.2 ± 0.1 (4)	No effect		No effect	
HU-210	0.27 ± 0.05	1.1 ± 0.1 (5)	0.090 ± 0.021	0.8 ± .03 (5)	0.050 ± 0.006	0.8 ± 0.1 (4)
HU-308	Weak inhibition		1.13 ± 0.30**	1.1 ± 0.2 (5)	0.097 ± 0.017	0.8 ± 0.1 (3)
WIN55,212-2	No effect		0.22 ± 0.05*	0.9 ± 0.1 (4)	0.086 ± 0.026	1.2 ± 0.3 (5)
NA-Gly	Complex effects – see text		3.03 ± 0.09**	1.4 ± 0.4 (4)	1.32 ± 0.10***	1.0 ± 0.1 (5)

n values for each experiment are given in brackets.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ relative to HU-210 using unpaired Student's *t*-test.

currents mediated by α1 GlyRs are shown in Fig. 1A. AEA and HU-210 both produced a concentration-dependent potentiation. The averaged dose–response relationships for both compounds are shown in Fig. 1B with the mean EC_{50} and n_H values of best fit summarised in Table 1. By contrast, 30 μM HU-308 inhibited EC_{10} α1 GlyR currents to 78 ± 4% ($n = 4$) of the original value (e.g., Fig. 1A). Fig. 1A also demonstrates that 30 μM WIN55,212-2 had no significant effect on current magnitude (+4 ± 4%, $n = 8$ cells), although in some cells a slow irreversible run-up in EC_{10} glycine current magnitude was apparent after prolonged WIN55,212-2 application. NA-Gly elicited complex effects which are described separately below.

The glycine-dependence of the cannabinoid effects was investigated by applying 30 μM of each cannabinoid at an EC_{80} (100 μM) glycine concentration. Examples of the effects of each compound are shown in Fig. 1C with averaged responses shown in Fig. 1D. For comparison, this panel also shows averaged effects of 30 μM of the same compounds at an EC_{10} glycine concentration. As previously shown [5], AEA had no significant effect at EC_{80} glycine. The efficacies of HU-308 and WIN55,212-2 were independent of glycine concentration, whereas the potentiating effect of HU-210 was significantly reduced at higher glycine concentrations (Fig. 1D). This is to be expected given that potentiating agents usually cannot increase current magnitude beyond the saturating glycine-activated amplitude.

AEA also produced a potent potentiation of heteromeric α1β GlyR currents activated by EC_{10} (10 μM) glycine, with a mean EC_{50} of 75 ± 20 nM an n_H of 1.4 ± 0.2 and a maximum potentiation magnitude of 128 ± 33% ($n = 4$ cells). Using an unpaired *t*-test, none of these parameters differed significantly from those seen at homomeric α1 GlyRs. This agrees with a previous study that suggested that the β subunit exerted no influence on AEA sensitivity or efficacy [5]. Effects of HU-210, HU-308 and WIN55,212-2 were tested at 30 μM concentrations only on EC_{10} currents in α1β heteromeric GlyRs. HU-210 potentiated currents by 78 ± 5% ($n = 3$), HU-308 modestly inhibited currents to 69 ± 8% ($n = 3$), whereas WIN55,212-2 had no effect in each of five cells. Using an unpaired *t*-test, none of these values differed significantly from their respective values at homomeric α1 GlyRs. Thus, the GlyR β subunit appears to have no influence on sensitivity to synthetic cannabinoids.

The effects of NA-Gly were complex. As shown in the example in Fig. 2A, 10 μM NA-Gly potently potentiated EC_{10} glycine currents in a rapidly reversible manner. The averaged NA-Gly dose–response was bell-shaped, reaching a maximum near 10 μM (Fig. 2B), although it was not possible to test concentrations greater than 30 μM due to solubility limitations. During the course of these experiments we noticed that potentiation magnitude progressively declined with successive 30 μM NA-Gly applications. This effect was quantified using continuous recordings as shown in Fig. 2C, where 10 s applications of 30 μM NA-Gly were applied at 20 s intervals in the presence of EC_{10} glycine. In addition to the potentiation observed at the onset of NA-Gly application (which we refer to as 'initial potentiation'), a sustained rebound potentiation was also observed immediately following the termination of the NA-Gly application (Fig. 2C). Interestingly, although the initial potentiation declined in magnitude to the extent that it virtually disappeared after four successive NA-Gly applications, the rebound potentiation magnitude remained almost unchanged. Results from five similar experiments, pooled in Fig. 2D, confirm these trends. Once lost, the initial potentiation did not reappear following a 5 min washout in 10 cells. This unusual effect most likely explains the bell shaped decline in the NA-Gly dose–response curve.

After several 30 μM NA-Gly applications had been applied to abolish the initial potentiation, it was possible to quantitate the NA-Gly inhibitory dose–response in the absence of contamination by potentiation. The inhibitory NA-Gly dose–responses thus determined were compared at EC_{10} and EC_{80} glycine concentrations, with averaged results presented in Fig. 2E. The mean IC_{50} and n_H values for both experiments, summarised in the legend to Fig. 2E, indicate that NA-Gly inhibitory potency significantly increased with glycine concentration.

3.2. *Effects on α2 GlyRs*

In contrast to their effects on the α1 GlyR, all three synthetic cannabinoids potently inhibited α2 GlyRs. Sample current recordings are shown in Fig. 3A, with averaged dose–responses for HU-210, HU-308 and WIN55,212-2 presented in Fig. 3B. Parameters of best fit to individual inhibitory dose–response curves, summarised in Table 1, reveal a potency sequence of HU-210 = WIN55,212-2 > HU-308. In contrast to the dramatic potentiation of α1 GlyRs, AEA had no significant effect on α2 GlyRs at concentrations up to 30 μM ($n = 5$ cells, Fig. 3A). NA-Gly also produced a dose-dependent inhibition at EC_{10} glycine concentrations, although a rebound potentiation was observed upon removal of high NA-Gly concentrations (e.g., Fig. 3A). This potentiation did not run down with

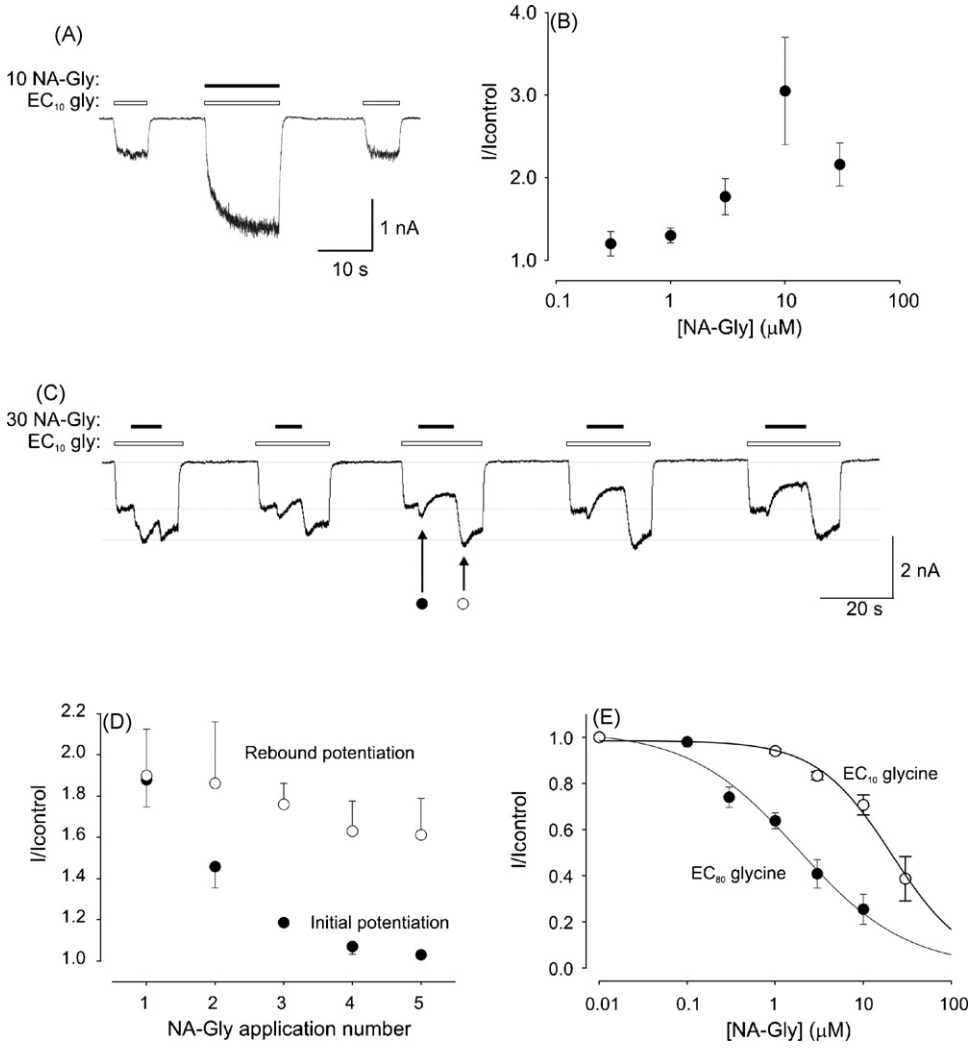

Fig. 2 – Effects of NA-Gly on α1 GlyRs. (A) Example of the potentiating effect of 10 μM (or 3.6 mg l^{-1}) NA-Gly on EC_{10} glycine current (left panel). Note the rapid reversibility. The right panel displays the mean potentiating NA-Gly dose–response in the presence of EC_{10} glycine, with each point averaged from 4–6 cells. (B) A sample recording showing that repeated 5 s applications of 30 μM (or 10.8 mg l^{-1}) NA-Gly every 20 s produce a progressive rundown in the initial potentiation magnitude of EC_{10} glycine currents, to uncover a sustained inhibitory effect. It also shows a rebound potentiation upon removal of NA-Gly which exhibits little rundown with repeated NA-Gly application. The displayed trace is a continuous recording and dashed lines are included to facilitate comparison of current magnitudes. Filled and unfilled circles indicate the time points at which initial and rebound potentiation magnitudes, respectively, were plotted in part (D). (D) Averaged magnitudes of initial potentiation and rebound potentiation plotted as a function of NA-Gly application number (with NA-Gly applied for 5 s every 20 s). All points were averaged from the same five cells. The initial potentiation magnitude reduced drastically with successive exposures to NA-Gly but rebound potentiation magnitude displays a slight downward trend that is not statistically significant. (E) Averaged NA-Gly inhibitory dose–responses at EC_{10} and EC_{80} glycine concentrations are plotted as unfilled and filled circles, respectively. The dose–response at EC_{80} glycine revealed a mean IC_{50} of 2.4 ± 0.7 μM (or 0.86 mg l^{-1}) and an n_H of 0.8 ± 0.03 (both n = 5 cells). The inhibitory dose–responses for NA-Gly at EC_{10} glycine (which were recorded following the elimination of initial potentiation by repeated 30 μM NA-Gly applications) revealed a mean IC_{50} of 24.1 ± 6.2 μM (or 8.6 mg l^{-1}) and an n_H of 1.1 ± 0.1 (n = 3 cells). The difference in IC_{50} values was highly significant ($p < 0.005$) using an unpaired t-test.

successive exposures to NA-Gly (not shown). Fig. 3B shows averaged dose–responses for both the inhibitory and rebound potentiating effects of NA-Gly, both averaged from the same four cells. Parameters of best fit to individual inhibitory dose–response curves are shown in Table 1. As the potentiating effect did not saturate, it was not possible to fit a Hill curve.

The effects of 30 μM HU-210, HU-308, WIN55,212-2 and NA-Gly were also investigated at an EC_{80} (500 μM) glycine concentration. Examples of the effects of each compound are shown in Fig. 3C with the averaged responses recorded at both EC_{10} and EC_{80} glycine concentrations compared in Fig. 3D. The inhibitory effects of HU-210, HU-308 and WIN55,212-2

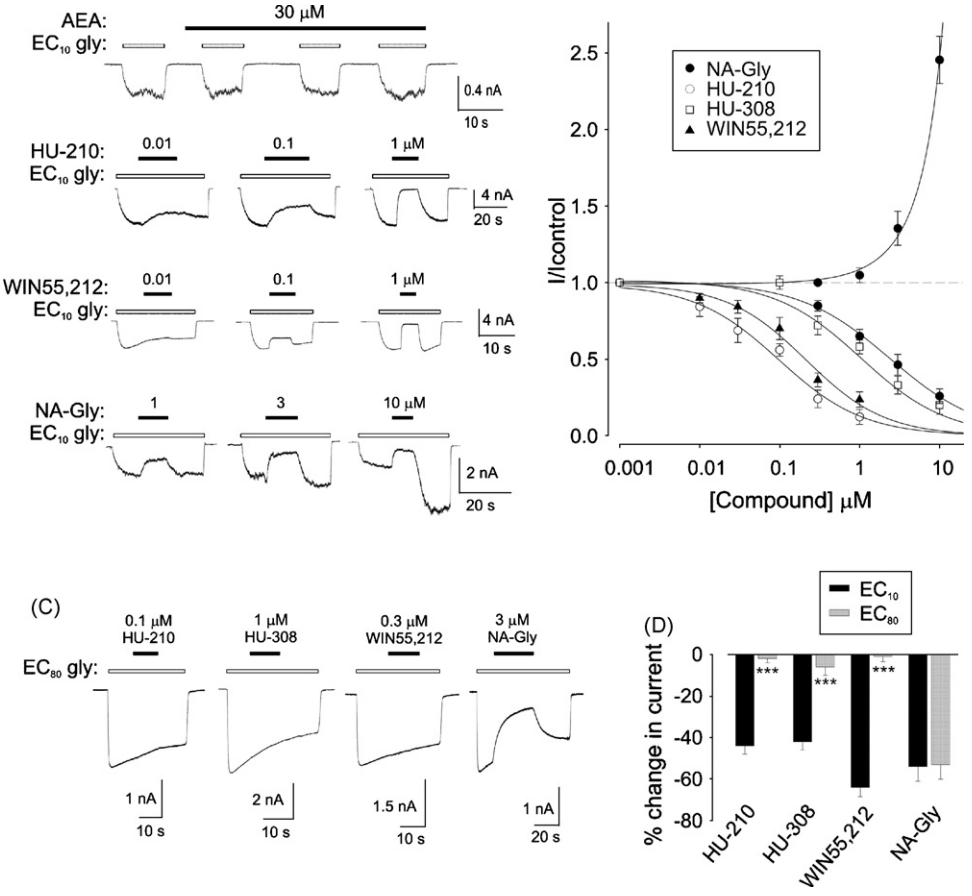

Fig. 3 – Effects of cannabinoids and NA-Gly on α2 GlyRs. (A) Examples of the effects of indicated compounds on currents activated by EC_{10} glycine. AEA has no effect even at a 30 μM concentration. HU-210, WIN55,212-2 and NA-Gly each produce a dose-dependent inhibition. Note the large rebound potentiation upon removal of 10 μM NA-Gly. (B) Averaged inhibitory dose–responses for NA-Gly, HU-210, HU-308 and WIN55,212-2. Parameters of best fit to individual dose–response curves are summarised in Table 1. The NA-Gly inhibitory and rebound potentiation dose–responses are both plotted as filled circles. (C) Effects of IC_{50} concentrations of each compound in the presence of an EC_{80} (500 μM) glycine concentration. (D) Averaged results of the experiments displayed in C ($n = 4$ for each compound). The inhibitory potencies of HU-210, HU-308 and WIN55,212-2 were significantly (*$p < 0.001$) reduced at the higher glycine concentration whereas inhibition by NA-Gly was not.**

were effectively abolished at the higher glycine concentration (Fig. 3D), implying a competitive mode of action. Inhibition by NA-Gly was, however, independent of glycine concentration.

3.3. *Effects on α3 GlyRs*

Sample traces and averaged dose–responses of the five test compounds on EC_{10} glycine currents in the α3 GlyR are shown in Fig. 4A and B. Parameters of best fit to the individual inhibitory dose–response relationships, summarised in Table 1, reveal a potency sequence of HU-210 = WIN55212 = HU-308 > NA-Gly. Again, AEA had no effect at concentrations up to 30 μM ($n = 5$ cells, Fig. 4A). The effects of 30 μM HU-210, HU-308, WIN55,212-2 and NA-Gly were also investigated at an EC_{80} (500 μM) glycine concentration. Examples of the effects of each compound are shown in Fig. 4C with averaged responses at both EC_{10} and EC_{80} glycine concentrations compared in Fig. 4D. As for α2 GlyRs, the higher glycine concentration dramatically reduced the potency of HU-210, HU-308 and WIN55,212-2 but had no effect on the potency of NA-Gly.

4. Discussion

4.1. *GlyRs exhibit novel cannabinoid pharmacological profiles*

This study has demonstrated that α1, α2 and α3 GlyRs have distinct cannabinoid pharmacological profiles. However, the profiles of α1 and α1β GlyRs were similar, strongly suggesting that the β subunit has minimal impact on cannabinoid pharmacology. The α1 GlyR exhibited a potentiating potency sequence of AEA > HU-210 which is the reverse of that seen at both CB1 and CB2 receptors [23]. By contrast, the α2 GlyR exhibited an inhibitory potency sequence of HU-210 > WIN > HU308 ≫ AEA. Although similar to the CB_2 receptor potency sequence [19–21], HU-210 is more than two

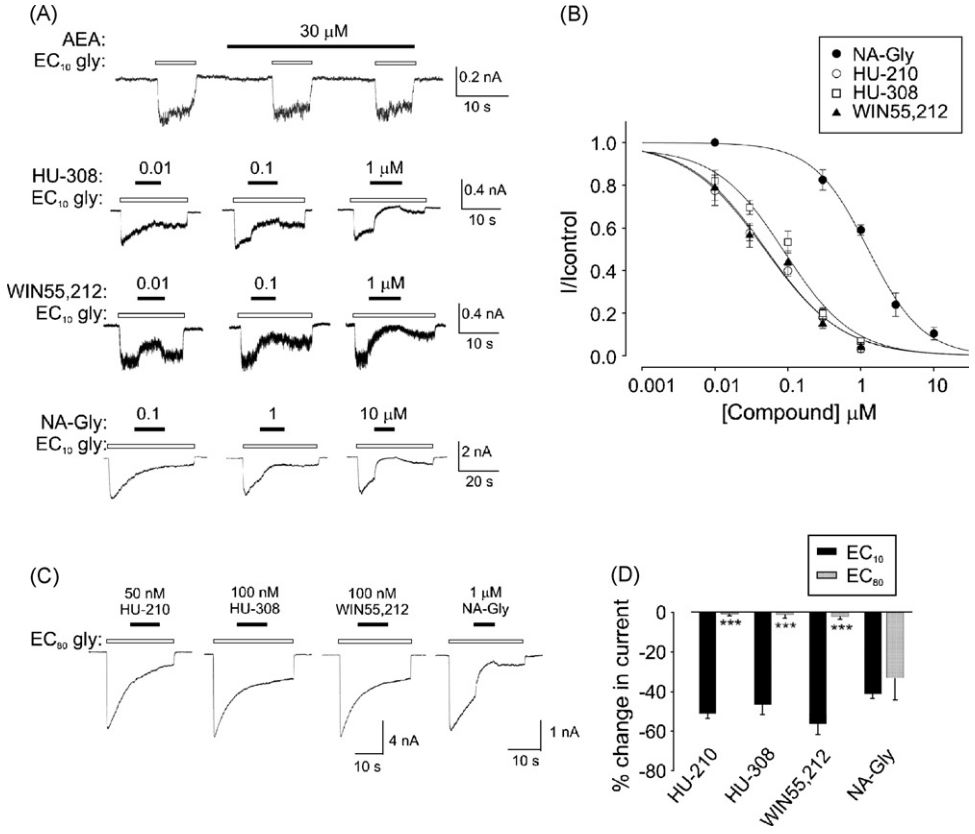

Fig. 4 – Effects of cannabinoids and NA-Gly on α3 GlyRs. (A) Examples of the effects of indicated compounds currents activated by EC_{10} glycine in α3 GlyRs. AEA has no effect at a 30 μM concentration and HU-308, WIN55,212-2 and NA-Gly produce dose-dependent inhibition. (B) Averaged inhibitory dose–responses for NA-Gly, HU-210, HU-308 and WIN55,212-2. Parameters of best fit to individual dose–response curves are summarised in Table 1. (C) Examples the effects of IC_{50} concentrations of HU-210, HU-308, WIN55,212-2 and NA-Gly on EC_{80} (500 μM) GlyR currents. (D) Averaged results of the experiments displayed in part (C) ($n = 4$ for each compound). As with α2 GlyRs, inhibition by HU-210, HU-308 and WIN55,212-2 were significantly (*$p < 0.001$) reduced at the higher glycine concentration whereas inhibition by NA-Gly was not.**

orders of magnitude more sensitive at the CB_2 receptor, and AEA potently activates CB_2 receptors at low micromolar concentrations whereas these concentrations are completely ineffective at α2 GlyRs [21,23]. The α3 GlyR was found to be equally sensitive to inhibition by HU-210, WIN55,212-2 and HU-308, but completely insensitive to AEA. However, the synthetic cannabinoid IC_{50} values at the α3 subunit were 50-fold lower than the K_i for HU-210 at both CB1 and CB2 receptors, only 4-fold lower than the K_{is} of HU-308 and WIN-55,212-2 at CB2 receptors, and similar to the affinity of WIN-55,212-2 at the CB1 receptor [21,23]. The characteristics to the glycine receptor subunits differ dramatically from the properties of both CB_1 and CB_2 receptors [23]. Thus, the cannabinoid pharmacological profiles α1, α2 and α3 GlyRs bear little or no resemblance to each other or to existing CB receptor subtypes.

Our results are in close agreement with [23] who also found that AEA potentiated recombinant α1 and α1β GlyRs at sub-micromolar concentrations. They also concur with the results of another study demonstrating that glycinergic postsynaptic currents in hypoglossal neurons (which are most likely mediated by α1β GlyRs) were not directly affected by WIN55,212-2 [18]. However, our results are at variance with a third report showing that AEA potently blocked a glycine-gated current in central neurons [17]. This discrepancy cannot be explained by the subunit or glycine concentration-dependent differences in AEA activity observed in the present study.

The effects of NA-Gly on all tested GlyR subtypes tested were significant at low micromolar concentrations, suggesting that GlyRs could be among the physiological targets of this endogenous compound [12,24]. As CB_1 and CB_2 receptors are insensitive to NA-Gly [15], the current findings further differentiate the pharmacological properties of GlyRs from those of CB_1 and CB_2 receptors.

4.2. *Mechanisms of action*

As cannabinoid agonists and NA-Gly are lipophilic, it is possible they may partition into the cell membrane where they may reach high local concentrations and thereby influence the actions of membrane proteins via a so-called 'receptor-independent' mechanisms [4]. Since the times required for cannabinoids to reach equilibrium in the cell membrane, and to deplete from the membrane following their

removal from the extracellular solution, are unknown, it is difficult to assess the likelihood of such a mechanism contributing their effects on the GlyR. However, despite the close structural similarities of AEA and NA-Gly, their drastically different sensitivities and modes of action at α1, α2 and α3 GlyRs strongly implies that these compounds interact via specific and distinct binding interactions.

The mechanism of action of NA-Gly at α1 GlyRs is highly complex, producing a potent potentiation which declines with exposure time at high NA-Gly concentrations. This suggests that NA-Gly progressively accumulates at a site closely associated with the GlyR, possibly the cell membrane, where it is able to progressively inactivate the potentiating mechanism. In the absence of potentiation, an inhibitory effect is uncovered, which is likely to be mediated by a distinct binding site since NA-Gly unbinds completely and rapidly from the inhibitory site. Upon removal of NA-Gly at EC_{10} glycine concentrations a rapidly reversible rebound potentiation is observed. This is possibly due to a third cannabinoid site as its sensitivity is lower than that of the inhibitory site in the α2 GlyR (Fig. 3B). Our data suggest that α3 GlyRs contain only the inhibitory site, whereas α2 GlyRs contain both the inhibitory site and the rebound potentiating site (Fig. 3).

Our data provide no direct clues as to the location of any of the cannabinoid binding sites. However, the dramatic subunit-specific differences in the modes of action of the cannabinoids suggest that weakly conserved regions among the three α GlyR subtypes might be promising sites to investigate. As the glycine ligand binding pocket residues are highly conserved in α1, α2 and α3 GlyRs, it is unlikely cannabinoids bind to this region despite exhibiting apparently competitive modes of inhibition. The regions of lowest sequence identity are in the large intracellular M3-M4 domain, the M4 domain and the short C-terminal tail. The rapid on and off rates of the cannabinoid effects suggest that an intracellular binding site is unlikely. It is more likely that the differential effects of cannabinoids are mediated by the relatively non-conserved M4 domain and C-terminal tail. Indeed, such a location fits well with the lipid solubility of these compounds.

4.3. *Possible physiological and pharmacological significance*

Since AEA potentiates α1β GlyRs with an EC_{50} of 75 nM, which is lower than its K_i at either CB_1 or CB_2 receptors [23], it is feasible that the α1β GlyR is endogenously modulated by this compound. Endocannabinoids are known to be released postsynaptically by depolarisation and to exert local presynaptic actions via CB_1 or CB_2 receptors to reduce neurotransmitter release probability [25]. Since a CB_1-specific antagonist increased glycinergic IPSC magnitude in hyoglossal neurons, it has been proposed that an endocannabinoid (possibly AEA) exerts a retrograde action to inhibit the presynaptic release of glycine [18]. Since AEA has a direct potentiating effect on α1β GlyRs at low glycine concentrations only ([5] and present study), it is likely to prolong the decay of glycinergic IPSCs rather than exert a significant effect on peak current magnitude. Indeed, such an effect has been observed in glycinergic IPSCs in hypoglossal neurons in response to a stimulus that increases presynaptic endocannabinoid release [18]. Further research is required to establish whether AEA is in fact the agent responsible for these effects.

As noted above, it is also possible that α1, α2 and α3 GlyRs may all be endogenous targets for NA-Gly. So far little is known about the physiological targets of NA-Gly or about the physiological stimuli that control its production and release. However, it has recently been shown that the glycine transporter, GlyT2, is inhibited by NA-Gly at low micromolar concentrations, similar to those that modulate the GlyR [24]. Because GlyT2 is responsible for the presynaptic accumulation of glycine, and inhibition of GlyT2 is expected to reduce the magnitude of glycinergic IPSCs [26], this action could be counteracted by the potentiating effects of low concentrations of NA-Gly on α1β GlyRs. Conversely, IPSCs at synapses incorporating α2 or α3 GlyRs should be strongly inhibited by NA-Gly. NA-Gly has also been shown to stimulate a G-protein coupled receptor, GPR92, which is highly expressed in dorsal root ganglia and has been postulated to play a role in sensory perception [27].

Heteromeric α1β GlyRs predominate at glycinergic synapses on spinal motor neurons [16,28]. Compounds that potentiate α1β GlyRs should dampen excessive motor neuron activity and thereby provide therapeutic leads for movement disorders including spasticity. Thus, HU-210 and AEA may provide useful starting points for developing novel GlyR-positive drugs. By contrast, α3 GlyRs are restricted to glycinergic synapses in the retina [29], superior colliculus, parietal cortex, cerebellum and hippocampus [30] as well as the peripheral laminae of the spinal cord dorsal horn [8]. In the latter area they are known to be co-localised with α1 GlyRs at inhibitory synapses on nociceptive sensory neurons [8]. A role for GlyRs in inflammatory pain sensitization is implied by the observation that the inflammatory mediator, prostaglandin E_2 (PGE_2) specifically inhibits α3 subunit-containing GlyRs in dorsal horn nociceptive neurons via a G protein-coupled receptor signalling pathway [8,9,31]. This process is likely to result in the disinihibition of firing activity in nociceptive projection neurons, thereby increasing the transmission of nociceptive stimuli to the brain [32,33]. This could explain the hyperalgesia and allodynia that is associated with chronic inflammation. It thus follows that compounds that increase current flux in GlyRs containing α1 or α3 subunits could produce anti-inflammatory analgesia. Spinal administration of cannabinoids and NA-Gly indeed produces analgesia in both inflammatory and neuropathic pain models [12–14,34,35]. The extent to which GlyRs may be targets of these actions are yet to be investigated, although data presented in this study suggest that NA-Gly, HU-210 and AEA could theoretically produce analgesia via this mechanism.

It is not known why different GlyR α subunits are present at particular synapses in retina and spinal cord [8,29,36,37]. Pharmacological elimination of a single GlyR subtype would permit the remaining glycinergic current to be analysed in isolation. Unfortunately, currently available pharmacological tools are not sufficiently selective to achieve this in α1, α2 and α3 subunit-containing GlyRs [38]. The results of the present study suggest that cannabinoid ligands such as WIN55,212-2, HU-210 and HU-308 should potently block synaptic α3 GlyRs while leaving α1 GlyR currents intact. These agents may therefore permit the dissection of the distinct properties of

α1- and α3-mediated glycinergic IPSCs in retinal and spinal pain sensory neurons.

Finally, the result that only synthetic cannabinoids inhibit the activity of α3 GlyRs should be considered when interpreting the anti-nociceptive or pro-nociceptive effects of endogenous and synthetic cannabinoids in CB receptor knockout animals. Furthermore, since synthetic cannabinoids inhibit α3 GlyRs, their role as analgesics in inflammatory pain should be assessed carefully. Indeed, a non-monotonic dose response of synthetic cannabinoids in humans was observed in situations were inflammation contributes to pain, such as third molar extraction (Cannabinor, Pharmos Ltd.) and capsaicin-induced pain [39], implying the existence of multiple synthetic cannabinoid targets.

Acknowledgements

This study was supported by project grants from the National Health and Medical Research Council of Australia to JWL and RJV, and from Medical Research Council of the United Kingdom to RJH (G0500833). JWL and RJV are both supported by National Health and Medical Research Council Research Fellowships.

REFERENCES

[1] Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, et al. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 1999;283:401–4.
[2] Ibrahim MM, Rude ML, Stagg NJ, Mata HP, Lai J, Vanderah TW, et al. CB2 cannabinoid receptor mediation of antinociception. Pain 2006;122:36–42.
[3] Pertwee RG. Pharmacological actions of cannabinoids. Handb Exp Pharmacol 2005;1–51.
[4] Oz M. Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids. Pharmacol Ther 2006;111:114–44.
[5] Hejazi N, Zhou C, Oz M, Sun H, Ye JH, Zhang L. Delta9-tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. Mol Pharmacol 2006;69:991–7.
[6] Sivilotti L, Woolf CJ. The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. J Neurophysiol 1994;72:169–79.
[7] Keller AF, Coull JA, Chery N, Poisbeau P, De Koninck Y. Region-specific developmental specialization of GABA-glycine cosynapses in laminas I–II of the rat spinal dorsal horn. J Neurosci 2001;21:7871–80.
[8] Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, et al. GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 2004;304:884–7.
[9] Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU. PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 2002;5:34–40.
[10] Chery N, de Koninck Y. Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. J Neurosci 1999;19:7342–55.
[11] Burstein SH. The cannabinoid acids: nonpsychoactive derivatives with therapeutic potential. Pharmacol Ther 1999;82:87–96.
[12] Huang SM, Bisogno T, Petros TJ, Chang SY, Zavitsanos PA, Zipkin RE, et al. Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. J Biol Chem 2001;276:42639–44.
[13] Succar R, Mitchell VA, Vaughan CW. Actions of N-arachidonyl-glycine in a rat inflammatory pain model. Mol Pain 2007;3:24.
[14] Vuong LA, Mitchell VA, Vaughan CW. Actions of N-arachidonyl-glycine in a rat neuropathic pain model. Neuropharmacology 2008;54:189–93.
[15] Sheskin T, Hanus L, Slager J, Vogel Z, Mechoulam R. Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. J Med Chem 1997;40:659–67.
[16] Lynch JW. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 2004;84:1051–95.
[17] Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, Burnashev N. Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. J Neurosci 2005;25:7499–506.
[18] Mukhtarov M, Ragozzino D, Bregestovski P. Dual Ca2+ modulation of glycinergic synaptic currents in rodent hypoglossal motoneurones. J Physiol 2005;569:817–31.
[19] Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, et al. Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. Mol Pharmacol 1995;48:443–50.
[20] Hanus L, Breuer A, Tchilibon S, Shiloah S, Goldenberg D, Horowitz M, et al. HU-308: a specific agonist for CB(2), a peripheral cannabinoid receptor. Proc Natl Acad Sci USA 1999;96:14228–33.
[21] Govaerts SJ, Hermans E, Lambert DM. Comparison of cannabinoid ligands affinities and efficacies in murine tissues and in transfected cells expressing human recombinant cannabinoid receptors. Eur J Pharm Sci 2004;23:233–43.
[22] Pribilla I, Takagi T, Langosch D, Bormann J, Betz H. The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. EMBO J 1992;11:4305–11.
[23] Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 2002;54:161–202.
[24] Wiles AL, Pearlman RJ, Rosvall M, Aubrey KR, Vandenberg RJ. N-Arachidonyl-glycine inhibits the glycine transporter, GLYT2a. J Neurochem 2006;99:781–6.
[25] Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. Science 2002;296:678–82.
[26] Gomeza J, Ohno K, Hulsmann S, Armsen W, Eulenburg V, Richter DW, et al. Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron 2003;40.
[27] Oh da Y, Yoon JM, Moon MJ, Hwang JI, Choe H, Lee JY, et al. Identification of farnesyl pyrophosphate and N-arachidonylglycine as endogenous ligands for GPR92. J Biol Chem 2008;283:21054–6.
[28] Legendre P. The glycinergic inhibitory synapse. Cell Mol Life Sci 2001;58:760–93.
[29] Haverkamp S, Muller U, Harvey K, Harvey RJ, Betz H, Wassle H. Diversity of glycine receptors in the mouse retina: localization of the alpha3 subunit. J Comp Neurol 2003;465:524–39.
[30] Meier JC, Henneberger C, Melnick I, Racca C, Harvey RJ, Heinemann U, et al. RNA editing produces glycine receptor

alpha3(P185L), resulting in high agonist potency. Nat Neurosci 2005;8:736–44.
[31] Reinold H, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, et al. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. J Clin Invest 2005;115:673–9.
[32] Lynch JW, Callister RJ. Glycine receptors: a new therapeutic target in pain pathways. Curr Opin Invest Drugs 2006;7: 48–53.
[33] Zeilhofer HU. The glycinergic control of spinal pain processing. Cell Mol Life Sci 2005;62:2027–35.
[34] Fox A, Kesingland A, Gentry C, McNair K, Patel S, Urban L, et al. The role of central and peripheral cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. Pain 2001;92:91–100.
[35] Conti S, Costa B, Colleoni M, Parolaro D, Giagnoni G. Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. Br J Pharmacol 2002;135:181–7.
[36] Haverkamp S, Muller U, Zeilhofer HU, Harvey RJ, Wassle H. Diversity of glycine receptors in the mouse retina: localization of the alpha2 subunit. J Comp Neurol 2004;477:399–411.
[37] Heinze L, Harvey RJ, Haverkamp S, Wassle H. Diversity of glycine receptors in the mouse retina: localization of the alpha4 subunit. J Comp Neurol 2007;500:693–707.
[38] Webb TI, Lynch JW. Molecular pharmacology of the glycine receptor chloride channel. Curr Pharm Des 2007;13:2350–67.
[39] Wallace M, Schulteis G, Atkinson JH, Wolfson T, Lazzaretto D, Bentley H, et al. Dose-dependent effects of smoked cannabis on capsaicin-induced pain and hyperalgesia in healthy volunteers. Anesthesiology 2007;107:785–96.