Effects of GABA Receptor Antagonists on Retinal Glycine Receptors and on Homomeric Glycine Receptor Alpha Subunits

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Running title: GABA antagonists at glycine receptors

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ABSTRACT

Glycinergic and GABAergic inhibition are juxtaposed at one retinal synaptic layer yet likely perform different functions. These functions have usually been evaluated using receptor antagonists. In examining retinal glycine receptors, we were surprised to find that commonly used concentrations of GABA antagonists blocked significant fractions of the glycine current. In retinal amacrine and ganglion cells, the competitive GABA_A receptor antagonists (bicuculline and SR95531) were also competitive GlyR antagonists. Picrotoxinin produced a non-competitive inhibition of retinal GlyRs. TPMPA, the GABA_C receptor antagonist, did not inhibit glycine receptors. All three GABA_A receptor antagonists were competitive inhibitors of homomeric α1 or α2 GlyRs expressed in HEK293 cells. Interestingly, bicuculline was much more effective at α2 GlyRs and might be used to separate glycine receptor subtypes. Thus, commonly used concentrations of GABA antagonists do not unambiguously differentiate GABA and glycine pathways. Picrotoxinin inhibition of GABA_C receptors requires two amino acids in the second transmembrane region (TM2): 2’serine and 6’ threonine. Although TM2 regions in GABA and glycine receptors are highly homologous, neither 2’ serine nor 6’ threonine is essential for picrotoxinin sensitivity in glycine receptors.

Keywords: inhibitory transmitters, picrotoxin, bicuculline and SR95531, GABA_C receptors, GABAP subunits
INTRODUCTION

The retina, like many other local neural networks, contains a direct polysynaptic excitatory pathway modulated by inhibitory interneurons. However, the abundance of intermingling of GABAergic and glycinergic synapses is unusual (Frumkes et al. 1981; Marc et al. 1995; Wassle et al. 1998; Zhang et al. 1997). The receptors for these transmitters have many properties in common (Hamill et al. 1983), but their co-localization within the retina’s inner plexiform layer presumably indicates that they have distinguishing features. A number of visual functions have been ascribed to each of these inhibitory transmitters (Ariel and Daw 1982; Belgum et al. 1984; Dong and Werblin 1998).

Studies on the distinct roles of synaptic glycine and GABA receptors have relied on the use of receptor antagonists such as strychnine and picrotoxinin, respectively. In studies of synaptic circuitry, high doses of antagonist are often used to completely eliminate one receptor pathway. For example, while the IC$_{50}$ of bicuculline is 1.7 µM (tested against 50 µM GABA in horizontal cell GABA$_A$ receptors), 100 µM bicuculline was required to fully block the GABA response. Similarly, 3 µM picrotoxin blocked most of the GABA current, but even 100 µM picrotoxin did not eliminate the GABA response (Feigenspan and Weiler 2004). Thus, in studies of glycine receptor function, high concentrations of GABA antagonists (e.g. 50 µM picrotoxin, 100 µM bicuculline, 20 µM SR95531) are used to eliminate GABA receptor function and ‘isolate’ the action of glycinergic synapses. This analysis depends on the selectivity of the antagonist. Yet many studies have shown that picrotoxin is not particularly selective and may act to block glycine receptors (Pribilla et al. 1992; Shan et al. 2001). There are also reports that
SR95531 and bicuculline can inhibit glycine receptors (Cohen et al. 1989; Dieudonne 1995; Sun & Machu, 2000). We therefore wanted to test these antagonists at commonly employed concentrations to determine their utility in separating GABA and glycine neural circuits in retina. We were surprised to find that GABA antagonists significantly suppressed the glycine receptor.

The action of GABA antagonists at the glycine receptor may cause confusion in studies of neural networks such as the retina. However, it may also provide an opportunity to investigate the mechanisms of action of these antagonists. We therefore determined the sensitivity of glycine receptor alpha subunits to these GABA antagonists. We found that the glycine receptors formed from \( \alpha_1 \) or \( \alpha_2 \) subunits had different antagonist sensitivity profiles. Thus, GABA antagonists may not only suppress glycine receptors, they may differentially affect glycine receptors depending upon their subunit composition.

Picrotoxin differentially inhibits GABA\textsubscript{A}, GABA\textsubscript{C}, and glycine receptors and these differences have been used to discern the site of action of this antagonist. One outcome of these studies is the hypothesis that picrotoxin inhibition requires a ring of five threonines in the pore forming TM2 region of the receptors (Zhang et al. 1995). Picrotoxin is a potent blocker of homomeric \( \alpha \) glycine receptors. It is about a thousand fold less potent in heteromeric \( \alpha/\beta \) receptors (Pribilla et al. 1992), presumably because the \( \beta \) subunits cannot contribute threonines (Shan et al, 2001). Another hypothesis, based on studies of subunits of the GABA\textsubscript{C} receptor, is that a serine in the TM2 region is required for potent picrotoxin inhibition (Wang et al. 1995). Our experiments show that neither of these hypotheses account fully for the action of picrotoxin on glycine receptors.
MATERIALS AND METHODS

Experiments were performed on transfected human embryonic kidney cells (HEK293) or retinal neurons isolated from aquatic larval tiger salamander (*Ambystoma tigrinum*). All procedures were performed in accordance with the USA Animal Welfare Act, National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication # 85-23), and approved by the University’s Animal Care Committee.

Salamanders, obtained from Kons Scientific (Germantown, WI) or Charles Sullivan (Nashville, TN) were kept on a 12-hour dark/12-hour light cycle in water tanks in a 4°C cold room. The retina was enzymatically dissociated using 50 µl papain (12 Unit/ml, Worthington Biochemicals) plus 300 µl of Ringer’s solution containing 5 mM L-cysteine, and 1 mM EDTA at pH 7.4. The isolated retinal neurons were plated on lectin-coated coverslips and placed in the recording chamber or stored in a 17°C incubator until used.

HEK293 cells were passed into 35mm culture dishes one day before transfection, so they could reach about 50% to 80% confluence for transfection. The plasmid containing GlyR subunit cDNA was transfected into HEK 293 cells using Fugene 6 (Roche Molecular Biochemicals). Fugene 6 transfection reagent (3 µl) was diluted in 100 µl serum-free DMEM medium. Glycine subunit cDNA (1 µg) and EGFP plasmid (0.1 µg) were then added. This solution was aliquoted into culture dishes. Studies on the HEK293 cells were conducted over the following 24-48 hours. Transfected cells were identified by fluorescence. Site-directed mutagenesis was performed using the QuickChange® mutagenesis kit (Stratagene). The successful incorporation of mutations was confirmed by sequencing (Roswell Park Cancer Institute, Buffalo, NY).
Whole-cell voltage clamp recordings were performed using an Axopatch 1C amplifier and pCLAMP software (Axon Instruments). The isolated retinal cells were superfused with amphibian Ringer’s solution containing (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 dextrose, and 5 HEPES, buffered to 7.8 with NaOH. The recording pipette was filled with a standard internal solution containing (in mM): 110 K-gluconate, 5 NaCl, 1 MgCl$_2$, 5 EGTA, and 5 HEPES, adjusted to pH 7.4 with KOH. Retinal third order neurons (amacrine and ganglion cells) were identified morphologically and electrophysiologically. All the dissociated third order retinal neurons had round cell bodies and few, short processes. The somas of third order neurons are generally bigger in size than the second order neurons. Third order neurons can be distinguished because they have large transient inward sodium currents (which are generally under 1 nA in amacrine cells and exceed 1 nA in ganglion cells) and lack inward rectifying potassium currents that are observed in second order neurons.

The external medium for HEK293 cell recordings was Kreb’s solution containing (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl$_2$, 2.5 CaCl$_2$, 10 HEPES, and 11 glucose, adjusted to pH 7.4 with NaOH. The recording pipettes contained (in mM): 140 K-gluconate, 5.4 NaCl, 2.0 MgCl$_2$, 1.0 CaCl$_2$, 11 EGTA, and 10 mM HEPES, adjusted to pH 7.4 with KOH. The pipettes had series resistances of about 5 MΩ and tip potentials of –10 mV in external solution. Drugs were applied using a DAD-12 fast superfusion system (ALA Scientific Instruments).

Glycine, picrotoxinin, imidazole-4-acetic acid (I4AA) and TPMPA ([1, 2, 5, 6-tetrahydropyridine-4-yl] methylphosphinic acid) were purchased from Sigma Aldrich. SR95531 and (-)-bicuculline methiodide were obtained from Research Biochemical
International. Picrotoxin is a mixture of picrotin and picrotoxinin. Both inhibit the glycine receptor; GABA receptors are selectively blocked by picrotoxinin. Since we were comparing inhibition of the two receptors, we used picrotoxinin exclusively. Drugs were made from stock solutions. Picrotoxinin was dissolved in DMSO to make 100 mM stock solution and kept away from light. DMSO concentrations were 0.1 ‰ v/v or less at the final picrotoxinin concentrations. This concentration of DMSO alone did not influence the effects described in this report. The water was double distilled and filtered (Millipore, 0.22 µm). Glycine was dissolved in stock Ringer’s or Kreb’s solution to make 100mM stock solution. Bicuculline, TPMPA and SR95531 were dissolved in double distilled water to make 10 mM stock solution. I4AA was dissolved in double distilled water to make 20 mM stock solution. All the stock solutions were kept at 4 ºC and used within a week.

Dose-response comparisons were generated by comparing effects of various concentrations of glycine against fixed concentrations of antagonist. In these experiments the antagonist concentration commonly used in previous studies of retinal synaptic circuitry was chosen. Alternatively, the effects of various antagonist concentrations on a fixed agonist concentration were tested. In these experiments, 100 µM glycine was used because it was frequently used in experiments on neurons. GABA at this concentration had no effect on expressed glycine receptors. Data are expressed as Means ± Standard Deviation (SD). Dose-response relationships were fitted to the Hill equation. The Wilcoxon matched-pairs signed-ranks and student t-tests were used to evaluate the significance of drug actions. It was sometimes difficult to obtain a full dose-
response curve from a single cell. Consequently, all responses were normalized to the effect of 2 mM glycine, except as noted.

We compared antagonists at concentrations that fully block GABA receptors, instead of comparing them at equal concentrations. Our protocol allows a direct comparison of responses to equal drug concentrations across subunits and native receptors. Therefore the percent suppression or the relative shift in EC$_{50}$ produced by the antagonists is not indicative of their relative potency.

RESULTS

Antagonist sensitivity in retinal neurons:

The effects of GABA antagonists on glycine responses were tested in isolated third order neurons, i.e. amacrine cells and ganglion cells of the tiger salamander retina. Neurons were voltage clamped at –60 mV. Under these conditions, 100 µM glycine produced large inward currents, which were suppressed by some GABA antagonists (figure 1). Throughout this study, we focused on GABA antagonist concentrations that are commonly used in retinal studies: 50 µM picrotoxinin, 100 µM bicuculline, 20 µM SR95531, 100 µM imidazole-4-acetic acid (I4AA) or 50 µM TPMPA ([1, 2, 5, 6-tetrahydropyridine-4-yl] methylphosphinic acid). Picrotoxinin suppressed glycine responses the most, inhibiting glycine current by 46 ± 2% (mean ± standard deviation, n=11). Other GABA antagonists also suppressed the glycine response. SR95531 reduced glycine currents by 29 ± 3% (n=7) and bicuculline suppressed 38 ± 2% (n=8) of the current. These effects were statistically significant (p < 0.01). All three antagonists reduced both the initial peak and the sustained portion of the glycine current, giving no evidence of use-dependent block. Several other GABA antagonists did not significantly
reduce the glycine current, including two GABAC-R antagonists: I4AA (figure 1) and TPMPA (not shown). Overall, GABA\textsubscript{A} receptor antagonists such as SR95531 and bicuculline were more effective antagonists of glycine currents than GABAC receptor antagonists such as I4AA and TPMPA (table 1).

Dose-response curves indicated that picrotoxinin had properties of non-competitiveness and shifted the glycine EC\textsubscript{50} to the right in retinal third order neurons (figure 1). The EC\textsubscript{50} of glycine alone was 39 ± 9 µM, but in the presence of 50 µM picrotoxinin, the glycine EC\textsubscript{50} was 71 ± 8 µM (n = 23). Characteristic of a non-competitive antagonist, the maximal response to glycine (2 mM) in the presence of picrotoxinin was only 66 ± 3 % of the maximal control glycine response. In contrast, both SR95531 and bicuculline behaved as competitive inhibitors of glycine. Bicuculline shifted the glycine EC\textsubscript{50} to 81 ± 16 µM (n = 20). SR95531 shifted it to 72 ± 7 µM (n = 18). Block by either antagonist could be fully overcome by 2 mM glycine, as expected of a competitive mechanism. The shifts in EC\textsubscript{50} were significant (p < 0.01) for all three antagonists (table 2). As anticipated from data in the right panel of figure 1, I4AA had little effect on the glycine dose-response curve (glycine EC\textsubscript{50} = 38 ± 2 µM, n= 15). Thus, these antagonists could block a third to a half of the glycine response if synaptic glycine reached 100 µM, and would have a greater effect if synaptic glycine was below 100 µM.

\textit{Antagonist sensitivity in \textalpha1 and \textalpha2 subunits}

The effects of GABA receptor antagonists on native glycine receptors justified a more detailed investigation of their interactions. The properties of retinal neurons may be complex because they can contain multiple glycine receptor subunits (Greferath et al. 1994) as well as GABA receptors. We initiated this investigation using single glycine
receptor alpha subunits (either $\alpha_1$ or $\alpha_2$) expressed in HEK293 cells. To make direct comparisons of the responses observed in retina, we used the same concentrations of agonists and antagonists. As in retina, several GABA antagonists inhibit glycine responses in this expression system and picrotoxinin was the most effective (figure 2, table 1). Interestingly, the antagonist profile was different for the two subunits.

Picrotoxinin produced a greater percentage block at the $\alpha_2$ subunit. When HEK293 cells were held at $-60$ mV, $50$ µM picrotoxinin blocked $55 \pm 3\%$ (n=8) of the response to $100$ µM glycine in cells expressing $\alpha_1$ glycine receptors and $93 \pm 1\%$ (n=12) in cells expressing $\alpha_2$ glycine receptors. The relative difference was greater for $100$ µM bicuculline, which blocked about $41 \pm 15\%$ (n=7) of the response in $\alpha_2$ glycine receptors, but only $4 \pm 9\%$ (n=10) in $\alpha_1$ GlyR. SR95531 (20µM) had similar effects on receptors formed from either of the two subunits, blocking the response to $100$ µM glycine by $17 \pm 11\%$ (n=11) in $\alpha_1$ and $20 \pm 6\%$ (n=9) in $\alpha_2$ GlyR. Surprisingly, $100$ µM I4AA, which had little effect in retina, blocked $35 \pm 6\%$ of the glycine current in $\alpha_2$ GlyR (n=6). It was less effective at the $\alpha_1$ GlyR ($12 \pm 5\%$ inhibition, n=10). As in retina, TPMPA was an ineffective antagonist at the $\alpha_1$ and $\alpha_2$ homomeric receptors. These results were statistically significant (p<0.01).

Thus, a variety of GABA receptor antagonists inhibit receptors formed by $\alpha_1$ or $\alpha_2$ GlyR subunits. All the GABA antagonists were more effective at the $\alpha_2$ homomeric receptor. Bicuculline (100 µM) was unusual in that it was almost totally ineffective at the $\alpha_1$ homomeric receptor and therefore might be useful in distinguishing between glycine receptors with these subunits. The sensitivity profiles of $\alpha_1$ and $\alpha_2$ differ so that
the relative effectiveness of picrotoxinin and bicuculline can also serve to distinguish between the two subunits. Strong inhibition by picrotoxinin and moderate inhibition by bicuculline is a property of α2 subunits, while moderate picrotoxinin inhibition coupled with very weak bicuculline inhibition is characteristic of α1 subunits. We have not yet tested retinal glycine receptors in different ganglion cells to determine if they revealed a differential sensitivity that would be indicative of distinct glycine receptor subunits.

Dose-response comparisons indicate that all the GABA receptor blockers are competitive antagonists at both α1 and α2 GlyRs (figures 3 and 4, table 2). In the α1 GlyR, picrotoxinin shifted the glycine dose response curve to the right (EC$_{50}$ shifted from 60 ± 2 µM to 108 ± 11 µM, n = 14) but the picrotoxinin antagonism could be completely overcome by high concentrations of glycine. SR95531 and I4AA produced smaller rightward shifts in the glycine dose-response curve (shift to EC$_{50}$ 80 ± 10 µM, n = 9 and 68 ± 5 µM, n = 10, respectively) and the antagonism could be fully overcome by high glycine concentrations. The shifts in EC$_{50}$ were significant (p < 0.01) for all these antagonists. The glycine dose-response curves in the α1 GlyR were not significantly changed by bicuculline (n = 8) or TPMPA (not shown).

At the α2 GlyR, several GABA antagonists also shifted the glycine dose-response curve to the right and inhibition was overcome by high glycine concentrations, indicative of competitive inhibition. Picrotoxinin shifted the glycine EC$_{50}$ from 111 ± 13 µM to 357 ± 11 µM (n = 12). Bicuculline shifted the EC$_{50}$ to 174 ± 34 µM (n = 9), I4AA shifted the EC$_{50}$ to 160 ± 3 µM (n = 9), and SR95531 shifted the EC$_{50}$ to 151 ± 8 µM (n = 10). All behaved as competitive antagonists. All the shifts were statistically significant (p < 0.01).
Models of picrotoxinin sensitivity:

Picrotoxin inhibition has been linked to the putative second transmembrane segment (TM2) of glycine and GABA receptors. This segment is thought to form the pore region of the receptor and is highly homologous in the two receptors (figure 5). With respect to GABA receptors, insights into the site of picrotoxin’s action have come from analysis of various subunits of the GABA$_C$ receptor, (GABA$p$) and among different species. For example, the human GABA$p$1 receptor is less sensitive to picrotoxin than the GABA$p$2 ($IC_{50}$ = 48.0 µM vs. $IC_{50}$ = 4.7 µM, against 20 µM GABA) (16). A 2’ serine in GABA$p$2, compared to a 2’ proline in GABA$p$1, accounts for the difference (Wang et al. 1995). On the other hand, the rat GABA $p$2 subunit is much less picrotoxinin sensitive than the human $p$2 subunit (Zhang et al. 1995). This can be explained by a 6’ threonine in human $p$2, while the rat $p$2 has a 6’ methionine (figure 5).

We performed the equivalent mutations in the $\alpha$1 GlyR, which contains a 6’ threonine in TM2 (figure 5). If we replaced threonine with methionine (T258M in the rat $\alpha$1 GlyR), then glycine $EC_{50}$ decreased approximately fivefold from 60 ± 2 µM to 14 ± 4 µM (figure 6). Unlike the GABA$p$ receptor, picrotoxinin inhibition was only slightly reduced. The effect of picrotoxinin was tested against 20 µM glycine, which is approximately as effective as 100 µM glycine used in antagonist studies of wild type $\alpha$1 GlyRs. Picrotoxinin $IC_{50}$ changed from 43 ± 11 µM to 97 ± 15 µM ($n = 8$, $p<0.05$). Thus, the efficacy of picrotoxinin decreased only about twofold in the glycine receptor, unlike the tenfold drop in the GABA receptor.

We also tested the importance of the amino acid at the 2’ position in the glycine receptor. Wang et al. (1995) found that mutating the 2’ serine in GABA$p$2 to a 2’ glycine
did not diminish picrotoxin sensitivity. The α1 GlyR has a 2’ glycine (G254, figure 5). We performed the reverse mutation, replacing the 2’ glycine in the α1 GlyR with 2’ serine. The glycine sensitivity of the α1(2’G → S) GlyR did not change significantly; the EC$_{50}$ was 60 ± 2 µM in wild type and 61 ± 2 µM in the mutant (figure 7, upper panel, n = 6, p < 0.05). The sensitivity to picrotoxinin was reduced; the IC$_{50}$ was 43 ± 11 µM in wild type and was 87 ± 17 µM in the mutated α1 subunit (n=7, p < 0.05). As in the GABA$_{\rho}$ receptor, the glycine-serine substitution did not produce a large alteration in agonist or antagonist action in the glycine receptor.

**Discussion**

Three main conclusions of this study are that many commonly used GABA$_{A}$ receptor antagonists block significant fractions of native glycine receptors, the GlyR α2 subunit is more sensitive to these antagonists than the GlyR α1 subunit, and the role of amino acids in the glycine receptor M2 region during picrotoxinin inhibition differ from that of the GABA receptor.

*Action of GABA receptor antagonists at retinal glycine receptors:*

Several GABA$_{A}$R antagonists suppressed glycine currents in amphibian retinal neurons. GABA$_{C}$R antagonists, TPMPA and I4AA, did not inhibit the glycine receptors in retina. Bicuculline and SR95531, competitive antagonists of GABA$_{A}$R, were also competitive glycine receptor antagonists in retinal neurons. Picrotoxinin, a non-competitive GABA$_{A}$ receptor antagonist but a competitive GABA$_{C}$ receptor antagonist (Zhang et al, 1995), was a non-competitive glycine antagonist in retina. It also shifted the glycine EC$_{50}$ in retina, which may represent a competitive inhibition as well. This would
be consistent with its effect in homomeric glycine receptors, where picrotoxinin was a competitive antagonist. All three GABA<sub>A</sub>R antagonists produced similar shifts in glycine EC<sub>50</sub> in retina. Antagonist concentrations that are employed to fully block the GABA receptor (50 µM picrotoxinin, 100 µM bicuculline, or 20 µM SR95531) blocked glycine responses by 46%, 38%, and 29%, respectively. This was a surprising degree of non-specific inhibition. Furthermore, some types of glycine receptor are more affected. Thus, use of picrotoxin and bicuculline would not only block GABA receptors, but also preferentially inhibit α2 containing glycine receptors.

This means that many previous experimental conclusions, which assumed unaltered activity of glycine receptors, are ambiguous. In particular, lack of evidence of glycine signals may have resulted from their unintentional block. This is also likely to be true for many studies throughout the central nervous system. Of the GABA<sub>A</sub>R antagonists, SR95531 at commonly used concentrations had the least crossover to glycine receptors. TPMPA, a GABA<sub>C</sub> antagonist, did not crossover to the glycine receptor.

Since the antagonists are competitive, the magnitude of their effects depends on the concentration of glycine in the synaptic cleft. In rat, 3 µM SR95531 or 3 µM bicuculline partially blocked GABAergic IPSCs with weak inhibition of glycinergic IPSCs. However, 10 µM bicuculline blocked about 15% of the glycine IPSC (Protti et al. 1997). Thus, it is likely that glycine in the synaptic cleft is not so high as to eliminate the effect of this competitive inhibitor.

Comparison of antagonists in native and heterologously expressed glycine receptors:

Picrotoxinin’s large inhibition of retinal glycine currents was unanticipated. In native glycine receptors, the β subunit is presumed to almost eliminate picrotoxinin’s effect
(Pribilla et al. 1992). They found that, with 100 µM glycine, the IC$_{50}$ of picrotoxinin was greater than 1000 µM in $\alpha_1\beta$ or $\alpha_3\beta$ GlyRs, and about 300 µM in $\alpha_2\beta$ GlyRs. Yet 50 µM picrotoxinin blocked almost half the response to 100 µM glycine in retinal neurons. This is especially surprising considering that the glycine EC$_{50}$ in retina is lower than in homomeric $\alpha_1$ or $\alpha_2$ GlyRs (or heteromeric $\alpha_1\beta$ or $\alpha_2\beta$ receptors – Li and Slaughter, personal observations). Retinal neurons may express an $\alpha_3$ subunit, but it has a picrotoxinin sensitivity that is similar to $\alpha_1$ and weaker than $\alpha_2$ (Gisselmann et al. 2002; Pribilla et al. 1992). It is likely that retinal neurons express glycine receptors containing $\beta$ subunits. The main conductance of glycine channels in goldfish ganglion cells is about 16 pS (Cohen et al. 1989), far below the ~80 pS expected in GlyRs made exclusively from alpha subunits. The presence of $\beta$ subunits makes the potency of picrotoxinin in retina, relative to alpha homomeric receptors, even more unexpected (Pribilla et al. 1992).

Another, and possibly associated phenomenon, is that picrotoxinin has a non-competitive inhibitory component in retina, that is not seen in expressed glycine receptors in our study or in heteromeric receptors GlyRs (Rajendra et al. 1997). The reason for the non-competitiveness in retina is unclear.

The requirement for the threonine ring at the TM2 6' position:

Picrotoxinin’s site of action is a topic of interest in GABA and glycine receptors. Two sites within the TM2 region, 2’ and 6’, have attracted particular interest. Human GABAr2 has a 6’ threonine and is picrotoxinin sensitive. Rat GABAr2 has a 6’ methionine and is picrotoxinin insensitive. A 6’ T→M mutation in human GABArRs results in picrotoxinin insensitivity (Zhang et al. 1995). Similarly, the $\alpha_1$ GlyR has a 6’ threonine and mutation to phenylalanine reduces picrotoxinin sensitivity in homomeric
α1 GlyRs over 30 fold (Shan et al. 2001). Alanine or cysteine substitutions also greatly reduced picrotoxinin sensitivity. This led Shan et al. (2001) to conclude that picrotoxinin sensitivity in the glycine receptor requires a ring of five 6’ threonines.

But we found that this five threonine ring was not required for picrotoxinin sensitivity. Shan et al (2001) did not evaluate the 6’ T→M mutation that accounted for the insensitivity of the rat GABARp2 receptor. We unexpectedly found that the GlyR α1 6’ T→M mutation had little effect on picrotoxinin sensitivity. Thus, picrotoxinin sensitivity remains intact in the α1 GlyR if the 6’ position contains threonine or methionine, but not phenylalanine, cysteine, or alanine.

Amino acids at the 2’ site of TM2 in GABA and glycine receptors:

The 2’ position of TM2 has also been associated with picrotoxinin block. A 2’ alanine increases picrotoxin potency in the invertebrate glutamate-gated chloride channel (Etter et al. 1999), and in the GABARp1 it drops the IC50 from 48 µm in WT to 0.1 µM (Wang et al. 1995). The α1 GlyR has a 2’ glycine; the α2 GlyR has a 2’ alanine. Perhaps the GlyR α1 subunit is less sensitive to picrotoxinin because it lacks a 2’ alanine. However, placing a 2’ alanine in the α1 GlyR (2’ G→A) results in only a minor change in picrotoxinin IC50, from 18 µM to 14.8 µM (Shan et al. 2001). Therefore, greater picrotoxinin sensitivity of α2 GlyR is not explained by the 2’ alanine.

There is also a question about the relative importance of proline at the 2’ position of TM2 in GABA and glycine receptors. In the GABARcR, a proline reduces inhibition, since a P→S mutation in human GABARp1 enhances picrotoxin IC50 from 48 µM to 4.8 µM. In the α1 GlyR a proline seems beneficial since a 2’ G→P mutation improves picrotoxin inhibition (WT IC50 = 18 µM; 2’ G→P IC50 = 2.6 µM) (Shan et al. 2001).
Thus, proline decreases picrotoxin inhibition in the GABA\textsubscript{C} receptor by an order of magnitude but improves inhibition at the glycine receptor by an order of magnitude. However, this indirect comparison leaves the possibility that proline is equally important at both GABA and glycine receptors, and that picrotoxin potency follows the order: 2’ serine > 2’ proline > 2’ glycine. If true, then it predicts that a 2’ serine substitution in \(\alpha_1\) GlyR (G→S) would greatly increase picrotoxin potency. However, the 2’ G→S mutation did not alter the picrotoxinin sensitivity of \(\alpha_1\) GlyRs. Therefore, the amino acid at the 2’ site in TM2 has very different effects on GABA and glycine receptors.

In summary, GABA antagonists are weak but significant antagonists at glycine receptors. This is particularly problematic for GABA\textsubscript{A}R antagonists. Despite high homology in the TM2 region, a variety of 2’ amino acid substitutions (proline, alanine, glycine) have different effects on picrotoxin inhibition of GABA and glycine receptors. At the 6’ position of TM2, the presence of a methionine or threonine has little effect on picrotoxinin inhibition of glycine receptors, while dramatically altering GABA\textsubscript{C} receptor inhibition.

Acknowledgements: We thank Jason Myers for construction of several glycine receptor point mutations. This work was supported by NEI grant EY014960.
Reference List


Sun H and Machu TK. Bicuculline antagonizes 5-HT (3A) and alpha2 glycine receptors expressed in Xenopus oocytes. *Eur J Pharmacol* 391:1109-1120, 2000.


Figure Legends

FIGURE 1. Glycine currents were measured in retinal third order neurons. The left panel shows current responses to 100 µM glycine alone and then in the presence of a GABAR antagonist. The antagonists are 50 µM picrotoxinin (PTX), 100 µM bicuculline (BIC), 20 µM SR95531 (SR) and 100 µM imidazole-4-acetic acid (I4AA). Cells were pretreated with antagonist before co-application with glycine. The solid bar above the trace indicates the time of glycine application, and the dashed line indicates the application time of antagonists. The right panel shows the normalized glycine dose response of a set of retinal third order neurons in the absence or presence of each of the antagonists.

FIGURE 2. Glycine currents were measured in HEK293 cells transfected with α1 (left) and α2 (right) GlyR subunits. The left trace in each set shows the effect of 100 µM glycine alone and the right trace of each set shows the effects of glycine plus 50 µM picrotoxinin, 100 µM bicuculline, or 20 µM SR95531 for each of the two subunits. Cells were pretreated with antagonist before co-application with glycine. The solid bar above the trace indicates the time of glycine application, and the dashed line indicates the application time of antagonists.

FIGURE 3. Glycine dose-response curves for the α1 GlyR with or without 50 µM picrotoxinin, 100 µM bicuculline, 20 µM SR95531, or 100 µM I4AA.

FIGURE 4. Glycine dose-response curves for the α2 GlyR with or without 50 µM picrotoxinin, 100 µM bicuculline, 20 µM SR95531, or 100 µM I4AA.

FIGURE 5. A comparison of the amino acid sequence of the TM2 region of rat glycine receptor α1 and α2 subunits, rat GABA ρ1 and ρ2 subunits, and human GABA
ρ1 and ρ2 subunits. The numbering system starts with 0 at the putative cytoplasmic edge of TM2. The amino acids at the 2’ and 6’ position are the focus of picrotoxinin action.

FIGURE 6. The upper panel shows the normalized dose response relationship of the wild type glycine α1 receptors and the 6’ T→M mutation. The lower panel shows normalized dose-response inhibition curves of glycine current with increasing concentrations of picrotoxinin. Note that the α1 T→M mutation makes the subunit five times more sensitive to glycine but the picrotoxinin IC50 increases only two folds.

FIGURE 7. The upper panel shows the normalized dose response relationship of the wild type glycine α1 receptors and the 2’ G→S mutation. The lower panel shows normalized dose-response inhibition curves of glycine (100 µM) current with increasing concentrations of picrotoxinin. The α1 G→S mutation does not change the subunit sensitivity to glycine or picrotoxinin.
Table 1  This lists the average percent inhibition of the current response to 100 µM glycine in retina and homomeric glycine receptors (formed from α1 or α2 subunits) produced by 50 µM picrotoxin (PTX), 100 µM bicuculline (BIC), 20 µM SR95531 (SR), 100 µM I4AA, or 50 µM TPMPA.
Table 2  This lists the glycine EC$_{50}$ in retina and in homomeric glycine receptors formed of either $\alpha$1 or $\alpha$2 subunits under control conditions and in the presence of 50 µM picrotoxinin (PTX), 100 µM bicuculline (BIC), 20 µM SR95531 (SR), 100 µM I4AA, or 50 µM TPMPA.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6