Characterization of $\alpha 3$ Glycine Receptors with Ginkgolide B and Picrotoxin

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Abstract

Ginkgolide B (GB) and picrotoxin (PTX) are antagonists of the major inhibitory receptors of the central nervous system: GABA and glycine receptors (GlyRs). GlyRs contain one or more of the four alpha subunit isoforms of which $\alpha 1$ and $\alpha 2$ have been extensively studied. This report compares GB and PTX block of $\alpha 3$ GlyRs expressed in HEK 293 cells, using whole-cell patch clamp techniques. In CNS, $\alpha 3$ exists as a heteropentamer in conjunction with beta subunits in a $2\alpha:3\beta$ ratio. Thus, the nature of block was also tested in $\alpha 3\beta$ heteromeric glycine receptors. GB and PTX blocked $\alpha 3$ GlyRs both in the presence (liganded state) and absence of glycine (unliganded state). This property is unique to $\alpha 3$ subunits; $\alpha 1$ and $\alpha 2$ subunits are only blocked in the liganded state. The GB block of $\alpha 3$ GlyRs is voltage-dependent (more effective when the cell is depolarized) and non-competitive, while the PTX block is competitive and not voltage-dependent. The heteromeric and homomeric $\alpha 3$ GlyRs recovered significantly faster from unliganded GB block compared to liganded GB block, but no such distinction was found for PTX block suggesting more than one binding site for GB. This study sheds light on features of the $\alpha 3$ GlyR that distinguish it from the more widely studied $\alpha 1$ and $\alpha 2$ subunits. Understanding these properties can help decipher the physiological functioning of GlyRs in the CNS and may permit development of subunit specific drugs.

Introduction

Glycine receptors (GlyRs) and GABA receptors are the major fast inhibitory receptors of the central nervous system. The GlyRs are ionotropic and conduct chloride ions. These cys-loop receptors are pentameric, stoichiometrically made of two α_{1-4} and three β subunits (Durisic et al., 2012). GlyR expression has been found extensively through autoradiography and immunohistochemistry techniques in the central nervous system including in the cerebellar cortex, brainstem, forebrain, retina, and hippocampus (Haverkamp et al., 2003; Crook et al., 2006; Baer et al., 2009; Xu and Gong, 2010). Despite studies on GlyR localization, deciphering the subunit composition of these receptors remains elusive. It is suggested that most GlyRs in native cells are alpha-beta heteromers; α_2 homomeric GlyRs expression in fetal rat being the only known exception although there has been speculation that α_3 or α_1 GlyRs exist in homomeric form in non-somatic locations (Lynch, 2009). The $\alpha_3\beta$ heteromers are often dominant, found in about half of the glycinergic synapses in rat retina and rat dorsal horn of the spinal cord (Haverkamp et al., 2003; Harvey et al., 2004). Relatively few studies have been conducted on α_4 GlyRs since it is a pseudo gene in humans (Lynch, 2009) although its expression has been shown in rat retina (Heinze et al., 2007). The unique expression of these GlyR subunits in different parts of the CNS suggests that each might have distinct functions. Thus, an important field of research is to develop tools to differentiate between the GlyRs subunits.

Our primary goal in this study was to characterize and compare the blocking action of picrotoxin (PTX) and ginkgolide B (GB) on recombinant homomeric and heteromeric α_3 GlyRs expressed in human embryonic kidney (HEK 293) cells. GB is a diterpene trilactone extracted from Ginkgo Biloba leaf (EGb 761). EGb 761 is widely used clinically as a treatment for neurodegeneration. GB, the most potent component of EGb 761, acts as an anti-convulsant, a platelet activating factor antagonist, and a neuroprotective agent (MacIennan et

al., 2002). In contrast, PTX induces convulsions (Hasan et al., 2014). Yet, both block GABA and glycine receptors. Thus the mechanism of block may contribute to the opposing outcomes of these GABA/Gly antagonists. Alternatively, the selectivity of each antagonist for different receptor subtypes may explain its systemic physiology.

Materials and Methods

Cell Culture

Human embryonic kidney cells (HEK293 cells, American Type culture collection, MD) used in the experiments were cultured in 35 mm plates with Dulbecco's Modified Eagle's Medium (DMEM) complete with 10 % (v/v) Fetal Bovine Serum and 1% (v/v) penicillin-streptomycin. After 24 hours they were transfected using XtremeGENE 9 DNA Transfection reagent (Roche Inc, Basel Switzerland) with either $\alpha 3$ or $\alpha 3 + \beta$ cDNAs. GFP was co-transfected. A total of 2.5 μ g DNA were used per 35 mm dish with a ratio of 20:1 GlyR:GFP. When the cells were transfected with both α and β subunits, they were mixed in a 10 β :1 α ratio. Cells were cultured for 18-24 hours before recording.

Electrophysiology:

Whole cell patch clamp recordings were conducted on the transfected HEK 293 cells (visually identified by green fluorescence) using Axopatch 200B amplifier and pCLAMP 9.0 software (Axon Instruments, Foster City, CA). The drugs were applied through the Octaflow II (ALA Scientific Instruments, Inc. NY) localized superfusion system. Krebs's external solution was used in recordings, containing (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 HEPES, 10 glucose (pH=7.4). Experiments were conducted using glass pipettes pulled by a multi-step horizontal puller (Sutter Instruments, CA) and fire polished. They had a tip resistance of ~5 M Ω and were filled with internal pipette solution containing (in mM): 140 CsMeSO₄, 10 EGTA, 10 HEPES, 4 MgCl, 2 ATP-Mg (pH=7.2).

Glycine and picrotoxin was purchased from Sigma-Aldrich Corporation (St.Louis, MO) and Ginkgolide B from Tocris Biosciences (Bristol, UK). Glycine was dissolved in Krebs's solution to make 5 mM solutions then further diluted to 500 μ M. GB and PTX were dissolved in DMSO (Fisher Scientific, Waltham, MA) to make 10 mM stock solution and

stored at 4 degree Celsius. The stock solutions were diluted with Krebs solution to the required concentrations before experiments.

Results

Glycine potency at the $\alpha 3$ GlyR

Initial experiments indicated that the $\alpha 3$ GlyRs, both homomeric and particularly heteromeric, are much less sensitive to glycine than GlyRs containing other alpha subunits. The glycine EC₅₀ for $\alpha 3$ homomers is 166 μ M, compared to 99 μ M for $\alpha 2$ GlyRs (figure 1A, D). Furthermore, addition of the β subunit raised EC₅₀ in $\alpha 3\beta$ to 395 μ M (figure 1B), while it had little effect in the $\alpha 2$ GlyR (figure 1C & D). In native tissue the glycine receptor is generally expressed as a heteromer and these results indicate that $\alpha 3\beta$ GlyRs might be distinguished by their low glycine sensitivity (figure 1E).

Antagonist potency at the $\alpha 3$ GlyR

Two antagonists of ligand-gated chloride channels, picrotoxin and ginkgolide, have been used to compare GlyR subtypes. While $\alpha 3$ GlyRs are less sensitive to glycine than other subtypes, they have comparatively high sensitivity to picrotoxin. When tested against 200 μ M glycine (approximate EC₆₀), the picrotoxin IC₅₀ was 0.9 μ M. This compares with picrotoxin IC₅₀ at $\alpha 1$ and $\alpha 2$ homomeric GlyRs of 37.3 and 12.6 μ M, respectively (figure 2A). Like other GlyR subtypes, addition of the β subunit reduced the potency of picrotoxin (IC₅₀ = 11.8 μ M tested against the EC₆₀ of glycine which is 468 μ M, figure 2B).

Ginkgolide B potency at the $\alpha 3$ GlyR was less than reported at $\alpha 1$ or $\alpha 2$ GlyRs, but like these other receptors the block was better in the heteromeric receptor (figure 2C). The GB IC₅₀ is 17 μ M in the $\alpha 3$ homomeric and 7 μ M in the $\alpha 3\beta$ heteromeric receptor. There is also a change in the Hill coefficient, from approximately 2 to 1. In comparison with $\alpha 1$ and $\alpha 2$ GlyRs, the relatively high potency of picrotoxin and low potency of ginkgolide B might be used to distinguish the $\alpha 3\beta$ GlyR.

GB is a non-competitive blocker and PTX is a competitive blocker of alpha 3 GlyRs

Studies have shown that GB is a non-competitive blocker of native GlyRs in hippocampal pyramidal rat neurons as well as in $\alpha 1$ and $\alpha 2$ GlyRs (Kondratskaya et al., 2004; Kondratskaya et al., 2005; Hawthorne et al., 2006a). In contrast, PTX is a competitive blocker in $\alpha 1$ and $\alpha 2$ GlyRs (Lynch et al., 1995; Wang et al., 2006). We tested GB and PTX block in $\alpha 3$ and $\alpha 3\beta$ GlyRs.

The peak current response (I_0) to application of 500 μ M or 5 mM glycine was recorded and compared to the peak response in the presence of the antagonist (I), with either 2 μ M PTX or 1 μ M GB in cells held at -50 mV (figure 3). In 8 cells expressing homomeric $\alpha 3$ GlyR subunits, 1 μ M GB on average blocked $12 \pm 9\%$ of 500 μ M glycine-induced current and $9 \pm 5\%$ of 5 mM glycine-induced current (Figure 3A). There was no statistically significant difference between the two blocks, indicative of non-competitive inhibition. In contrast, 2 μ M PTX blocked $43 \pm 9\%$ of the 500 μ M, but only $16 \pm 10\%$ of 5 mM glycine-induced current. This difference in fractional block was statistically significant ($p < 0.01$), suggesting that PTX is a competitive blocker.

In heteromeric $\alpha 3\beta$ GlyR, there was no significant difference in the block of 500 μ M or 5 mM glycine produced by 1 μ M GB, but 10 μ M PTX produced a greater block of 500 μ M than 5 mM glycine (Figure 3B). 1 μ M GB blocked $21 \pm 13\%$ of 500 μ M and $12 \pm 7\%$ of 5 mM glycine-induced current. But 10 μ M PTX blocked $65 \pm 6\%$ of 500 μ M and only $16 \pm 10\%$ of 5 mM glycine-induced current. Higher PTX concentrations were used in the experiments on heteromeric receptors because they are less sensitive than the homomeric glycine receptor (see figure 2). Overall, block of $\alpha 3$ -containing GlyRs by picrotoxin and ginkgolide B show the same competitive/non-competitive differences seen in the $\alpha 1$ and $\alpha 2$ containing GlyRs.

GB block is voltage sensitive in $\alpha 3$ GlyR

We tested the voltage sensitivity of GB and PTX block in homomeric and heteromeric $\alpha 3$ GlyR. Two measurements were taken during a 10 s application of 500 μ M Glycine + antagonist: first the block of peak current and then after 5.5 sec. These responses were compared to the peak of the initial glycine current without antagonist (only the currents in the presence of antagonists are shown in figure 4). For homomeric $\alpha 3$ GlyRs, the measurements were taken at -10 mV and -50 mV, which were on either side of the reversal potential of -30 mV. A high concentration of GB (100 μ M) was used in $\alpha 3$ GlyRs because of its comparatively low potency (Figure 4)

In $\alpha 3$ GlyRs the action of GB was voltage sensitive. GB blocked $58 \pm 14\%$ of the glycine current at -50 mV in contrast to $81 \pm 5\%$ at -10 mV. After 5.5 sec, GB blocked $75 \pm 15\%$ at -50 mV and $98 \pm 2\%$ at -10 mV (figure 4, top). Thus, at both time points $\alpha 3$ GlyRs were blocked significantly better at -10 mV compared to -50 mV ($p < 0.001$). This finding is consistent with studies in rat hippocampal pyramidal neurons (Kondratskaya et al., 2002). Top right of Figure 4 shows sample traces of 10 s application of 500 μ M Gly + 100 μ M GB at the two voltages. The control glycine trace, without antagonist, is not shown because the control peak was of equal magnitude at both voltages. The histogram at top left summarizes the voltage sensitive block by GB of the peak current and the current after 5.5 seconds.

In $\alpha 2$, PTX is not a voltage sensitive blocker (Wang et al., 2006). In order to test how PTX behaves in $\alpha 3$, we used the same protocol employed for GB. At block peak, 2 μ M PTX blocked $55 \pm 11\%$ of the current evoked by 500 μ M Gly at -10 mV and $52 \pm 13\%$ at -50 mV. Since the differences in blocks are not significant, we conclude that PTX is not a voltage sensitive blocker in $\alpha 3$ GlyRs (data not shown).

Similar experiments were repeated on $\alpha 3\beta$ GlyRs, but using a lower GB (1 μ M) concentration because of its greater potency in the heteromers. Here we made the

measurements at -10 mV, -50mV and -90 mV. At -50mV and -10 mV this batch of cells showed outward current, so we also tested at -90 mV, where an inward current was evoked.

In $\alpha 3\beta$ GlyRs, GB blocked $25 \pm 8\%$ of 500 μ M Gly induced peak current at -10 mV, compared to $10 \pm 4\%$ at -90 mV and $11 \pm 4\%$ at -50 mV. Thus, like homomeric $\alpha 3$ GlyRs, there was a significantly better GB block at -10 mV in $\alpha 3\beta$ GlyRs. Since there was almost no difference between -90 mV and -50 mV block, we conclude that direction of ion flow is not an important factor in GB block. There was not a statistically significant difference in block at the two voltages after 5.5 s. GB block of glycine in $\alpha 3\beta$ GlyRs was $61 \pm 16\%$ at -10 mV, $53 \pm 15\%$ at -90 mV, and $50 \pm 16\%$ at -50 mV. The right panel of Figure 4B shows sample traces and the left panel shows a summary of the data at -10 mV and -90 mV. Difference in the rate of desensitization in $\alpha 3\beta$ and $\alpha 3$ GlyRs is a potential reason for the lack of voltage sensitivity after 5.5 s in $\alpha 3\beta$.

The $\alpha 3\beta$ GlyRs showed no statistically significant difference in PTX block at -10 mV, -50 mV and -90 mV. At the peak of the glycine current, PTX blocked $30 \pm 13\%$ at -10 mV, $30 \pm 13\%$ at -50mV and $38 \pm 15\%$ at -90 mV.

Differences from other GlyRs

a. Pre-treatment with GB and PTX blocks $\alpha 3$ GlyR currents

Both GB and PTX are thought to block the channel pore. Many pore blockers are use-dependent because they require the agonist to open the pore to allow access to the blocker. Hence, two blocking mechanisms were compared: 1) Liganded or use-dependent block that occurs when glycine and antagonist are applied together and 2) Unliganded or non-use-dependent block in which the action of the antagonist does not depend on the presence of the agonist. PTX block has been linked to amino acids in the glycine channel pore but has been shown to be non-use dependent in $\alpha 1$ (Lynch et al., 1995), but use-dependent in $\alpha 2$ (Wang et

al., 2006). GB has been proposed to block in a use-dependent manner in native hippocampal neuron (Kondratskaya et al., 2002) and in $\alpha 2$ GlyRs (Hawthorne et al., 2006a). In our experiments, the protocol used to test for unliganded block was to: 1) apply 500 μ M glycine for 2 s (first glycine response), wait for the current to return to baseline, and then 2) apply antagonist GB or PTX (without glycine) for 10 s and then after a wash period of 3 s, 3) apply only 500 μ M glycine for 2 s (second glycine response). If the antagonist blocked the unliganded receptor, then the second glycine peak would be reduced. To test for liganded block: 1) 500 μ M glycine was first applied for 2 s (first glycine response) then after recovery to baseline 2) GB or PTX was co-applied with 500 μ M glycine for 10 s, followed by a 3 s wash and then 3) 500 μ M glycine (without antagonist) was reapplied for 2 s (second glycine peak). The results are expressed as the percentage of control peak glycine current that was blocked ($\frac{I_0 - I}{I_0} \times 100\%$).

An example of this protocol is shown in Figure 5. The experiment was performed on $\alpha 3$ homomers and PTX fully blocked the effect of concomitantly applied glycine (top trace). After washout of both drugs, brief reapplication of glycine caused progressively larger currents, indicating a slow removal of the bound PTX block. When this protocol was repeated with the application of PTX alone (bottom trace) there was a similar slow unblocking. Thus, regardless of whether PTX was applied in the presence (liganded) or absence (unliganded) of glycine, PTX produced a similar level of block. Each of the secondary glycine applications represents a new sequence of the three steps and all sequences shown were performed in the same cell. The results indicate that, like the $\alpha 1$ and $\alpha 2$ GlyRs, PTX block of the $\alpha 3$ homomer was not use-dependent.

To test the validity of our experimental protocol, we compared the GB liganded and unliganded block of $\alpha 2$ GlyRs, using the protocol shown in figure 5 (Figure 6, top left). The first pair of superimposed traces shows that glycine alone (black) and glycine after

pretreatment with GB (gray) produced similar currents. There was no evidence of $\alpha 2$ GlyR inhibition in the unliganded-block protocol. In contrast, $\sim 27\%$ of $\alpha 2$ GlyRs were blocked in liganded receptors. This experiment agrees with studies indicating that $\alpha 2$ GlyRs show only liganded block and also supports our experimental design.

Surprisingly, GB blocked unliganded $\alpha 3$ GlyRs and this block showed voltage dependence similar to the liganded receptor block (Figure 6, top right). Each superimposed pair of current traces in Figure 6 shows the response to glycine alone in black and the second glycine current, 3s after GB removal, in gray. The bar graph below each pair of superimposed traces shows the fractional remaining current in the step 3 protocol for all cells tested. The first two sets of superimposed traces in Figure 6 top right show that, for cells held at -10 mV, GB blocks on average $61 \pm 11\%$ of the unliganded receptor after 3s and $65 \pm 5\%$ of the liganded receptor. The middle two traces show that GB is less effective in cells held at -50 mV, where the unliganded block is $20 \pm 5\%$ and the liganded block is $34 \pm 5\%$. Thus, GB could bind and block the closed channel. PTX could also bind to the closed, unliganded $\alpha 3$ GlyR as shown in the last two pair of superimposed traces. In the unliganded protocol, $55 \pm 9\%$ of the current remained blocked by PTX after 3s, (measurements only conducted at -50 mV since PTX block is not voltage sensitive). PTX blocked $44 \pm 11\%$ of the GlyR current in the liganded receptor. In all three sets of responses there was no statistically significant difference in the fraction of block produced in the liganded vs the unliganded state.

These experiments were repeated in the heteromeric GlyR, but drug concentrations were 500 μM glycine, 1 μM GB and 10 μM PTX, because the heteromeric receptor was more sensitive to GB and less to PTX and glycine (Figure 6, lower panel). These receptors were also found to be blocked in unliganded and liganded state by PTX and GB. Again, we tested $\alpha 3\beta$ GlyRs at -10 mV, -50 mV (not shown) and -90 mV to examine whether direction of ion flow has any effect on recovery. At -10 mV and -50 mV the currents were outward and at -90

mV the current was inward. For the unliganded receptor, the remaining GB block after 3s was $29 \pm 13\%$ at -10 mV, $40 \pm 15\%$ at -50 mV and $36 \pm 14\%$ at -90 mV. When these receptors were tested in the liganded state, the GB block was $61 \pm 16\%$ at -10mV and $40 \pm 9\%$ at both -90 mV and at -50 mV. The liganded PTX block after 3s was $36 \pm 11\%$, measured at -90 mV holding potential.

The $\alpha 3$ homomeric and heteromeric glycine receptors are blocked in the unliganded and liganded state by GB and PTX. Only -10 mV and -90 mV GB blocks are shown in figure 7 because there were no differences between GB blocks at -50 mV vs -90 mV. Thus, it follows that direction of ion flow does not affect recovery. Based on these results, the next set of experiments were conducted at -10 mV and at -90 mV.

4. Recovery Rate from Liganded and Unliganded block

Next, we compared the rate of recovery of the GlyRs from the liganded and unliganded block described above. The same drug concentrations were used (i.e, 500 μ M Glycine, 100 μ M GB, 1 mM PTX for $\alpha 3$ and 500 μ M Glycine, 1 μ M GB and 10 μ M PTX for $\alpha 3\beta$) but the wash time was prolonged to 10 s. The results express GB or PTX block remaining after 10s wash. Note that the peak of the second glycine current after 10 sec includes recovery from both block and desensitization. Preliminary experiments, without antagonists, indicated that recovery from desensitization was complete at 3s. However, it is unclear if antagonists will affect this rate of recovery from desensitization, so the recovered current of the second glycine peak may not only measure of antagonist unbinding.

At -10 mV, homomeric $\alpha 3$ GlyRs recovered faster from GB unliganded block compared to GB liganded block (Figure 8A). There was a $30 \pm 12\%$ block remaining in the liganded condition after 10s and the receptors fully recovered in ~ 60 s. Unliganded GB block fully recovered in 10 sec. There were no significant difference in recovery after 10s of liganded and unliganded blocks with GB at -50 mV or for PTX at -50 mV. After 10 s wash,

at -50 mV, about $17 \pm 17\%$ block remained in unliganded GB condition and about $28 \pm 11\%$ block remained in liganded GB condition. Similarly, block remaining 10 s after unliganded PTX treatment was $28 \pm 9\%$ and after liganded PTX was $23 \pm 11\%$.

A similar trend was found in $\alpha 3\beta$ heteromers. As shown in Figure 8B, liganded GB block at -10 mV produced a $34 \pm 17\%$ block after 10s wash while only $5 \pm 5\%$ block remained 10s after unliganded GB block at -10mV. No significant difference between recovery from liganded and unliganded block was found with GB at -90 mV or with PTX. At -90 mV, block remaining 10s after liganded GB block was $15 \pm 7\%$ and after unliganded GB block was $22 \pm 9\%$. And $20 \pm 17\%$ block remained 10s after liganded PTX block and $12 \pm 7\%$ block remained after unliganded PTX block.

These results suggest mechanistic differences between GB block of the glycine bound and glycine free receptor. It is possible that there is more than one voltage sensitive binding site for GB. For simplicity if we assume there are two sites, one site is accessible in the liganded or unliganded state and the other site is only accessible when glycine is present. At holding potential of -10 mV, GB is bound efficiently to the second site and dissociates more slowly compared to the same liganded receptors at more negative voltages or to unliganded receptors at -10 mV.

Discussion

Distinct Pharmacology of $\alpha 3$ GlyR

This study reveals a number of unique features conferred by the $\alpha 3$ subunit of the glycine receptor. It is relatively insensitive to glycine, the heteromeric $\alpha 3\beta$ receptor EC_{50} is close to 400 μM . This is almost an order of magnitude less sensitive than heteromeric $\alpha 1$ GlyRs. The β subunit contributes to this low glycine sensitivity, which is unusual in that the β subunit of $\alpha 1$ or $\alpha 2$ GlyRs has little effect on sensitivity. The low glycine sensitivity might expand the dynamic range of the glycine response, since the $\alpha 3$ -containing receptor can respond to glycine levels that have saturated the other GlyR types.

Another interesting property of the $\alpha 3$ GlyR is the high sensitivity to picrotoxin. With an IC_{50} of $\sim 1 \mu M$, the homomeric $\alpha 3$ GlyR is as sensitive, or more sensitive, than GABA receptors. The $\alpha 3\beta$ GlyR has an PTX IC_{50} of $\sim 12 \mu M$, which is still close to that of GABA receptors. This indicates that PTX does not uniquely identify GABA receptors in pharmacological experiments and adds to the questionable utility of PTX based on studies of $\alpha 2$ GlyRs.

PTX Block of $\alpha 3$ GlyR

Mechanism of PTX inhibition in ligand-gated ion channels has not been fully elucidated. A single mutation in M2 reduces PTX sensitivity in GABA and glycine receptors (Pribilla et al., 1992; Zhang et al., 1995). The ring of threonines in the 6' position is associated with PTX sensitivity in GABA, 5-HT₃ (Gurley et al., 1995; Das and Dillon, 2005) and glycine receptors (Shan et al., 2001a; Hawthorne and Lynch, 2005), which suggests a pore binding model for PTX. However, PTX inhibition is competitive and voltage insensitive (Lynch et al., 1995; Wang et al., 2006) which are inconsistent with a pore blocker. This has led to an allosteric model of PTX inhibition (Lynch et al., 1995; Shan et al., 2001a). PTX is

competitive, not use-dependent, and voltage insensitive in $\alpha 3$ GlyRs. Furthermore, recovery rate of $\alpha 3$ GlyR from liganded and unliganded block by PTX is indistinguishable. Thus, PTX binding site in $\alpha 3$ GlyRs is in a region that is freely accessible in both open and closed state of the receptor. Glycine is not necessary for the $\alpha 3$ GlyR to recover from liganded block, which argues against the trap mechanism that is proposed in $\alpha 2$ GlyRs (Wang et al., 2006) and $\alpha 1$ GlyRs (Hawthorne and Lynch, 2005).

Comparison of PTX and GB at $\alpha 3$ GlyR

PTX and GB have similarities in molecular structure (Ivic et al., 2003a) and are both thought to bind near the pore region of the channel (Hawthorne et al., 2006b; Heads et al., 2008b). Support for this comes from mutations of T6, an amino acid in the middle of the M2 transmembrane segment, that reduce the effectiveness of PTX and GB (Shan et al., 2001b; Hawthorne et al., 2006b). In our experiments the potency of GB was similar to PTX, although repeated exposure can increase the apparent potency of a use-dependent ligand. The heteromer is more sensitive to GB and less sensitive to PTX, which is also true in $\alpha 2\beta$ GlyRs. The most distinctive differences between the two antagonists is that GB is voltage-dependent and non-competitive. These are properties that the $\alpha 3$ GlyR shares with the $\alpha 2$ receptor. In heterologously expressed $\alpha 2$ GlyRs, and in native rat hippocampal neurons, GB potency does not depend on the glycine concentration but is less effective when the cell is hyperpolarized. These two properties, along with use-dependence, are hallmarks of pore blockers.

But GB block at $\alpha 3$ GlyRs is not exclusively use-dependent; GB can bind and block the unliganded, closed state of the receptor. This is unlike GB's effect in hippocampal pyramidal cells or in heterologously expressed $\alpha 2$ GlyRs and does not fit with the classic pore blocker model which acts only on open channels.

Although $\alpha 1$ GlyRs has been reported to be blocked in unliganded state by PTX, none of the other receptors are blocked by either antagonist in the unliganded state. The unique antagonism profile of $\alpha 3$ GlyRs may help in pharmacologically identifying the subunit composition in native neurons.

Most studies agree that homomeric $\alpha 3$ GlyRs are not expressed in somatic regions of adult neurons (Lynch, 2009), but may be present in presynaptic nerve terminals of adult auditory brainstem neurons (Turecek and Trussell, 2002) and neurons of rat supraoptic nucleus (Deleuze et al., 2005). The $\alpha 3\beta$ GlyRs were seen in the inner plexiform layer of the retina and dorsal horn of the spinal cord neurons using immunohistochemistry (Haverkamp et al., 2003; Harvey et al., 2004), but these findings need validation (Elliott et al., 2015). Use of GB and PTX as pharmacological tools can help provide further support for this identification.

$\alpha 3$ GlyRs have been implicated in tactile allodynia (Huang et al., 2017) and $Gla3^{-/-}$ knockout mice have reduced prostaglandin E2 induced pain sensitization. (Harvey et al., 2004). These findings identify $\alpha 3$ GlyRs as molecular pain target and since GB and PTX block $\alpha 3\beta$ GlyRs, they might be useful in pain therapy if their action can be localized.

We sought to compare PTX antagonism with GB antagonism in $\alpha 3$ GlyRs due to the postulated structural similarity between the two compounds (Ivic et al., 2003b). Although the mechanism of GB inhibition is unresolved, there is evidence that GB binds to the 2'-6' pore lining region of the M2 transmembrane segment of GlyRs (Kondratskaya et al., 2005; Hawthorne et al., 2006a; Heads et al., 2008a). This is further supported by the non-competitive, use-dependent and voltage sensitive nature of the block (Kondratskaya et al., 2002; Kondratskaya et al., 2004; Kondratskaya et al., 2005; Hawthorne et al., 2006a). In addition to non-competitive, voltage sensitive and use-dependent properties, our data show an unliganded GB block in $\alpha 3$ GlyRs. We have also shown that recovery from the liganded block is slower than the unliganded block. These data might be explained by two GB binding

sites in the $\alpha 3$ GlyR. Site 1 is outside the pore and responsible for unliganded block. It can even be in the extracellular domain since a recent study identifies a novel allosteric site above the glycine binding site (Huang et al., 2004). Site 2 is in the pore and accessible only when the channel is open. Recovery from liganded GB block requires GB to fall off from both Sites 1 and 2 and hence takes longer than unliganded recovery. Either or both of these sites can be voltage sensitive. A similar competitive, unliganded inhibition of $\rho 1$ GABA_C receptors by GB has been reported (Huang et al., 2012) and they also suggested the possibility of two ginkgolide binding sites. The apparent competitive inhibition in GABA_C receptors may be due to an allosteric effect on agonist binding rather than a physical competition for the binding site.

Acknowledgement: The work was funded by National Eye Institute grant to M.M.S. S.C. was supported by scholarships from University at Buffalo.

Figure 1: Comparative agonist sensitivity of the alpha subunits in glycine receptors. A) Relative response of homomeric alpha2 and alpha3 GlyRs. B) Glycine-elicited response in homomeric and heteromeric alpha3 GlyRs. C) Response of alpha2 homomeric and

heteromeric GlyRs. D) EC50 values of homomeric and heteromeric alpha2 and alpha3

GlyRs. E) Comparison of mean EC50 values of various heteromeric GlyRs.

Figure 2 Antagonist effectiveness at GlyRs. A) Comparison of the IC₅₀ values of picrotoxin block of glycine for homomeric GlyRs (see text for detailed description). B) Comparison of picrotoxin block of glycine activity in homomeric and heteromeric alpha3 GlyRs. C) Block of 500 uM glycine by ginkgolide in homomeric and heteromeric alpha3 GlyRs.

Figure 3: Picrotoxin is a competitive antagonist, GB is non-competitive. In the bar graphs, dashed bars represent I/I_0 (where I is the peak glycine current in the presence of the antagonist and I_0 is the peak current in glycine alone) for 5mM gly and solid bars represent I/I_0 for 500 μ M Gly. In $\alpha 3$ GlyR (left graph), blocking effects of 1 μ M GB and 2 μ M PTX were recorded at -50 mV. With GB, I/I_0 (5 mM Gly) = 0.91 ± 0.05 , $n=8$ and I/I_0 (500 μ M Gly) = 0.88 ± 0.09 , $n=8$. With PTX, I/I_0 (5 mM Gly) = 0.84 ± 0.10 , $n=8$ and I/I_0 (500 μ M Gly) = 0.57 ± 0.09 , $n=8$. The second graph shows the effect of 1 μ M GB and 10 μ M PTX on $\alpha 3\beta$ GlyR at -90 mV. With GB, I/I_0 (5 mM Gly) = 0.88 ± 0.07 , $n=8$ and I/I_0 (500 μ M Gly) = 0.79 ± 0.13 , $n=10$. With PTX, I/I_0 (5 mM Gly) = 0.84 ± 0.10 , $n=5$ and I/I_0 (500 μ M Gly) = 0.35 ± 0.06 , $n=5$.

Figure 4: GB block is voltage-dependent in $\alpha 3$ GlyRs. Points marked 1,2,3 and 4 in the bar graph (left) correspond to the time points marked in the raw traces (right). The bar graph depicts I/I_0 where I_0 is the control 500 μ M Glycine trace (not shown in raw trace). Top panel – Voltage sensitive GB block in $\alpha 3$ GlyRs – Points 1 and 3 are block of peak currents at -10 mV and -50 mV respectively. I/I_0 values for the points marked 1 and 3 in the bar graph are 0.42 ± 0.14 , $n=13$ and 0.19 ± 0.05 , $n=17$. Points 2 and 4 are GB block after 5.5 sec

at -10 mV and -50 mV respectively. I/I_0 value for point 2 is 0.02 ± 0.02 , $n=17$ and for point 4 is 0.25 ± 0.15 , $n=13$. The peak of the control glycine current was same at -10 mV and -50 mV.

Bottom Panel - Voltage sensitive GB block in $\alpha 3\beta$ GlyRs - Points 1 and 3 are block of peak currents at -10 mV and -90 mV respectively. I/I_0 value at point 1 is 0.75 ± 0.08 , $n=9$ and at point 3 is 0.90 ± 0.04 , $n=7$. Points 2 and 4 are GB block after 5.5 sec at -10 mV and -90 mV respectively. I/I_0 value for point 2 is 0.39 ± 0.16 , $n=9$ and for point 4 is 0.47 ± 0.15 , $n=7$.

Figure 5 Picrotoxin block of liganded and unliganded homomeric $\alpha 3$ glycine receptors. Glycine was applied before and then again after treatment with picrotoxin plus glycine (top) or picrotoxin alone (bottom). Each of the four post-picrotoxin traces represents a separate experiment in which glycine was applied sometime after picrotoxin removal, as indicated.

Figure 6: GB can block the unliganded glycine receptor. The figure shows blocking effects of antagonists GB and PTX in liganded and unliganded state on $\alpha 2$, $\alpha 3$ and $\alpha 3\beta$ GlyRs. For all parts dashed bars represent unliganded block conditions while solid bar represents

liganded block conditions. The raw traces above dashed bars show relative peak of the first glycine peak (black) to second glycine peak after 3s wash (grey). The bars represent I/I_0 values where I_0 is the first glycine peak and I is the second glycine peak. Error bars represent SD.

In $\alpha 2$ part of the figure, I/I_0 value for liganded state at -10 mV is 0.74 ± 0.08 , $n=12$. The unliganded block was fully recovered by 3s ($I/I_0=0.95 \pm 0.06$, $n=15$).

In $\alpha 3$ GlyRs, I/I_0 values at -10 mV GB unliganded was 0.39 ± 0.1 , $n=5$ and at -10 mV GB liganded was 0.35 ± 0.05 , $n=5$. The plotted value at -50 mV GB unliganded was 0.80 ± 0.1 , $n=3$ and liganded was 0.66 ± 0.05 , $n=4$. For PTX unliganded block, I/I_0 value was 0.45 ± 0.09 , $n=8$ and for PTX liganded block it was 0.56 ± 0.1 , $n=7$.

In $\alpha 3\beta$ GlyRs, I/I_0 values at -10 mV GB unliganded was 0.71 ± 0.1 , $n=8$ and for liganded GB at the same voltage it was 0.39 ± 0.16 , $n=11$. At -90 mV, plotted I/I_0 values for unliganded GB was 0.64 ± 0.1 , $n=8$ and liganded GB was 0.60 ± 0.08 , $n=7$. For PTX block, I/I_0 values at unliganded was 0.67 ± 0.1 , $n=6$ and at liganded it was 0.64 ± 0.20 , $n=6$.

Figure 7: Recovery from GB block of glycine receptors is voltage dependent. Shows recovery from unliganded and liganded blocks of GB and PTX after 10s wash. For both

$\alpha 3$ and $\alpha 3\beta$ GlyRs dashed bars and the corresponding traces above that represent unliganded block while solid bars along with the raw traces above represent liganded block. In the raw traces, black shows the first glycine response and grey shows the glycine response after 10s wash. The plotted values in the bar graph is I/I_0 values where I_0 is the first glycine peak and I is the second glycine peak. Error bars represent SD.

$\alpha 3$ GlyRs – Plotted I/I_0 values for unliganded GB block at -10 mV = 0.99 ± 0.05 , $n=5$ and liganded GB block = 0.70 ± 0.10 , $n=5$ ($p < 0.05$). At -50 mV, unliganded GB block was 0.83 ± 0.16 , $n=3$ and liganded GB block was 0.71 ± 0.10 , $n=4$. With unliganded PTX block I/I_0 value was 0.72 ± 0.09 , $n=7$ and with liganded PTX I/I_0 was 0.77 ± 0.11 , $n=8$.

$\alpha 3\beta$ GlyRs – At -10 mV, I/I_0 values for unliganded GB block was 0.96 ± 0.08 , $n=6$ and liganded GB block was 0.66 ± 0.07 , $n=6$ $p < 0.001$. At -90 mV, I/I_0 values for unliganded GB block was 0.85 ± 0.09 , $n=7$ and liganded GB block was 0.78 ± 0.07 , $n=6$. I/I_0 values for unliganded PTX block was 0.88 ± 0.07 , $n=6$ and liganded PTX block was 0.80 ± 0.16 , $n=6$.

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