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Identification and characterization of heptapeptide modulators of the glycine receptor

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Abstract

The glycine receptor is a member of the Cys-loop receptor superfamily of ligand-gated ion channels and is implicated as a possible therapeutic target for the treatment of diseases such as alcoholism and inflammatory pain. In humans, four glycine receptor subtypes ($\alpha 1$, $\alpha 2$, $\alpha 3$, and β) co-assemble to form pentameric channel proteins as either α homomers or $\alpha\beta$ heteromers. To date, few agents have been identified that can selectively modulate the glycine receptor, especially those possessing subtype specificity. We used a cell-based method of phage display panning, coupled with two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes, to identify novel heptapeptide modulators of the $\alpha 1\beta$ glycine receptor. This involved a panning procedure in which the phage library initially underwent subtractive panning against Human Embryonic Kidney (HEK) 293 cells expressing alternative glycine receptor subtypes before panning the remaining library over HEK 293 cells expressing the target, the $\alpha 1\beta$ glycine receptor. Peptides were identified that act with selectivity on $\alpha 1\beta$ and $\alpha 3\beta$, compared to $\alpha 2\beta$, glycine receptors. In addition, peptide activity at the glycine receptor decreased when zinc was chelated by tricine, similar to previous observations of a decrease in ethanol's enhancing actions at the receptor in the absence of zinc. Comparisons of the amino acid sequences of heptapeptides capable of potentiating glycine receptor function revealed several consensus sequences that may be predictive of a peptide's enhancing ability.

Keywords

Glycine Receptor; Phage Display; Allosteric Modulator; Electrophysiology; *Xenopus* oocytes

1. Introduction

The Cys-loop receptor superfamily constitutes a major class of ligand-gated ion channels involved in fast inhibitory and excitatory neurotransmission throughout the central nervous

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system and periphery. Members include excitatory cation-selective channels such as the nicotinic acetylcholine receptor and the serotonin type 3 receptor, as well as inhibitory anion-selective channels such as the γ -aminobutyric acid type A and glycine receptors (Thompson et al., 2010). Due to their involvement in a variety of central nervous system disorders, several members of this receptor superfamily serve as targets both for compounds in clinical use, as well as investigational agents (Dineley et al., 2015; Nys et al., 2013). In particular, the glycine receptor has been identified as a potential target for a variety of therapeutic applications, including the treatment of inflammatory pain and alcoholism (Foster et al., 2015; Lynch and Callister, 2006; Molander et al., 2005; Molander and Söderpalm, 2005a).

Although glycine receptors predominate in the brain stem and spinal cord, they are also expressed in higher brain regions such as the nucleus accumbens, frontal cortex, and hippocampus (Jonsson et al., 2012; 2009; Molander and Söderpalm, 2005b). A variety of drugs of abuse, including alcohol, inhalants and volatile anesthetics enhance glycine receptor function at concentrations that are achieved *in vivo* (Lynch, 2004; Molander et al., 2005; Xiong et al., 2009). Individual receptor subunits of this Cys-loop superfamily form pentamers around central ion-conducting pores. In humans, there are 4 glycine receptor subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, and β) that can form α -homomeric or $\alpha\beta$ -heteromeric channels. Enhanced activation of these channels through positive allosteric modulation effectively treats inflammatory pain in rodents and may aid in the treatment of addictions such as alcoholism, through modulation of dopamine release (Molander et al., 2005; Molander and Soderpalm, 2005 a,b; Xiong et al., 2012). However, while $\alpha 3$ -containing receptors are thought to predominate in glycine receptor-mediated analgesia, it is not entirely clear which subtypes are responsible for the behavioral and dopamine-modulating effects of alcohol. While $\alpha 1$ - and $\alpha 2$ -containing receptors seem to be the most likely *in vivo* targets of alcohol (Blednov et al., 2015), there would be considerable utility in the development of glycine receptor modulators with subunit selectivity, to definitively determine the relative contributions of each particular subtype to alcohol's pharmacological effects. Our previous work validated the use of phage display for the discovery of novel peptide modulators of the glycine receptor (Tipps et al., 2010). In the present report, we expand on these studies by identifying and characterizing the action of peptides with selectivity for $\alpha 1\beta$ and $\alpha 3\beta$ over $\alpha 2\beta$ -containing glycine receptors and identify several possible amino acid consensus sequences within the heptapeptides. Interestingly, the actions of these peptides appear to be zinc-dependent. This zinc dependence was also previously shown to affect alcohol modulation of the glycine receptor (McCracken et al., 2010), suggesting that the presence of zinc may be necessary for efficient modulation of glycine receptor activity by allosteric modulators.

2. Materials and Methods

2.1 Reagents

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Peptides were synthesized as HCl salts at 98% purity by Peptide 2.0 Inc. (Chantilly, VA). Peptides were received as a lyophilized powder and suspended at a concentration of 10 mM

in ultrapure H₂O, based on the theoretical peptide molecular weight and the dry weight of lyophilized peptide material. Suspended peptides were stored as single-use aliquots at –20°C for up to 4 weeks before use.

2.2 Human Embryonic Kidney (HEK) Cell Culture and Expression of Glycine Receptors

HEK 293 cells were obtained from the American Type Culture Collection and grown according to standard procedures (Freshney, 2002). Briefly, cells were cultured at 37°C and 5% CO₂ in Gibco® Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate, and 10% fetal bovine serum (Thermo-Fisher Scientific, Waltham, MA). Cells were split every 5 days with Gibco® trypsin-EDTA (Thermo-Fisher Scientific) up to 25 times, after which new aliquots of early-passage cells were started. Cells were transfected with 4 µg of glycine receptor α1β, α2β, or α3β cDNA (1:20 α:β ratio) in modified pBK-cytomegalovirus vectors (Mihic et al., 1997) using PolyFect reagent (Qiagen, Valencia, CA). All cells were incubated for at least 48 h before use in panning.

2.3 Phage Display

The Ph.D.-7 phage library was purchased from New England Biolabs (Ipswich, MA) and the panning procedures followed were the same as described in Tipps et al. (2010), except as noted below. The phage display procedure consisted of two separate panning series, D7.1 and D7.2. Each series was identical except for the subtractive panning step against negative selection cells. Subtractive panning for series D7.1 consisted of washing the phage library over negative selection HEK 293 cells expressing α2β glycine receptors while D7.2 consisted of a pair of washes of library over negative selection HEK 293 cells expressing α2β, followed by HEK 293 cells expressing α3β glycine receptors.

Amplified phage were tittered and diluted in phosphate-buffered saline containing 1.5% bovine serum albumin and 0.1% Tween to ~10¹¹ PFU/ml for each round of panning. After four rounds of panning, individual plaques from the final round were isolated and incubated overnight in LB broth containing a 1:100 dilution of an overnight culture of *E. coli* ER2738, at 37°C with gentle agitation. Phage DNA was isolated from overnight cultures using the S.N.A.P. MiniPrep kit (Thermo-Fisher Scientific) and sequenced using a –96gIII sequencing primer in order to identify the peptides displayed on the N-terminal portions of the pIII coat protein, presumably responsible for glycine receptor binding.

2.4. Oocyte isolation and cDNA Injection

Xenopus laevis were obtained from Nasco (Fort Atkinson, WI) and housed at a 19°C on a 12 h light/dark cycle. A portion of the ovaries was removed surgically, in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations, and placed in isolation media (108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES). Forceps were used to manually remove the thecal and epithelial layers from stage V and VI oocytes before removal of the follicular layer by a 10 min incubation in 0.5 mg/ml type 1A collagenase in buffer containing 83 mM NaCl, 2 mM MgCl₂ and 5 mM HEPES. A 32.2 nl sample of 50 ng/µl α1β, α2β, or α3β (at a 1:20 α:β ratio) cDNA, in a modified pBK-cytomegalovirus vector (Mihic et al., 1997), was injected into the animal pole of each oocyte using a micropipette (10–15 µm tip size) attached to an electronically active microdispenser.

Oocytes were stored individually at room temperature in 96-well plates containing modified Barth's saline (MBS) [89 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄•7H₂O, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂, pH 7.5] supplemented with 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin and 50 mg/l gentamicin, and sterilized by passage through a 0.22 µm filter.

2.5. Two-electrode voltage-clamp electrophysiology

Electrophysiological recordings were conducted 1–5 days post cDNA injection. Oocytes were placed in a 100 µl bath with the animal poles facing upwards and impaled with two high-resistance (0.5–10 MΩ) glass electrodes filled with 3 M KCl. Oocytes were perfused with MBS at a rate of 2 ml/min through 18-gauge polyethylene tubing using a Masterflex peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) and voltage-clamped at –70 mV using an OC-725 C oocyte clamp (Warner Instruments, Hamden, CT). All solutions were prepared in MBS or MBS + 2.5 mM tricine. Peptides were diluted in MBS or MBS + 2.5 mM tricine to final working concentrations from frozen 10 mM stocks. When maximally-effective concentrations of agonists were applied, applications lasted for 15 s and were followed by 10–15 min washouts with MBS to allow for receptor re-sensitization. For experiments using submaximal concentrations of glycine, concentrations that yielded 5–10% of the maximally-effective glycine response (EC_{5–10}) were applied for 45 s followed by 3–5 min washouts with MBS to allow for receptor re-sensitization. Peptides were co-applied with agonist following a 45 s preincubation of peptide alone. Data were acquired at a rate of 1 kHz using a Powerlab 4/30 digitizer with LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia).

2.6. Determination of Zinc Concentration in Peptide Stocks

Zinc concentrations in peptide stocks were determined using a quadrupole-based Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS) at the Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin. 10 mM stocks of peptide were diluted 10-fold in 2% HNO₃ before analysis. Electrophysiological and ICP-MS data were collected in parallel from the same batch of each peptide.

3. Results

3.1. Selection of α1β glycine receptor-binding peptides

Phage display was conducted using the Ph.D.-7 Phage Display Library from New England Biolabs containing approximately 10⁹ unique heptapeptide sequences displayed pentavalently on M13 phage. In an attempt to obtain peptides selective for the α1β glycine receptor subtype, we employed a modified version of our previous phage display procedure (Tipps et al., 2010) in which the phage library was first subjected to a subtractive panning step against α2β-expressing (D7.1), or α2β- and α3β-expressing (D7.2) HEK 293 cells. In total, 40 peptides were identified via phage display as capable of binding to the α1β glycine receptor; 15 from panning series D7.1 and 25 from D7.2 (Table 1).

3.2 Electrophysiological characterization of $\alpha 1\beta$ glycine receptor-binding peptides

Of the 40 peptide sequences initially identified, 25 were synthesized and characterized for their actions on $\alpha 1\beta$ glycine receptors via two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes. Peptides were tested at 30 μM , a concentration previously shown to be sufficient to affect glycine receptor function (Tipps et al., 2010). A glycine concentration that yielded 5 to 10 percent of the maximally-effective glycine response (EC_{5-10}) was initially identified in each oocyte and applied several times for 45 s, followed by 3–5 min washouts to allow for receptor re-sensitization. Peptide was applied for 30 s alone followed by a 45 s co-application with EC_{5-10} glycine. Peptide applications resulted in a range of effects from 26.8 $\pm$ 4.9% inhibition (D7.1-115) to 93.3 $\pm$ 12.2% potentiation (D7.2-123) of the EC_{5-10} glycine response (Fig. 1). None of the peptides had effects in the absence of glycine, implying that they lack both glycine receptor agonist activity as well as effects at any endogenous oocyte proteins that could influence the holding current of the oocyte. During the course of conducting experiments, we sometimes noticed variations in the degrees of modulation produced by peptides synthesized in different batches (Fig. 2). This was not entirely unexpected as net-peptide content can vary between batches of synthetic peptides and peptide concentrations were calculated based on the dry weight of the lyophilized powder provided by the supplier. In order to prevent batch-to-batch variations from confounding results in characterization experiments, all data presented below were obtained from single batches of peptides.

To determine if peptides were capable of affecting glycine receptor-mediated currents at lower concentrations, six different peptides of the initial screen were applied at 1, 3, 10, and 30 μM concentrations to $\alpha 1\beta$ glycine receptors activated by EC_{5-10} glycine. The threshold concentrations of activity for the three most efficacious of these peptides were between 3 and 10 μM (Fig. 3). Previous work in our lab illustrated the abilities of allosteric modulators to differentially affect currents produced by saturating concentrations of agonists varying in efficacy (Kirson et al., 2012, 2013). In order to test if this was also true for peptide modulation, 30 μM D7.2-122 was tested for its ability to modulate $\alpha 1\beta$ glycine receptors activated by maximally-effective concentrations of the partial agonist taurine and the higher efficacy agonist glycine (Fig. 4). As with other allosteric modulators, D7.2-122 had no effects on currents produced by saturating glycine concentrations but did enhance currents produced by a saturating concentration of taurine.

In order to determine whether negative selection during phage panning against glycine receptors of different subunit composition allows for the identification of peptides with subunit selectivity, the most effective peptides were applied to $\alpha 1\beta$, $\alpha 2\beta$, and $\alpha 3\beta$ glycine receptors activated by EC_{5-10} glycine (Fig. 5). Interestingly, all peptides had higher activity on $\alpha 1\beta$ and $\alpha 3\beta$ receptors compared to $\alpha 2\beta$ [$F(2,44) = 4.24$, $P < 0.025$; Two-way ANOVA]. However, peptides appeared to be equally or slightly more effective in enhancing $\alpha 3\beta$ than $\alpha 1\beta$ glycine receptor function, despite inclusion of the $\alpha 3\beta$ receptor subunits in the D7.2 negative selection process.

3.3 Identification of possible consensus sequences for glycine receptor-enhancing peptides

While there is no obvious consensus sequence among all of the 40 peptides identified by phage panning, analysis of their amino acid compositions revealed selection both for and against particular amino acids within the identified peptides, compared to the observed frequencies of amino acids within the library as a whole, as characterized by the manufacturer (Fig. 6A,B). Fig. 6A shows the observed frequencies of individual amino acids expressed as the percentage of all 280 amino acids present within the 40 identified heptapeptides, while Fig. 4B expresses the percent change in the observation frequency of amino acids in the 40 identified peptides compared to the observed frequency of amino acids in the whole library, as characterized by New England Biolabs. Overall, it is clear that amino acids T, P, and G were strongly selected for while K, W, F, E were selected against (30% increase or decrease in observed frequency, respectively). A more striking selection is seen when analyzing the amino acid compositions of only the eight glycine receptor-enhancing peptides that produced 20% potentiation of EC₅₋₁₀ glycine responses (Table 1, bold; Fig. 6C,D). Here T, P, S, D, Q, and W are all strongly selected for (45% increase in observed frequency) while L, N, V, Y, F, E, and C were strongly selected against (38% decrease in observed frequency).

Further analysis of the positions of the amino acids that were strongly selected for in the eight glycine receptor-enhancing peptides revealed several possible consensus sequences that may contribute to their enhancing abilities (Table 2). First, serines are present towards the C-terminal regions of peptides, at positions 6 or 7 in six of eight enhancers. Further, four out of eight peptides contain the sequences PxxS within their sequences and the two most effective peptides, D7.2-122 and D7.2-123, contain the sequences TxPxxS, where 'x' could be any amino acid. The two peptides that do not contain serines at the C-terminal region (D7.1-107 and D7.2-111) contain the consensus xxxQxPx. In addition, D7.1-107, D7.1-118, and D7.2-123 all contain TxT while D7.2-122 and D7.2-123 both contain TTxxxxx as well as sharing the sequence TxPxxS.

3.4 Characterizing the role of zinc in peptide action

Zinc affects modulation of the glycine receptor by ethanol, with zinc chelation markedly decreasing alcohol enhancement of receptor function when tested using submaximal glycine concentrations (McCracken et al., 2010). In order to determine what role, if any, zinc plays in the activities of these peptides at the glycine receptor, potentiating concentrations of select peptides were co-applied with the zinc-chelating agent tricine. As seen in Fig. 7, removal of zinc by tricine almost completely abolishes modulation by all peptides except D7.2-123. Interestingly, the inhibitory action of D7.1-115 was also blocked in the presence of tricine, implying a possible zinc-dependence of peptide action. Moreover, zinc is a known contaminant in many biological buffers and labware at concentrations capable of affecting glycine receptor function (Cornelison and Mihic, 2013; Kay, 2004). To determine whether the variable effects of the different peptides we tested could be due to varying levels of zinc contamination within the peptides themselves, samples of a variety of peptides were submitted for ICP-MS analysis to quantify total zinc content. Fig. 8 compares peptide effects

on EC₅₋₁₀ glycine-mediated currents relative to zinc concentrations measured from those specific peptide stocks. No correlation between the two measures was seen.

4. Discussion

The glycine receptor is a putative target for the treatment of inflammatory pain and alcoholism (Blednov et al., 2015; Jonsson et al., 2014; Molander et al., 2007, 2005; Molander and Söderpalm, 2005a; Tipps et al., 2010; Xiong et al., 2012); novel specific modulators of glycine receptor function could thus have potential therapeutic applications. Of particular interest would be modulators capable of enhancing receptor activity, as several studies have shown that glycine receptor activation in the nucleus accumbens and ventral tegmental area decreases ethanol drinking in rodents (Molander and Soderpalm, 2005a; Molander et al., 2005; Li et al. 2012). Tipps et al. (2010) demonstrated the feasibility of utilizing phage display to identify novel peptide modulators of the glycine receptor. In the present report, we expand on these studies, identifying heptapeptides acting on the glycine receptor with subunit selectivity (Figs. 1 & 5), and characterizing peptide actions on receptor function. To achieve this, we incorporated a negative selection step into the phage selection process, in which the phage library was first washed over HEK 293 cells expressing glycine receptor subunits we wished to select against before panning against the glycine receptor target of interest. Peptides that bound non-specifically to the endogenous membrane components of HEK 293 cells, or to our expressed negative selection receptor subunits would, in principle, be eliminated from the population of the library upon transfer of the supernatant to the positive selection plates containing HEK 293 cells expressing the $\alpha 1\beta$ glycine receptor. Through this negative selection step peptides capable of acting selectively on $\alpha 1\beta$ compared to $\alpha 2\beta$ glycine receptors were identified. However, we did not identify peptides that differentiated between $\alpha 1\beta$ and $\alpha 3\beta$ glycine receptors (Fig. 5) despite the inclusion of the latter in the negative selection procedure of panning series D7.2. This may be due to a higher level of similarity between $\alpha 1$ - and $\alpha 3$ -containing glycine receptors at the peptide binding areas. Why then did any $\alpha 3\beta$ -binding peptides survive the panning procedure if they were negatively selected against? One reason could be that the panning is somewhat 'leaky'. If some phage capable of binding in the negative selection do not bind, but do bind in the subsequent positive selection, they would be amplified and re-introduced into the next round of panning.

For molecules with potential for therapeutic use, high potency is desired, preferably in the nM or lower concentration range. However, the glycine receptor specific peptides identified with the New England Biolabs Ph.D. libraries, both in this study (Fig. 3) and by Tipps et al. (2010), acted at low μ M concentrations. This could be due to the pentavalent display of these libraries. Multivalent display can lead to an avidity effect where phage displaying multiple copies of a particular peptide, each binding to the target, increase the apparent affinities of the multivalent phage-peptide fusions, compared to when peptides are tested alone. This phenomenon has been well documented in the phage display literature (Lowman, 1997). In order to overcome this, one can utilize a monovalent display system, such a phagemid display system, when performing panning procedures (Lowman, 1997). Alternatively, peptide potency could be increased through affinity maturation by using the sequence of the most promising peptides from initial screens, such as D7.2-122 or D7.2-123 in this case, as a

scaffold for the creation of a new phage library in which all displayed peptides would be some variation of the originally-identified sequences. We are investigating both possible methods to identify peptides that act with greater potency at the glycine receptor.

The variety of amino acid sequences seen in the 40 identified peptides (Table 1) reflects the large and diverse nature of the pentameric glycine receptor as it is likely that such a large molecular structure presents many potential binding sites for the various peptides contained within the library. Further, it should be noted that phage display can only identify peptides capable of interacting with a particular target, and does not provide information about any possible functional effect of the peptides at that target. Therefore, we narrowed our consensus search to only the eight peptides that enhanced EC₅₋₁₀ receptor currents 20% and revealed several possible amino acid consensus sequences that may contribute to their abilities to positively modulate glycine receptor function (Table 2). The most prevalent consensus was the sequence PxxS, found in four of eight peptides, where the proline residue is located at either positions 3 or 4 and the serine is located at the C-terminal positions 6 or 7. Further, this consensus is expanded within the top two enhancing peptides, D7.2-122 and D7.2-123, to TxPxxS. These two peptides also both contained TTxxxxx, which may also be responsible for their robust abilities to enhance glycine receptor function. Additionally, these two peptides share the sequence TxP with D7.2-118, which just missed the cutoff for 20% receptor enhancement (19.1% potentiation of EC₅₋₁₀ glycine response). In addition to these sequences, we found that three of eight allosteric enhancers contained the sequence TxT, and two contained xxxQxPx. Of the 17 of 25 characterized peptides that did not enhance receptor function 20%, TxT was found in three (D7.1-104, D7.1-112, and D7.2-015), QxP was found in one (D7.2-120) and PxxS was found in one other (D7.1-110). It may be that these three consensus sequences allow for interaction with the glycine receptor, and enhancing activity relies on the presence of other specific sequences within the peptide; alternatively, other amino acids within these peptides may interfere with the glycine receptor-enhancing activity that these consensus sequences contribute. Further, it is possible that these peptides could enhance receptor activity at higher concentrations, but are less potent than the eight that did.

Zinc is a biphasic modulator of the glycine receptor, capable of enhancing receptor function at concentrations below approximately 10 μ M, and inhibiting it at higher concentrations (Bloomenthal et al., 1994; Harvey et al., 1999; Laube et al., 1995). It is clear that some peptides, such as D7.2-123-B, contain sufficient contaminating zinc to affect glycine receptor function. However, ICP-MS measures total zinc, and there is no way to tell how much of this value reflects free zinc concentrations acting at the glycine receptor. Further, the relationship between the degree of receptor modulation observed and zinc concentration of the various peptides indicates that peptide effects cannot solely be due to contaminating zinc (Fig. 8). For example, peptides that range in activity between -24.19% to 46.60% change in EC₅₋₁₀ glycine responses contain between 12.38 – 15.36 nM zinc, a very small range that could not account for the degree of variance observed in the peptide effects. Further, D7.2-123-B had very similar effects at 30 and 100 μ M concentrations, despite there being over a 3-fold difference in zinc concentration (108.68 vs. 362.38 nM zinc) in those two peptide preparations. Glycine receptor potentiation by zinc typically peaks around 1 μ M zinc (Miller et al., 2005), with significantly different effects between 100 and 300 nM zinc.

Therefore, the similarity in effects between the different concentrations of D7.2-123-B must be due to some interaction between both zinc and peptide effects at the glycine receptor.

Previous studies have shown that zinc interacts with other allosteric modulators at the glycine receptor (Kirson et al., 2013; McCracken et al., 2010, 2013). Of note, the chelation of zinc via tricine decreases the effects of alcohol at $\alpha 1$ -, $\alpha 2$ -, and $\alpha 3$ -containing glycine receptors, essentially blocking the actions of ethanol at concentrations producing under 100% potentiation of EC₅₋₁₀ glycine currents (100 mM ethanol), while significantly inhibiting alcohol effects at higher concentrations (200 mM ethanol) (McCracken et al., 2010, 2013). It is possible that zinc acts as a co-factor necessary to observe appreciable modulation of receptor function by allosteric modulators at concentrations that produce little effect on their own. Nanomolar concentrations of zinc sufficient to enhance glycine receptor function are ubiquitous *in vivo* and *in vitro*, and the receptor may have evolved to respond optimally to glycine when zinc is bound. Thus it is probable that peptide selection occurs to zinc-bound glycine receptors during the panning procedure. Regardless, free or rapidly exchangeable forms of zinc are maintained in the brain at levels capable of potentiating receptor currents, indicating that zinc-dependence would not necessarily hinder the potential use of these peptides *in vivo* (Frederickson et al., 2006; Frederickson and Bush, 2001).

5. Conclusions

We have extended our initial observations demonstrating the utility of using a modified phage display procedure in the discovery of novel allosteric modulators of the glycine receptor. Expression of target channels in HEK 293 cells ensured the proper conformation of multimeric channels for phage display libraries to be panned against, yielding numerous heptapeptide sequences. The addition of a negative selection step in which libraries are first washed over cells expressing receptors similar to the target can help in obtaining peptides with subunit selectivity, but may require a more stringent selection if the targets are molecularly similar. Additionally, the zinc-dependence of peptide activity seen here (Fig. 7), taken together with previous observations of the zinc-dependence of ethanol actions on the glycine receptor, indicates a possible function of zinc as an endogenous co-modulator necessary for efficient allosteric modulation of this receptor by some modulators.

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Fig. 1. Screen for peptide activity at the $\alpha 1\beta$ glycine receptor

(A) Sample two-electrode voltage-clamp tracing of the effects of 30 μ M D7.1-115 on $\alpha 1\beta$ glycine receptors activated by EC₅₋₁₀ glycine. Peptide D7.1-115 had no effect on oocytes expressing $\alpha 1\beta$ receptors in the absence of glycine. However, D7.1-115 inhibited the effects of glycine when co-applied with EC₅₋₁₀ glycine. (B) Sample tracing of the effects of 30 μ M D7.1-123 on $\alpha 1\beta$ receptors activated by EC₅₋₁₀ glycine. Once again, D7.2-123 had no effect on oocytes expressing $\alpha 1\beta$ glycine receptors in the absence of glycine, although it enhanced receptor function when co-applied with EC₅₋₁₀ glycine. (C) The percent change in EC₅₋₁₀

glycine responses produced by 30 μ M concentrations of peptides that were pre-incubated for 30 s followed by a 45 s co-application with EC₅₋₁₀ glycine. Peptides exhibited varying degrees of activity at the $\alpha 1\beta$ glycine receptor, but none had an effect in the absence of glycine. Data are expressed as the mean $\pm$ S.E.M. across all batches of peptides tested on 3–15 oocytes isolated from at least two frogs.

Fig. 2. Batch-to-batch variation of peptide modulation of the $\alpha 1\beta$ glycine receptor

Four peptides were synthesized multiple times to acquire enough material for characterization experiments. The different batches displayed varying degrees of activity on $\alpha 1\beta$ glycine receptor when 30 μ M concentrations of peptides, calculated based on dry peptide weight, were pre-incubated for 30 s followed by a 45 s co-applications with EC₅₋₁₀ glycine. This is likely due to varying net peptide content between batches. Data are expressed as the mean $\pm$ S.E.M. of 3–7 oocytes isolated from at least two frogs.

Fig. 3. Concentration dependence of the effects of several glycine receptor enhancing peptides
 The percent change in EC_{5-10} glycine current produced by 1, 3, 10, and 30 μM concentrations of peptides pre-incubated for 30 s followed by 45 s co-applications with EC_{5-10} glycine. Data are represented as the mean $\pm$ S.E.M. of 3–5 oocytes obtained from at least 2 frogs.

Fig. 4. Peptide activity on $\alpha 1\beta$ glycine receptors activated by maximally-effective agonist concentrations

(A) Sample tracings showing the effects of 30 μ M D7.2-122-A on a saturating concentration of the high efficacy agonist glycine and (B) the partial agonist taurine. Peptide was pre-incubated 30 s followed by a 45 s co-application with saturating agonist concentrations. (C) Summary of the effects of 30 μ M D7.2-122-A on $\alpha 1\beta$ receptor currents generated by maximally effective concentrations of glycine or taurine. Peptide D7.2-122-A had no effect on currents generated by saturating glycine concentrations while enhancing currents

generated by saturating taurine concentrations. Data are represented as the mean $\pm$ S.E.M. of 3 oocytes isolated from two frogs.

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Fig. 5. Subunit specificity of select peptides

Percent change in EC_{5-10} glycine responses on $\alpha 1\beta$, $\alpha 2\beta$, and $\alpha 3\beta$ glycine receptors produced by 30 s pre-incubations of 30 μM concentrations of peptides followed by 45 s co-applications with EC_{5-10} glycine. All peptides had greater effects on $\alpha 1\beta$ and $\alpha 3\beta$, compared to $\alpha 2\beta$, glycine receptors. Activity at $\alpha 3\beta$ receptors was the same or slightly increased for all peptides relative to $\alpha 1\beta$, despite inclusion of $\alpha 3\beta$ subunits in the negative selection step of the D7.2 panning series. Data are expressed as the mean $\pm$ S.E.M. of 3–6 oocytes isolated from at least two frogs.

Fig. 6. Selection for specific amino acids during phage display panning

(A) The observed frequencies of individual amino acids expressed as the percent of all 280 amino acids within the 40 peptides identified in panning series D7.1 and D7.2. (B) The percent change in observation frequency of amino acids in the 40 identified peptides compared to the observed frequency of amino acids in the library as a whole. (C) The observed frequency of individual amino acids expressed as the percent of all 56 amino acids within the eight glycine receptor-enhancing peptides (those that produce 20% potentiation of EC₅₋₁₀ glycine mediated currents). (D) The percent change in observation frequency of amino acids in the 8 receptor enhancing peptides compared to the observed frequency of amino acids in the library as a whole. Observation frequency of amino acids in the library, as a whole, was provided by NEB.

Fig. 7. Zinc chelation by tricine inhibits the effects of peptides at $\alpha 1\beta$ glycine receptors
The percent change in EC₅₋₁₀ glycine responses produced by peptides that were pre-incubated for 30 s followed by a 45 s co-application with EC₅₋₁₀ glycine in MBS or MBS + 2.5 mM tricine. All peptides were applied at a concentration of 100 μ M except D7.2-111-B, which was applied at 30 μ M. Zinc chelation by tricine blocked or strongly inhibited the effects of all peptides tested. Data are expressed as the mean $\pm$ S.E.M. of 3–4 oocytes isolated from at least two frogs.

Fig. 8. Correlation between peptide activity on $\alpha 1\beta$ receptors and zinc contamination as determined by ICP-MS of peptide solutions

Aliquots of the same batch of peptide were used for the determination of glycine receptor modulation, via two-electrode voltage clamp, and quantitation of total zinc content in peptide stock by ICP-MS. Electrophysiological and ICP-MS data represent the values for 30 μ M peptide except where noted, when multiple concentrations were tested. The symbols for peptides D7.2-111-B and D7.2-122-B overlap.

TABLE 1

List of peptide sequences identified after panning with the Ph.D-7 library. D7.1 phage: positive selection = $\alpha 1\beta$ glycine receptor, negative selection = $\alpha 2\beta$ glycine receptor. D7.2 phage: positive selection = $\alpha 1\beta$ glycine receptor, negative selection = $\alpha 2\beta$ and $\alpha 3\beta$ glycine receptor. Peptides in bold are those that enhance $\alpha 1\beta$ glycine receptor function by 20% potentiation of EC₅₋₁₀ glycine mediated currents.

Phage	Sequence
D7.1-102	SWLAMEF
D7.1-103	GGNPTIY
D7.1-104	MNPTYTM
D7.1-105	NVAPAMS
D7.1-106	QVRSVVH
D7.1-107	DQTQTPR
D7.1-109	DSKHNFS
D7.1-110	MGPPRTS
D7.1-111	TPIANPP
D7.1-112	YTSTGPA
D7.1-113	GTLWSSM
D7.1-114	SHGRINS
D7.1-115	HVLVPLH
D7.1-117	NMIPSHG
D7.1-118	TDTASRS
D7.2-011	STIHGST
D7.2-012	SIRLDSQ
D7.2-013	HAPKSDT
D7.2-014	DRMPHYF
D7.2-015	STFTKSP
D7.2-016	TGADLNT
D7.2-017	YTMPGEL
D7.2-018	VIPHVLS
D7.2-019	GVQIMGR
D7.2-110	NANAALP
D7.2-111	SWQQGPY
D7.2-112	GSSCCKT
D7.2-113	SILPYPY
D7.2-115	KLPGWSG
D7.2-116	NQLTTLN
D7.2-118	AAPTVP

Phage	Sequence
D7.2-119	QETRAPG
D7.2-120	NQLPLHA
D7.2-121	GPMLARG
D7.2-122	TTMPIDS
D7.2-123	TTPTKSA
D7.2-124	VQTYARG
D7.2-125	TSLNRYP
D7.2-126	SHTAPLR
D7.2-128	DVPVPQV

TABLE 2

Possible consensus motifs for $\alpha 1\beta$ glycine receptor enhancing peptides (those that produce 20% potentiation of EC₅₋₁₀ glycine mediated currents).

Peptide	Sequence
Motif	PxxS
D7.1-105	NV A P A M S
D7.2-115	KL P GW S G
D7.2-122	T T M P I D S
D7.2-123	T T P T K S A
Motif	TxPxxS
D7.2-122	T T M P I D S
D7.2-123	T T P T K S A
Motif	xxx Q x P x
D7.1-107	D Q T Q T P R
D7.2-111	SW Q Q G P Y
Motif	TxT
D7.1-107	D Q T Q T P R
D7.1-118	T D T A S R S
D7.2-123	T T P T K S A
Motif	TT xxxxxx
D7.2-122	T T M P I D S
D7.2-123	T T P T K S A