The Thesis Committee for Garrett Lee Cornelison
Certifies that this is the approved version of the following thesis:

Zinc Interactions with Allosteric Modulators at the Glycine Receptor

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:  
S. John Mihic

Richard W. Aldrich
Zinc Interactions with Allosteric Modulators at the Glycine Receptor

by

Garrett Lee Cornelison, B.S.

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Abstract

Zinc Interactions with Allosteric Modulators at the Glycine Receptor

Garrett Lee Cornelison, M.A.
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Supervisor: S. John Mihic

The glycine receptor (GlyR) is a ligand-gated ion channel member of the Cys-loop receptor superfamily, responsible for inhibitory neurotransmission in the brain and spinal cord. Zinc is a potent allosteric modulator of GlyR function, enhancing GlyR activity at low nM to 10μM concentrations while inhibiting GlyR activity at higher concentrations. We investigated sources of contaminating zinc, identifying low nM levels of zinc in ultrapure H2O, powdered reagents used in the preparation of common electrophysiological buffers, and in polystyrene pipets. These low levels of zinc were capable of enhancing GlyR function. These findings suggest that without checking for this effect using a zinc-chelator such as tricine, one cannot assume that responses elicited by glycine applied alone are not necessarily also partially due to some level of allosteric modulation by zinc. Taurine-activated GlyR may have a role in the rewarding effects of drugs of abuse. Zinc is found at GlyR-potentiating concentrations throughout the nervous system, so we examined the combinatorial effects of zinc with drugs of abuse on taurine-activated GlyR to mimic in vivo conditions. Whole cell recordings revealed that zinc potentiation of saturating taurine-generated currents decreased further potentiation...
by drugs of abuse, indicating no synergistic effects on efficacy when receptors are saturated with taurine as may be seen during synaptic events in vivo. Finally, we utilized phage display to identify novel peptide modulators of the GlyR. We tested 26 peptides against α1β GlyRs, identifying peptides with various levels of activity on GlyR function. We demonstrated that these modulators were zinc-dependent, as their effects on GlyR activity were abolished in the presence of the zinc-chelating agent tricine. Together, these data indicate the importance of accounting for the effects of zinc when studying the function of the GlyR, as even low levels of zinc that can be found as contaminants in labware and buffers can affect GlyR function and responses to various allosteric modulators, including drugs of abuse.
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1.0 | Introduction

1.1 - The Glycine Receptor

Receptors of the Cys-loop superfamily constitute a major class of ligand-gated ion channels. This family of receptors includes excitatory cation-selective channels such as the nicotinic acetylcholine receptor (nAChR), and the serotonin type 3 receptor (5-HT3), as well as inhibitory anion-selective channels such as the γ-aminobutyric acid type A receptor (GABAAR) and the glycine receptor (GlyR) (Thompson et al., 2010). Cys-loop receptors share several common structural elements and are composed of five subunits, forming a pentamer around a central ion-conducting pore. Each subunit consists of a large extracellular N-terminal domain (ECD), 4 transmembrane domains (TM1-4), and a large, poorly conserved intracellular domain between TM3-4 (Lynch, 2004). The TM2 region of each subunit lines the pore while the ECD contains both the ligand binding site and the characteristic Cys-loop formed by a conserved region of 13 amino acids flanked by cysteine residues that form a disulfide bond (Lynch 2004; Thompson et al., 2010).

The GlyR is responsible for the majority of fast inhibitory neurotransmission in the brainstem and spinal cord and is also found in many higher brain regions including the hippocampus, nucleus accumbens (NAcc), and prefrontal cortex (Baer et al., 2009; Jonsson et al., 2012, 2009; Lynch, 2004). GlyR can exist either as pentameric homomers made solely of α subunits or heteromers in the form of α & β subunits arranged around a central chloride-conducting pore. To date, three α and one β subunits have been described in humans (Baer et al., 2009). The α-subunits display high levels of homology with 80-90% primary amino acid sequence identity while the β subunit displays ~47%
similarity with the α1-subunit (Lynch, 2004). GlyR α2 homomers dominate during development (Becker et al., 1988) while α1β heteromers predominate in adult spinal cord neurons (Lynch, 2004). Recent studies have identified preferential expression of α2 and β in higher brain regions such as the NAcc, frontal cortex, and hippocampus (Jonsson et al., 2012; 2009).

1.2 - The Glycine Receptor: Activation and Modulation

The GlyR, like all Cys-loop receptors, is designed to mediate fast neuronal transmission. In the absence of ligand, channels exist in a closed state with little to no spontaneous activity. Ligand binding occurs at the interface of the ECDs of adjacent subunits, allowing for 5 possible ligand-binding sites per homomeric receptor (Brejc et al., 2002). Agonist binding at a single site can result in simultaneous conformational change of all subunits to the activated state (Corringer et al., 2000), with maximal receptor activation occurring when three or more glycine molecules are bound (Lewis et al., 2003; Beato et al., 2004). Amino acids such as taurine and β-alanine are also capable of activating GlyR, albeit with lower efficacies than glycine, lending to their classification as partial agonists of the GlyR (Lynch et al., 1997). However, studies of taurine levels in some brain regions have led to speculation that it may act as the primary ligand of GlyR in these areas (Mori et al., 2002; Ericson et al., 2006).

GlyR activity is modulated by a wide variety of compounds including alcohols, inhaled drugs of abuse, anesthetics, neurosteroids, divalent cations such as Zinc (Zn), tropeins, and others (Beckstead et al., 2000; Cheng and Kendig, 2002; Downie et al., 1996; Harvey et al., 1999; Laube et al., 1995; Molander et al., 2007, 2005; Yamashita et
al., 2001; Yevenes and Zeilhofer, 2011). These compounds are capable of modulating ligand-activated GlyR currents, but have no effect when applied to receptors in the absence of ligand. Most of these compounds, particularly the drugs of abuse, enhance GlyR function, resulting in a left-shift of glycine concentration response curves (Yevenes and Zeilhofer, 2011). Allosteric modulators exert their greatest enhancing effects on low concentrations of glycine that are unlikely to be seen at GlyR in vivo except at the tail-end of synaptic events, or perhaps at extrasynaptic receptors (Scimemi and Beato, 2009). Indeed these compounds have negligible effects when tested with saturating concentrations of glycine (Kirson et al., 2012; McCracken et al., 2010). The large number of drugs of abuse capable of modulating GlyR have led to the emergence of the GlyR as a possible pharmacological target for the treatment of substance abuse, particularly alcoholism (Tipps et al., 2010; Molander et al., 2007; 2005).

1.3 - Zinc Modulation of the Glycine Receptor

Trace metals play various vital roles in human physiology and pathology. Zinc (Zn) is one of the most prevalent nutritionally-essential elements in the human body and is the most abundant trace metal in the brain (Watt et al., 2013; Tapiero et al., 2003). Zn is essential to the structure and function of a large number of macromolecules and is essential for over 300 enzymatic reactions (Tapiero et al., 2003). Long known as a potent modulator of GlyR functioning, Zn is interesting in that it serves as a bi-phasic modulator of GlyR, enhancing GlyR activity at concentrations in the low nM to 10 µM range and inhibiting GlyR activity at higher concentrations (Bloomenthal et al., 1994; Laube et al., 1995; Harvey et al., 1999).
Zn’s effects at the GlyR have been extensively studied due to its possible physiological relevance. Zn is ubiquitous in the brain at low nM concentrations capable of enhancing GlyR functioning (Frederickson et al., 2006b). However, synaptic release of Zn has also been observed, particularly at glutamatergic mossy fiber synapses in the hippocampus (Vogt et al., 2000; Qian and Noebels, 2005; Frederickson et al., 2006a). While early studies indicated synaptically-released Zn may reach concentrations that are inhibitory to GlyR (Lynch, 2004), more recent studies have indicated that this level does not exceed 10 μM (Frederickson et al., 2006a).

Recent studies have shed light into the interactions of Zn with various drugs of abuse at the GlyR (McCracken et al., 2010; Kirson et al., 2013). McCracken et al. (2010) demonstrated that low nM concentrations of Zn could enhance the effects of ethanol on α1 GlyR activated by sub-maximal concentrations of glycine. Further, it was demonstrated that contaminating levels of Zn in buffer solutions used during electrophysiological recordings contain sufficient free Zn to affect GlyR functioning. This was evident as treatment of buffers with the Zn-chelating agent tricine decreased glycine-activated GlyR currents as well as ethanol enhancement at the GlyR. Kirson et al. (2013) demonstrated that physiological concentrations of Zn actual decrease the effects of drugs of abuse on GlyR activated by saturating concentrations of taurine while having no effect on GlyR activated by saturating concentrations of glycine. This study will be discussed in detail in chapter 3 of this thesis.

Zn is an extremely ubiquitous molecule, both as a contaminant in experimental settings and physiologically in the brain. It is therefore important to determine the effects of physiological and contaminating levels of Zn on the GlyR. This will assist in the
interpretation of previous studies that may not have accounted for the effects of contaminating Zn and can help in the design of future studies of the GlyR. Further, it is important to obtain a greater understanding of the physiology of the GlyR in regards to the effects of zinc and drugs of abuse, as the GlyR is potential therapeutic target for the treatment of drug abuse. The rest of this thesis will focus on a series of studies illustrating various sources of Zn contamination in common labware, how this contamination can effect studies of the GlyR, the effects of Zn on GlyRs activated by saturating concentrations of the full agonist glycine versus the partial agonist taurine, and the effects of Zn on the modulation of GlyR currents by novel peptide modulators recently discovered via phage display.
2.0| Materials and Methods

2.1 - Reagents

Except NaOH [Cat. No. SS255] obtained from Fisher (Pittsburgh, PA), Ph.D.TM 7 phage display reagents from New England Biolabs (Ipswitch, Massachusetts), and peptides obtained from Peptide2.0 (Chantilly, Virginia), all other chemicals were purchased from Sigma-Aldrich (St Louis, MO) including NaCl [Cat. No. S9625], CaCl₂ [C7902], KCl [P5405], MgSO₄ • 7H₂O [2030391], Ca(NO₃)₂ • 4H₂O [C1396], HEPES [H3375], NaHCO₃ [S3817], tricine [T5816] and glycine [G7126]. Distilled water was purified further using a Barnstead E-Pure Ultrapure D4641 Water Purification System.

2.2 - Oocyte isolation and cDNA injection

Xenopus laevis were obtained from Nasco (Fort Atkinson, WI) and housed at room temperature on a 12-hour light/dark cycle. Oocytes were obtained via surgery, performed in accordance with AAALAC regulations, and placed in isolation media containing 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 followed by removal of the follicular layer using a 10 minute incubation in 0.5 mg/mL Sigma type 1A collagenase in buffer containing 83 mM NaCl, 2 mM MgCl₂, and 5 mM HEPES. Oocytes were injected through their animal poles with 30 nL of α₁, α₂, α₁β, or α₂β glycine receptor subunit cDNA (at a 1:20 α₁:β ratio) in a modified pBK-cytomegalovirus vector (Mihic et al., 1997), using a micropipette (10-15 μm tip size) attached to an electronically-activated microdispenser. Oocytes were stored in the dark at
room temperature for 24 hours followed by subsequent storage in the dark at 19°C for up to 5 days post-injection in 96-well plates containing modified Barth’s saline (MBS) [88 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 at 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.

### 2.3 - Two-electrode voltage-clamp electrophysiology

Oocytes expressed GlyRs within 48h and all electrophysiological recordings were made within 5 days of 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 3M KCl. Cells were voltage-clamped at –70mV using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT) and perfused with MBS at a rate of 2mL/min. using a Masterflex USA peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) through 18-gauge polyethylene tubing. All glycine solutions were prepared in MBS or MBS + 2.0 or 2.5 mM tricine. When maximally-effective concentrations of glycine were applied, applications lasted for 15s and were followed by 10 minute washouts with MBS to allow for complete receptor resensitization. For experiments using submaximal concentrations of agonist, concentrations that yielded 5 percent of the maximally-effective glycine response (EC₅) or 5 to 10 percent of the maximally-effective glycine response (EC₅₋₁₀) were applied for 45 s followed by 3-5 minute washouts with MBS to allow for complete receptor resensitization. All modulators and chelators used were applied for 30 s alone prior to co-application with agonist. Data were acquired at a
rate of 1kHz using a Powerlab 4/30 digitizer using LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia).

2.4 - Cadmium and Zn Concentration Determination

Zn and cadmium concentrations were determined in MBS, distilled water, and peptides 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. Solutions were diluted as necessary in 2% HNO₃ before analysis.

2.5 - Phage Display and Peptide Preparation

Two separate phage display screens were performed, denoted as the D7.1 and the D7.2 series. Phage display was performed using the Ph.D™-7 phage display kit from New England Biolabs (Ipswitch, Massachusetts) according to the manufacturer’s protocol. Five consecutive rounds of panning were performed. Each round consisted of washing the Ph.D™-7 library over negative selection HEK cells expressing either α2β GlyR (D7.1-1 series) or α2β and α3β GlyR (D7.2 series). Following negative selection, phage that did not bind the negative selection cells were collected and washed over positive selection HEK cells expressing the α1β GlyR. Phage that did not bind to the positive selection cells were discarded and the remaining phage were amplified and subjected to further rounds of panning. Phage remaining after 5 rounds of panning were sequenced to identify the peptides expressed on their coats.

Peptides were obtained as HCl salts from Peptide 2.0 Inc. (Chantilly, Virginia). Lyophilized peptides were stored at -20°C until use. Just prior to use, lyophilized
peptides were suspended in ultrapure H$_2$O at a concentration of 10mM and split into single use aliquots that were stored at -20°C until the time of each experiment, when they were brought to a 1mM working-stock with 1X MBS. Peptides were applied to oocytes at concentrations ranging from 30µM to 100µM for 30s alone prior to co-application with agonist. Aliquot peptides were stored for no longer than 2-4 weeks prior to use in experimentation.

**2.6 - Data Analysis**

Peak currents were measured and used in data analysis. Currents generated under the various experimental conditions were normalized against currents generated by the indicated control applications and expressed as mean ± S.E.M. of the percent of control generated current (sections 3.2.1 and 3.2.2) or percent change from control generated current (section 3.2.3). Statistically significant differences among experimental conditions were determined using one-way, two-way or three-way ANOVAs and post hoc tests, as indicated. SigmaPlot version 11.0 (Systat Software, San Jose, CA) was used for statistical testing.
3.0 | Contaminating levels of Zn found in commonly-used labware and buffers affect glycine receptor currents\textsuperscript{1}

3.1 - Introduction

Recent studies have shown that Zn is a contaminant present at low nM concentrations in physiological solutions and in commonly-used labware (Kay, 2004; McCracken et al., 2010). Due to an increasing concern that some of the results of our studies may have been due to an effect of glycine in conjunction with Zn, rather than glycine itself, we utilized an electrophysiological approach to examine the effects of background contaminating Zn levels and to distinguish among several possible sources of Zn contamination in reagents and labware used in the preparation of solutions for electrophysiological recordings.

\textsuperscript{1}Portions of this chapter have previously been published in \textit{Brain Research Bulletin}. Cornelison G.L., Mihic S.J. 2014. Contaminating levels of zinc found in commonly-used labware and buffers affect glycine receptor function. \textit{Brain Res Bull} 100, 1-5.Copyright © 2014 Elsevier Ltd. All rights reserved. Experiments were planned by Cornelison and Mihic; Experiments were performed by Cornelison; Paper was written by Cornelison and Mihic.
3.2 - Results

3.2.1 - Type of vial containing glycine does not affect degree of contaminating Zn-mediated GlyR enhancement

To determine if various vials commonly used for the preparation of agonist solutions contain different amounts of contaminating Zn sufficient to affect GlyR currents, glycine solutions were prepared in three different vials in the presence of the Zn-chelating agent tricine or without: Fisher glass screw-thread vials (Cat. No. 03-339-22J), National Scientific silanized glass vials (Cat. No. B7999-S3), or BD Falcon™ polypropylene tubes (Cat. No. 352096). Low concentrations of Zn (below 10 μM) left-shift glycine concentration-response curves of α1-containing GlyR, with the greatest enhancing effects of Zn seen at low concentrations of glycine (Laube. et al. 1995). We therefore tested, in different types of vials, concentrations of glycine that elicited 5 percent of maximally-effective glycine responses (EC₅).

EC₅ concentrations of glycine in buffer containing 2.5 mM tricine were determined in Fisher glass vials and used as controls against which all other experimental conditions were normalized (Figs. 3.1 A,B). Fig. 3.1A shows a sample tracing of successive 45 s applications of EC₅ glycine from solutions made up in glass, silanized glass, or polypropylene vials, with or without 2.5 mM tricine. Control applications of EC₅ glycine + 2.5 mM tricine in glass were interspersed throughout to account for any drift in EC₅ glycine-mediated responses. For all vials, GlyR currents mediated by EC₅ glycine in MBS without tricine were consistently higher than currents generated by EC₅
glycine in MBS + 2.5 mM tricine \([F(4,19) = 21.77, p < 0.001]\) (Fig. 3.1B). However, Student-Newman-Keuls posthoc tests revealed no significant differences in currents generated by EC$_5$ glycine in MBS among the different vials used, in either the absence or presence of tricine (Fig. 3.1B). As previously shown by McCracken et al. (2010), chelation of Zn by tricine does not affect currents mediated by saturating concentrations of glycine (Fig. 3.1C). Further, vial choice does not significantly affect currents mediated by saturating (10 mM) concentrations of glycine \([F(4, 8) = 1.77, p > 0.19]\) (Fig. 3.1C).
Figure 3.1. Contaminating Zn-mediated enhancement of GlyR function is not affected by the type of vial in which glycine solutions are prepared. A) Sample tracing showing submaximal α1β GlyR currents elicited by glycine solutions prepared in glass, silanized glass, and polypropylene vials in the absence or presence of 2.5 mM tricine (tri). B) Summary data of experiments depicted in panel A. EC5 glycine was determined in the presence of 2.5 mM tricine in glass vials and its effect was used as the control against which all other conditions were normalized in each oocyte. Control applications of EC5 glycine + tricine solutions in glass vials were tested throughout each experiment to account for possible drift in GlyR responses over time. For all vials, EC5 glycine-mediated currents were consistently higher for solutions prepared in the absence of 2.5 mM tricine but, among vial types, there were no significant differences in currents generated by EC5 glycine solutions prepared in either the presence or absence of 2.5 mM tricine. C) Summary data showing that choice of vial type does not affect GlyR currents mediated by a saturating (10 mM) concentration of glycine. Data are presented as the mean ± S.E.M. of 3 - 5 oocytes.
3.2.2 - Washing agonist-solution vials does not affect the degree of contaminating Zn-mediated GlyR enhancement

We next wished to determine whether Zn was present on the surfaces of vials and whether washing them prior to the preparation of EC₅ glycine solutions would decrease contaminating Zn-mediated GlyR enhancement. Glass and polypropylene vials were either: (1) not washed, (2) washed 5 times with de-ionized H₂O (diH₂O), (3) washed fifty times with diH₂O, or (4) soaked in MBS + 2.5 mM tricine for 10 minutes before immediate drying and preparation of EC₅ glycine. GlyR responses elicited by EC₅ glycine prepared in these were compared with EC₅ glycine solutions prepared in corresponding unwashed vials with MBS + 2.5 mM tricine. The various washing procedures did not affect the degree of contaminating Zn-mediated GlyR enhancement for polypropylene vials [F(3,19) = 0.03, p > 0.99] or glass [F(3,11) = 0.02, p > 0.99] (Figs. 3.2A,B).
Figure 3.2. Washing vials does not affect the degree of contaminating Zn-mediated GlyR enhancement. EC₅ glycine was determined in the presence of 2.5 mM tricine in polypropylene or glass vials and used for solutions prepared in the absence of tricine in vials that were either not washed, washed five times with diH₂O, washed 50 times with diH₂O, or submitted to a 10 min. soak in MBS + 2.5 mM tricine prior to drying and use. Data are reported as percent current of that elicited by EC₅ glycine in the presence 2.5 mM tricine. A) Summary data for currents elicited by EC₅ glycine solutions prepared in polypropylene tubes under the various wash conditions. B) Summary data for currents elicited by EC₅ glycine solutions prepared in glass vials under the various wash conditions. Data are presented as the mean ± S.E.M. of 3 - 5 oocytes.
3.2.3 - Polystyrene but not glass serological pipets contain contaminating Zn that significantly affects EC₅₀ glycine-mediated GlyR currents

During the course of conducting these studies we observed that the first EC₅₀ glycine solution made using a polystyrene serological pipet to transfer the MBS buffer to vials seemed to produce larger GlyR currents than subsequent EC₅₀ glycine solutions made using the same pipet to transfer the MBS. Further, it appeared that this phenomenon did not occur when glass pipets were used for the transfer of MBS. To quantify this, experiments were conducted in which three successive EC₅₀ glycine solutions were prepared using the same serological pipet to transfer MBS to the working-solution vial (either Fisher glass [Cat No. 13-678-31J], Fisher polystyrene (Cat. No. 13-678-12E], or VWR polystyrene [Cat. No. 89130-898]). GlyR currents generated by solutions prepared with the second and third use of each pipet were compared to currents generated by solutions made with the first use of a given pipet. Subsequently, an amount of tricine equal to a final concentration of 2 mM was added to each of those vials and the experiment was repeated to determine if Zn contamination was responsible for the observed effects. All data shown in Fig. 3.3 obtained with the second and third uses of pipets were normalized against the responses observed during the first use of the pipet, in either the absence or presence of tricine, as appropriate. Tricine always decreased absolute currents as expected. Fig. 3.3 shows the results for glycine solutions prepared using glass as well as two different polystyrene serological pipets. A three-way ANOVA illustrates a significant effect of the type of pipet used [F(2,59) = 48.85, p < 0.001] and an effect of tricine [F(1,59) = 128.53, p < 0.001] but no difference between the second and third repeated uses of each pipet [F(1,59) = 0.48, p > 0.49].
Figure 3.3 Polystyrene, but not glass serological pipets contain Zn contamination sufficient to affect submaximal GlyR currents. EC₅ glycine solutions were prepared in glass vials with either Fisher glass, Fisher polystyrene, or VWR polystyrene serological pipets. Each pipet was used to prepare 3 identical EC₅ glycine solutions. The first application was the control and the results of the subsequent two applications were normalized against the first and presented as percent change relative to the first. After application of all three solutions, a final concentration of 2mM tricine was added to each vial and the solutions are re-applied. The vertical dashed line separates responses observed in the absence (left of line) and presence (right of line) of tricine. A) Summary data for currents elicited by EC₅ glycine solutions prepared with Fisher glass pipets. B) Summary data for currents elicited by EC₅ glycine solutions prepared with Fisher polystyrene pipets. C) Summary data for currents elicited by EC₅ glycine solutions prepared with VWR polystyrene pipets. Data are presented as the mean ± S.E.M. of 4 - 6 oocytes.
3.2.4 - ICP-MS determination of Zn and cadmium concentrations.

Distilled water and MBS samples were submitted for determination of Zn and cadmium levels via ICP-MS. Cadmium was below the detection limit in all water samples tested but was detected at a concentration of 7.5 ± 0.1 nM (n=3) in MBS. The Zn concentration in distilled water was 8.0 ± 1.7 nM (n=4), increasing to 44.4 ± 2.2 nM (n=13) in MBS. Water initially dispensed from a Fisher polystyrene pipet had a Zn concentration of 129.7 ± 15.2 nM (n=4), decreasing to 28.9 ± 5.4 nM (n=4) the second time it was used, indicative of Zn washout from the pipet.

3.3 - Discussion

Due to its enhancing effects on glycine receptor function in the low nM concentration range, Zn may complicate experimental measurements that are presumed to be due solely to an effect of exogenously-applied glycine. Our previous studies (Kirson et al., 2013; McCracken et al., 2010) showed that tricine decreases the magnitudes of currents elicited by low but not maximally-effective concentrations of glycine, as well as all concentrations of the partial agonist taurine. The most parsimonious explanation of these findings is that, by chelating Zn, tricine allows one to measure the true effects of agonists in isolation on the GlyR. This has implications for previously published studies in which it was tacitly assumed that background contaminating levels of Zn were insufficient to affect experimental results. This appears not to be the case and in this study we attempted to determine the sources of Zn in our electrophysiological assays.

The choice of type of vial in which experimental solutions are made before application to oocytes does not appear to play a major role in Zn contamination (Fig. 3.1),
since currents elicited by solutions made up in all three vials were similar to one another. In all three cases, addition of tricine decreased currents elicited by low but not high concentrations of glycine, most likely via chelation of this allosteric modulator of the GlyR. These data suggest that either all three types of vials have similar contaminating levels of Zn on their surfaces or, more likely, that the Zn contamination is coming from either the deionized water or buffer constituents. We ruled out the former possibility by washing vials repeatedly, or even soaking them for 10 min. in the presence of tricine, before using them in experiments and still saw greater effects of glycine, compared to those seen when tricine was added to vials just before use (Fig. 3.2). However, even though there appears to be no surface contamination of vials with Zn, this is not necessarily the case with pipets used to make solutions. Both Fisher and VWR polystyrene pipets appear to be contaminated with Zn since the first use of each pipet led to solutions that elicited markedly higher currents than subsequent uses of each pipet (Fig. 3.3); the presence of Zn in the Fisher polystyrene pipets was confirmed by ICP-MS measurements. This was not true of the Fisher glass pipets tested. As in previous studies, the presence of tricine prevented contaminants from having modulatory effects. This last study also argues against the remote, but admittedly not disproved, possibility that all of these findings are simply due to an allosteric effect that tricine itself has on the GlyR; i.e., that tricine acts as an inverse allosteric modulator. This seems unlikely since we found that another Zn chelator, N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN), also produces the same decreases in glycine-mediated currents as tricine, and that tricine has no significant effects if applied with TPEN (data not shown).
In addition, tricine does not act as an allosteric modulator at GABA<sub>A</sub> receptors (Wilkins and Smart, 2002).

Kay (2004) used the Zn-sensitive fluorimetric probe FluoZin-3 with a detection threshold of 50 pM to measure Zn contamination in commonly used labware. He found it in a wide range of materials, ranging from several plastic labware sources such as transfer pipettes, Tygon tubing, stainless steel, and glass. Even transient contact with contaminated labware could lead to concentrations in the ~100 nM concentration range, at which half maximal enhancement of GlyR function can be observed using an EC<sub>50</sub> concentration of glycine (Miller et al., 2005). In our studies, Zn contamination appeared to come from a variety of sources including the distilled water, solutes used to make the MBS buffer, and the polystyrene pipets.

Cadmium (Cd) is a transition metal falling just below Zn on the element periodic table and could conceivably be thought responsible for some of the effects we observed. Kay (2004) reported that FluoZin-3 cannot distinguish between Cd and Zn. We found cadmium in the MBS at a concentration of about 7 nM. It is unlikely that Cd is the responsible agent in our studies since cadmium inhibits rather than enhances GlyR-mediated currents in dissociated rat hippocampal neurons and has low potency, with an IC<sub>50</sub> of 1.27 mM (Wang et al., 2006). It was also found to act at a site distinct from that mediating Zn’s actions.

In conclusion, our results suggest that Zn is found as a contaminant in laboratory buffers and some labware in concentrations sufficient to enhance glycine receptor function. Without checking for this effect using a chelator such as tricine, one cannot assume that responses elicited by the application of glycine alone are not necessarily also
partially due to some level of allosteric modulation by Zn. This issue may be especially
important to those attempting to glean detailed mechanistic information of glycine
receptor function, such as in single channel electrophysiology studies.
4.0| Physiological concentrations of Zn reduce taurine-activated GlyR responses to drugs of abuse²

4.1- Intro

The sulfonic acid taurine acts as a partial agonist at the GlyR, possessing approximately 50% of the efficacy of glycine (Lape et al., 2008). Taurine is the second most abundant amino acid in the brain, and has been implicated as an endogenous ligand of the GlyR in multiple brain regions (Albrecht and Schousboe, 2005; Dahchour et al., 1996; Ericson et al., 2006; Mori et al., 2002; Rodríguez-Navarro et al., 2009). Although GlyR are found synaptically in a variety of brain stem nuclei (Ferragamo et al., 1998; Lim et al., 2000) and in cerebellum (Dieudonne et al., 1995) they are also found extrasynaptically, where taurine and β-alanine may be acting as the endogenous agonists (Mori et al., 2002). Taurine may reach concentrations as high as 20 mM in astrocytes, from which it is released by osmoregulatory mechanisms (Albrecht and Schousboe, 2005). Extracellular taurine concentrations measured by microdialysis range from 1-100 μM but, by their nature, likely underestimate concentrations found locally around astrocytes.

²Portions of this chapter have previously been published in Neuropharmacology. Kirson D., Cornelison G.L., Philpo A.E., Todorovic J., Mihic S.J. 2013. Physiological concentrations of zinc reduce taurine-activated GlyR responses to drugs of abuse. Neuropharmacol 75:286–294. Copyright © 2013 Elsevier Ltd. All rights reserved. Experiments were planned by Mihic and Kirson; Experiments were performed by Kirson, Cornelison, Philpo, and Todorovic; Paper was written by Kirson, Cornelison, and Mihic.
Taurine plays a role in the effects of ethanol in the NAcc (Ericson et al., 2011). Although allosteric modulators have no effects when tested using saturating concentrations of full agonists at the GlyR, this is not true when saturating concentrations of partial agonists are tested (Albrecht and Schousboe, 2005; Kirson et al., 2012; Scimemi and Beato, 2009). In this chapter we examined the possible interactions of these allosteric modulators with the ubiquitous GlyR modulator, Zn, on glycine- and taurine-activated GlyR. Zn modulation of the glycine-activated GlyR has been extensively studied but the interactions between Zn and the taurine-activated GlyR have not been characterized to the same extent, especially in conjunction with ethanol or other modulators. Previous studies of Zn modulation of taurine-activated GlyR responses focused on adding Zn at both enhancing and inhibiting concentrations, without controlling for the background Zn likely to be found at biologically-relevant concentrations (Laube et al., 2000). In this set of studies we further characterize Zn modulation, as well as compare Zn’s interactions with other allosteric modulators on the glycine- vs. taurine-activated GlyR.

4.2 - Results

4.2.1 - Chelation of endogenous Zn decreases responses to taurine

We previously showed that various allosteric modulators of GlyR, such as ethanol and some anesthetics, have different effects on currents generated by saturating concentrations of glycine versus taurine (Kirson et al., 2012). In order to compare Zn modulation of GlyR currents generated by maximally-effective concentrations of glycine and taurine, we compared currents generated by co-applications of agonist and either 100
nM Zn or 2.5 mM of the Zn chelator tricine with currents generated by agonist alone. Fig. 4.1A shows a sample tracing of successive 15 s applications of 10 mM glycine in the presence of a background concentration of Zn, 2.5 mM tricine, or 100 nM Zn. Fig. 4.1B shows the same experimental protocol as in 4.1A but with 100 mM taurine instead employed as the agonist. Addition of 100 nM Zn enhanced saturating taurine currents almost 60% (Fig. 4.1C) while leaving saturating glycine-mediated currents unchanged. Elimination of background levels of Zn in the perfusion buffer by co-application of tricine decreased saturating taurine currents while having no effects on saturating glycine currents. A two-way ANOVA showed a significant interaction effect between the concentration of Zn in the buffer and the agonist tested \([F(1,37) = 34.4, p < 0.001]\). A Student-Newman-Keuls (SNK) multiple comparison post-hoc test showed significant differences between glycine and taurine both in the presence of 2.5 mM tricine \([q = 4.6, p < 0.01]\) and 100 nM Zn \([q = 7.1, p < 0.001]\), as well as a significant effect of Zn concentration when taurine was the agonist \([q = 12.3, p < 0.001]\).
Figure 4.1. Zn affects currents elicited by maximally-effective concentrations of taurine but not glycine. A) Sample tracings showing the effect of a maximally-effective concentration of glycine applied in the presence or absence of Zn. The tracing shows 15 s co-applications of 10 mM glycine with either 2.5 mM tricine or 100 nM Zn following a 30 s preincubation with tricine or Zn. Each of these applications was preceded and followed by applications of 10 mM glycine alone in buffer containing background levels of Zn. Horizontal bars over tracings indicate time of exposure to glycine, tricine, or Zn. Washouts 10 min in duration separated agonist applications. B) Sample tracing showing the effect of a maximally-effective concentration of taurine applied in the presence or absence of Zn. The tracing follows the same protocol as in panel A. Horizontal bars over tracing indicate time of exposure to taurine, tricine, or Zn. C) Summary of the effects of Zn on brief applications of maximally-effective concentrations of glycine or taurine. The y-axis represents the percent current potentiation observed in the presence or absence of Zn compared with that produced by glycine or taurine in background levels of Zn. Data are shown as mean ± S.E.M. of 9-11 oocytes. *, p < 0.05.
We next looked at the effects of Zn modulation using saturating agonist concentrations that were applied continuously for 10 min, followed by 2 min co-applications of agonist and either 2.5 mM tricine or 100 nM Zn. This continuous agonist application approach allows for channels to equilibrate between the open and desensitized states (Figs. 4.2A,B). Under these conditions all receptors have bound agonist and are either activated (opening) or desensitized. In this experimental paradigm the effects of modulators can thus only be due to their effects on channel opening/closing kinetics (Popen) or desensitization/resensitization rates and not on possible effects on agonist affinity. As seen in Fig. 4.2C, the effects exhibited by Zn trended in the same directions as those seen in Fig. 4.1C, but to a smaller degree. Co-application of 100 nM Zn produced greater potentiation of saturating taurine currents compared to saturating glycine currents. Co-application of tricine showed a decrease in saturating taurine responses compared to a negligible increase in saturating glycine. Similar to the short application experiments, a two-way ANOVA showed a significant interaction effect between the concentration of Zn in the buffer and the agonist tested [F(1,16) = 34.3, p < 0.001]. A SNK multiple comparison post-hoc test revealed significant differences between glycine and taurine both in the presence of 2.5 mM tricine [q = 7.1, p < 0.001] and 100 nM Zn [q = 4.6, p < 0.01], as well as a significant effect of Zn concentration when taurine was the agonist [q = 11.6, p < 0.001].
Figure 4.2. Zn affects currents elicited by long exposures to maximally-effective concentrations of taurine but not glycine.  A) Sample tracing showing the effects of 2.5 mM tricine or 100 nM Zn co-applied with saturating concentrations of glycine after 10 min of continuous glycine application. Two min applications of 10 mM glycine in either 2.5 mM tricine or 100 nM Zn were preceded and followed by 10 mM glycine in buffer containing background levels of Zn. Horizontal bars over the tracing indicate time of exposure to glycine, tricine, or Zn.  B) Sample tracing showing the effects of 2.5 mM tricine or 100 nm Zn co-applied with saturating concentrations of taurine, after 10 min of continuous taurine application. The tracing follows the same protocol as in panel A. Horizontal bars over the tracing indicate time of exposure to taurine, tricine, or Zn.  C) Summary of the effects of maximally-effective concentrations of glycine or taurine in the presence or absence of Zn during continuous agonist exposures. The y-axis represents the percent current enhancement observed in the presence or absence of Zn compared with the glycine or taurine current level immediately preceding tricine or Zn co-application. Data are shown as mean ± S.E.M. of 4-6 oocytes. *, p < 0.05.
4.2.2 - Zn and ethanol interactions

Physiologically-relevant low nM concentrations of Zn enhance ethanol modulation of GlyR currents generated by submaximal but not maximally-effective concentrations of glycine (McCracken et al., 2010). As these two modulators are likely to be present concurrently at GlyR in vivo, we compared the effects of enhancing concentrations of Zn on ethanol modulation of GlyR activated by maximally-effective glycine or taurine concentrations. We first looked at the effects of 15 s co-applications of maximally-effective concentrations of agonist and 200 mM ethanol in the presence of background levels of Zn and also in the presence of 2.5 mM tricine or 100 nM Zn. Fig. 4.3A shows that ethanol modulation of the maximally-effective taurine response is present in all three different Zn concentrations. However, 100 nM Zn significantly decreased [Two-way Repeated Measures (RM) ANOVA with SNK multiple comparison procedure; q = 5.0, p < 0.01; q = 4.6, p < 0.01, respectively] the degree of ethanol percent potentiation of a saturating taurine concentration compared to the potentiation seen in either the background Zn level or after Zn chelation. In background levels of Zn, or in the presence of 2.5 mM tricine, or 100 nM Zn, 200 mM ethanol produced significantly greater enhancement of responses in saturating taurine than glycine [q = 12.9, p < 0.001; q = 11.6, p < 0.001; q = 8.3, p < 0.001, respectively], as there was negligible inhibition of glycine currents observed instead (Fig. 4.3B). The same trends were observed using 50 mM ethanol, just with a lower degree of ethanol potentiation (Fig. 4.3C) [q = 3.85, p < 0.05, comparing taurine alone with taurine plus Zn].
Figure 4.3. Zn affects ethanol potentiation of currents elicited by maximally-effective concentrations of taurine but not glycine. A) Sample tracings showing the effect of Zn on brief applications of maximally-effective concentrations of taurine. Taurine (100 mM) was co-applied with 200 mM ethanol for 15 s following a 30 s preincubation with 200 mM ethanol. Ethanol applications were flanked by 15 s applications of maximally-effective taurine applied alone. Series of applications were carried out in buffer containing background levels of Zn, 2.5 mM tricine or 100 nM Zn. Horizontal bars over tracings indicate time of exposure to taurine, tricine, Zn or ethanol. B) Summary of the effects of 200 mM ethanol on brief applications of maximally-effective concentrations of glycine or taurine in the presence of a background level of Zn, the absence of Zn produced by tricine, or the addition of 100 nM Zn. The y-axis represents the percent current potentiation observed with ethanol co-application, in background Zn, 2.5 mM tricine or 100 nM Zn. Data are shown as mean ± S.E.M. of 6 oocytes. C) Summary of the effects of 50 mM ethanol on brief applications of maximally-effective concentrations of taurine in the presence of a background level of Zn, the absence of Zn produced by tricine, or the addition of 100 nM Zn. The y-axis represents the percent current potentiation observed with ethanol co-application, in background Zn, 2.5 mM tricine or 100 nM Zn. Data are shown as mean ± S.E.M. of 8 oocytes. *, p < 0.05.
We next looked at the combined effects of Zn and ethanol on receptors comprised of different subunits. The heteromeric α1β GlyR is the predominant adult form found in brainstem and spinal cord of mammals and is likely to be found synaptically, as the β subunit is involved in anchoring the GlyR via its interactions with gephyrin (Kirsch and Betz, 1995). As shown in Fig. 4.4A, the effects of combined ethanol and Zn on the heteromeric channel exhibit the same trends as those seen on the homomeric channel but with slightly increased enhancement of taurine currents for the heteromeric channel. A Two-way RM ANOVA with SNK multiple comparison procedure showed ethanol potentiation of currents in 100 nM Zn significantly decreased from both background Zn [q = 3.8, p < 0.05] and 2.5 mM tricine [q = 3.1, p < 0.05], as well as significantly greater ethanol percent potentiation of taurine vs glycine currents [q = 7.5, p = 0.001]. We also performed equivalent experiments on α2 homomeric (Fig. 4.4B) and α2β (Fig. 4.4C) heteromeric receptors. Two-way RM ANOVAs with SNK multiple comparison procedures showed significantly greater ethanol percent potentiation of taurine vs glycine currents [α2, q = 7.14, p < 0.01; α2β, q = 4.6, p < 0.05].
Figure 4.4. Zn/ethanol interactions on GlyR composed of a variety of different subunits. Summaries of the effects of ethanol on brief applications of maximally-effective concentrations of glycine or taurine in the presence of a background level of Zn, the absence of Zn produced by tricine, or the addition of 100 nM Zn on the α1β GlyR (A) α2 GlyR (B) or α2β GlyR (C). The y-axes represent the percent current potentiation observed with ethanol co-application, in background Zn, 2.5 mM tricine or 100 nM Zn. Data are shown as mean ± S.E.M. of 5-8 oocytes. *, p < 0.05.
4.2.3 - Zn and isoflurane interactions

Because biologically-relevant concentrations of Zn affect ethanol potentiation of saturating taurine currents, we investigated other allosteric modulators of the GlyR for Zn-modulator interactions. The inhaled volatile anesthetic isoflurane was tested using the same experimental protocols as those used for ethanol. Fig. 4.5A shows the results of 15s co-applications of 0.55 mM isoflurane with either 10 mM glycine or 100 mM taurine in the presence of background levels of Zn, 2.5 mM tricine or 100 nM Zn. As for ethanol, isoflurane potentiation of saturating taurine currents in either background Zn or 2.5 mM tricine were very similar, while the addition of 100 nM Zn significantly decreased the isoflurane percent potentiation from those levels [Two-way RM ANOVA with SNK multiple comparison procedure; q = 4.1, p < 0.02; q = 4.4, p < 0.05, respectively]. Saturating glycine-mediated currents were minimally affected by isoflurane whether in background levels of Zn, 2.5 mM tricine, or 100 nM Zn. Isoflurane enhancement was significantly different between glycine and taurine in the presence of a background level of Zn [q = 7.4, p < 0.001] and in 2.5 mM tricine [q = 7.8, p < 0.001].
Figure 4.5. Zn decreases isoflurane and toluene potentiation of currents elicited by maximally-effective concentrations of taurine but not glycine. These experiments were carried out in the same manner as the ethanol experiments described in Fig. 3. A) Effect of isoflurane on currents elicited by brief applications of maximally-effective glycine (10 mM) or taurine (100 mM) concentrations in the presence or absence of Zn. Agonist was co-applied with 0.55 mM isoflurane for 15 s following a 30 s preincubation with 0.55 mM isoflurane. Data are shown as mean ± S.E.M. of 6 oocytes. B) Effect of toluene on currents elicited by brief applications of maximally-effective concentrations of glycine (10 mM) or taurine (100 mM) in the presence or absence of Zn. Agonist was co-applied with 0.42 mM toluene for 15 s following a 30 s preincubation with 0.42 mM toluene. Data are shown as mean ± S.E.M. of 4 oocytes. *, p < 0.05.
4.2.4 - Zn and toluene interactions

The next allosteric modulator tested was the inhaled drug of abuse toluene. Fig. 4.5B shows the results of the brief co-applications of 0.42 mM toluene with either 10 mM glycine or 100 mM taurine in the presence of background levels of Zn, 2.5 mM tricine, or 100 nM Zn. Toluene had negligible effects on 10 mM glycine currents and its effects on 100 mM taurine currents were similar to effects seen with ethanol and isoflurane. Again, similar to ethanol and isoflurane, toluene percent potentiation of saturating taurine-mediated currents was significantly reduced in the presence of 100 nM Zn, compared to the other two Zn conditions [Two-way RM ANOVA with SNK multiple comparison procedure; q = 6.3, p < 0.002; q = 5.7, p < 0.002, respectively]. Toluene enhancement of saturating taurine was significantly greater than effects on saturating glycine in background levels of Zn [q = 8.8, p < 0.001], 2.5 mM tricine [q = 7.2, p < 0.001], and 100 nM Zn [q = 3.3, p < 0.05].

4.2.5 - Zn & ethanol interactions at a low taurine concentration

We previously found that Zn enhances ethanol potentiation of low concentrations of glycine, and that the degree of ethanol enhancement is reduced when Zn is chelated by tricine (McCracken et al., 2010). Although we saw minor ethanol effects with changing Zn levels at saturating concentrations of glycine, we did see changes in ethanol potentiation of saturating concentrations of taurine based on the level of Zn present (Fig. 4.2B). Thus we extended the observations made in the McCracken et al. (2010) paper, this time using EC₅₀ concentrations of taurine. We first tested whether ethanol potentiation of EC₅₀ taurine currents in α1 GlyR depends on the concentration of Zn. Fig.
4.6A shows that when Zn is chelated with tricine, the degree of ethanol potentiation of EC₅ taurine-mediated currents is the same as EC₅ glycine-mediated currents for both 50 mM and 200 mM ethanol. We next tested if chelation of Zn significantly reduces ethanol potentiation of EC₅ taurine-mediated currents as it does for glycine-mediated currents. Fig 4.6B shows the results of 200 mM ethanol effects on EC₅ glycine and taurine currents, with and without the addition of 2.5 mM tricine. With no differences between agonists, ethanol potentiation was significantly lower in 2.5 mM tricine compared to background levels of Zn [Two-way ANOVA with SNK multiple comparison procedure; q = 3.7, p < 0.05].
Figure 4.6. Zn affects ethanol potentiation of low concentrations of full and partial agonists. A) Ethanol potentiation of the effects of 5% maximal (EC$_5$) glycine- or taurine-mediated currents in the presence of 2.5 mM tricine (i.e., in the absence of Zn). EC$_5$ glycine and taurine were co-applied with either 50 mM or 200 mM ethanol following a 30 s preincubation with ethanol. Data are shown as mean ± S.E.M. of 5 oocytes. B) Ethanol potentiation of EC$_5$ glycine and taurine is enhanced by Zn. EC$_5$ glycine or taurine were co-applied with 200 mM ethanol following preincubation with ethanol, in the presence of either background Zn (left two bars) or 2.5 mM tricine (right two bars). Data are shown as mean ± S.E.M. of 9 oocytes. *, p < 0.05.
4.3 - Discussion

The dopaminergic projection from the ventral tegmental area (VTA) to the NAcc is critical to the perception of the rewarding properties of drugs of abuse, and ethanol’s actions on the GlyR may be particularly important in these brain regions. For example, extracellular dopamine levels increase in the NAcc following ethanol (Imperato and Di Chiara, 1986) or glycine (Molander et al., 2005) administration and glycine perfusion into the NAcc of rats decreases alcohol consumption (Molander et al., 2005). Many volatile agents such as isoflurane and toluene also have abuse liability (Johnston et al., 2012; Wilson et al., 2008). Toluene also increases extracellular dopamine levels in the VTA and NAcc when perfused into the former (Riegel et al., 2007). Most studies examining the effects of these agents on GlyR functioning do so by determining the effects of single modulators in isolation. However, since Zn is ubiquitous in cerebrospinal and interstitial fluids at GlyR-potentiating levels, the effects of drugs of abuse on GlyR in vivo will always be seen in combination with Zn effects. Since taurine and glycine may act as GlyR agonists in brain regions such as the NAcc, we examined the effects of combinations of allosteric modulators on GlyR activated by these agonists. Maximally-effective concentrations of taurine but not glycine were affected by Zn chelation (Figs. 4.1 & 4.2). This is likely the result of glycine producing a very high probability of channel opening (intra-cluster Po ~ 1), with maximally-effective concentrations keeping the channel open almost 100% of the time before it desensitizes, regardless of the presence of any allosteric modulator. As a partial agonist, taurine has a much lower Po (~ 0.5) at maximally-effective concentrations (Lape et al., 2008), allowing for Zn to exhibit its enhancing effects. The Zn potentiation seen after 10 min. of
continuous taurine perfusion could be attributable to Zn: (1) increasing Po; (2) enhancing conductance; (3) decreasing the desensitization rate or; (4) increasing the rate of GlyR resensitization. Previous studies performed using low agonist concentrations show that Zn does increase Po but it does not affect conductance (Laube et al., 2000). The decrease in responses seen when Zn is chelated by tricine suggests that submaximal glycine responses and all taurine responses are overestimated when not controlling for background Zn concentrations in buffer solutions.

Because Zn is ubiquitous in the CNS, the effects of any drugs of abuse or other allosteric modulators of interest on GlyR functioning require comparison in the presence and absence of potentiating concentrations of Zn. In the presence of a maximally-effective concentration of glycine, changing the Zn concentration had no effect on responses for any of the modulators tested, since 10 mM glycine has already produced a maximal response. However, effects on maximally-effective concentrations of taurine were expected and seen. If Zn and other positive modulators were acting in an additive or synergistic manner, we would expect the 100 nM Zn condition to give a higher degree of potentiation than our standard buffers, with chelation of Zn by 2.5 mM tricine giving a lower degree of potentiation than both other conditions. However we did not find this to be the case for maximally-effective concentrations of taurine with alcohol, isoflurane, or toluene.

Chelation of Zn with tricine did not change the alcohol, isoflurane, or toluene potentiation of a maximally-effective taurine concentration when compared to the effects seen in the presence of background levels of Zn (Figs. 4.3B, 4.5A, & 4.5B). However, at submaximal concentrations of glycine or taurine, chelation of background Zn does
decrease ethanol potentiation of GlyR responses (Fig. 4.6B). The most parsimonious explanation for the lack of change seen at higher levels of taurine would be that background levels of Zn during those particular experiments were low. However, the addition of 100 nM Zn to buffers, ensuring that the concentration of Zn is in the potentiating range, led to a significant reduction in the ethanol, isoflurane, and toluene potentiation of taurine-activated GlyR responses (Figs. 4.3B, 4.5A & 4.5B). This finding cannot be explained by simple competition between modulators as Zn is not believed to bind the receptor at the same locations as alcohols, anesthetics, and inhalants (Beckstead et al., 2000; Laube et al., 2000; Lynch et al., 1998; Mihic et al., 1997; Yamakura et al., 1999). However, Zn does increase the Po of the taurine-activated GlyR (Laube et al., 2000), in effect mimicking the glycine-bound GlyR. When this occurs and the GlyR Po approaches 1 any other positive modulator present will as a result produce decreased percent enhancement compared to when less Zn is present.

Since the α2 subunit appears to be expressed at higher levels than α1 in higher brain regions (Jonsson et al., 2012) we studied ethanol/Zn interactions on a variety of different GlyR subtypes activated by taurine vs glycine (Figs. 4.3B, 4.4A-C). The α2-containing receptors were previously shown to be less sensitive to ethanol (Mascia et al., 1996), and Zn (Miller et al., 2005), than those that contain α1 subunits and this was also reflected in our experiments using a maximally-effective concentration of taurine. We performed these experiments in the absence and presence of the β subunit, which anchors the GlyR to gephyrin in synapse (Kirsch and Betz, 1995).

In summary, low concentrations of Zn potentiated GlyR responses from maximally-effective concentrations of taurine but not glycine. Chelation of Zn reduced
GlyR responses produced by maximally-effective concentration of taurine but not glycine, and thus any experiments conducted in the absence of Zn chelation will tend to overestimate taurine efficacy. This would also be true when submaximal glycine concentrations are used. At the low concentrations tested, Zn decreased the enhancing effect of taurine-mediated responses for all drugs of abuse tested when applied in combination with them. As taurine is likely to be an endogenous GlyR ligand in brain regions affected by these compounds, the presence of low concentrations of Zn in these regions results in GlyR responses that are not the result of a simple summation of responses to single modulators, and this needs to be taken into consideration when investigating the role of the GlyR in vivo. It is highly likely that previously published studies of this receptor have in reality been studying the Zn-modulated GlyR. To truly understand how allosteric modulators act at the GlyR, even when they may be found concomitantly with Zn, it is important to first characterize their effects in isolation, before studying their interactions in combination.
5.0 Zn interactions with novel peptide modulators of the GlyR
discovered via phage display

5.1- Introduction

The GlyR has been identified as a potential therapeutic target for the treatment of substance abuse, particularly alcoholism (Tipps et al., 2010; Molander et al., 2007; 2005). Novel modulators of GlyR function may have potential therapeutic applications. Tipps et al. (2010) demonstrated the feasibility of utilizing phage display to identify novel peptide modulators of the GlyR. Here, we expand on that work, identifying peptides with subunit specificity and investigating the role of Zn on their activity at the GlyR. We utilized the New England Biolabs Ph.D™-7 phage display library consisting of genetically modified M13 bacteriophage that express random heptapeptides fused to minor coat protein pIII. Each library represents around $10^9$ unique 7 amino acid sequences present at around 100 copies each. The phage display the peptides on their outer coat, allowing for the screening of physical interactions between these peptides and protein targets of interest. This is accomplished by washing the libraries over protein targets either coated on the surface of custom made plates or expressed in the membranes of host cells, such as human embryonic kidney (HEK) cells. Phage that do not bind the protein target are discarded in the supernatant while those that express peptides that can interact sufficiently with the protein target remain bound. These phage can be sequenced to identify the peptide presumably responsible for interaction between the phage and the protein target.

Two separate phage display screens were performed, denoted as the D7.1 and the D7.2 series. For each screening procedure, five consecutive rounds of panning were
performed. Each round consisted of washing the Ph.D\textsuperscript{TM}-7 library over (negative selection) HEK cells expressing either $\alpha_2\beta$ GlyR (D7.1 series) or $\alpha_2\beta$ and $\alpha_3\beta$ GlyR (D7.2 series). Phage that did not bind the negative selection cells were collected and washed over positive selection HEK cells expressing the $\alpha_1\beta$ GlyR. Phage that did not bind to the positive selection cells were discarded and the remaining phage were amplified and subjected to further rounds of panning. In this way, any phage that express peptides that would bind to the negative selection receptors or nonspecifically to HEK cells would be eliminated in the negative selection while those that bind to the target, $\alpha_1\beta$ GlyR, will be obtained in the positive selection step. Receptors with high levels of homology to the target $\alpha_1\beta$ GlyR (e.g. the $\alpha_2\beta$ and $\alpha_3\beta$ GlyR) were incorporated into the negative selection in order to identify peptides with high specificity for $\alpha_1\beta$ GlyR. Identified peptides were synthesized and screened for their ability to affect $\alpha_1\beta$ GlyRs via the two-electrode voltage clamp method in \textit{Xenopus laevis} oocytes.

5.2 – Results

5.2.1- Identified Peptides Effect on GlyR

In total, 40 peptide sequences were identified as interacting with $\alpha_1\beta$ GlyR via phage display, 15 D7.1 peptides and 25 D7.2 peptides (Table 1). Of these peptides, 26 were synthesized (12 D7.1 and 14 D7.2) and tested for their activities on $\alpha_1\beta$ GlyR via the two-electrode voltage clamp technique in \textit{Xenopus laevis} oocytes (figure 5.1). Not all of the identified peptides were synthesized and/or tested due to time restraints or solubility issues. Peptides were first tested on $\alpha_1\beta$ GlyR activated by EC\textsubscript{5-10} glycine at peptide concentrations of 30\textmu M, a concentration previously shown to be sufficient for previously
identified peptide modulators to affect GlyR functioning (Tipps et al., 2010). Figure 5.1 shows the high variability in the effects of these peptides at α1β GlyR, ranging from 78.38 ± 10.11 % potentiation of EC5-10 glycine responses by D7.2-122 to 32.89 ± 9.37 % inhibition of EC5-10 glycine responses by D7.1-115. In order to test the specificity of peptides identified in these phage display procedures, we tested the most effective peptide, D7.2-122, on α1β, α2β, and α3β GlyR (Figure 5.2). 30μM D7.2-122 had a significantly greater effect on α1β and α3β GlyR compared to α2β GlyR, indicating some degree of subunit specificity can be obtained utilizing this method.
<table>
<thead>
<tr>
<th>Phage</th>
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<tr>
<td>D7.1-102</td>
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<tr>
<td>D7.1-103</td>
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</tr>
<tr>
<td>D7.1-104</td>
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<td>D7.1-109</td>
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<td>D7.1-110</td>
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<tr>
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</table>

**Table 5.1.** In total, 40 peptides were identified through the two phage display procedures. 15 D7.1 peptides targeting the $\alpha_1\beta$ GlyR and negatively selected against the $\alpha_2\beta$ GlyR and 25 D7.2 peptides targeting the $\alpha_1\beta$ GlyR and negatively selected against the $\alpha_2\beta$ and $\alpha_3\beta$ GlyR.
Figure 5.1. The effects of 30μM concentrations of peptides on α1β GlyR currents elicited by EC5-10 glycine. D7.1 peptides are represented by black bars and D7.2 peptides are grey bars. N= 2-8 oocytes.
Figure 5.2. The effects of $30\mu M$ D7.2-122 on $\alpha_1\beta$, $\alpha_2\beta$, and $\alpha_3\beta$ GlyR activated by EC$_{50}$ glycine. N= 4-6 oocytes.
5.2.3- Peptide activity is Zn-dependent

In order to determine what role, if any, Zn plays in the activity of these peptides at the GlyR, 100 μM concentrations of select peptides were co-applied with the Zn-chelating agent tricine. As seen in figure 5.3, the co-application of tricine with peptides inhibits their enhancing effects at the GlyR. Interestingly, the inhibitory action of D7.1-115 was also blocked in the presence of tricine. To determine whether the variable effects of the peptides were due solely to varying levels of Zn contamination within the peptides themselves, samples of D7.1-122 and D7.1-123 were sent for ICP-MS analysis at Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin. ICP-MS analysis indicated that both peptides contain a similar amount of Zn contamination. D7.2-122 contained 14.55 ± 0.16 nM Zn while D7.2-123 contained 12.38 ± 0.16 nM Zn.
Figure 5.3. Tricine blocks the effects of 100µM D7.1 peptides on α1β GlyR activated by EC5-10 glycine.
5.3 – Discussion

Tipps et al. (2010) developed a method by which phage display can be utilized to find novel peptide modulators of the GlyR. Here we expanded on this work by incorporating the α2β and α3β GlyRs in a negative selection step in an attempt to identify peptides highly specific to the α1β GlyR subtype. We synthesized and tested 26 of 40 identified peptides for activity on the α1β GlyR via two-electrode voltage clamp in *Xenopus laevis* oocytes. Peptides had varying effects on α1β GlyR with the most effective enhancing peptide, D7.2-122, potentiating EC5-10 glycine responses by 78.38 ± 10.11 % and the most inhibitory peptide, D7.1-115, causing 32.89 ± 9.37 % inhibition of EC5-10 glycine responses. When tested for specificity, D7.2-122 was significantly more effective at the α1β and α3β GlyRs than the α2β GlyR, indicating some degree of subunit specificity. The α1 and α3 GlyR subunits have a higher level of homology than the α1 and α2, therefore it may be more difficult to identify peptides with specificity between receptors containing α1 or α3 GlyR subunits.

Since Zn plays a role in the activity of other modulators of the GlyR (McCracken et al., 2010; Kirson et al., 2013), several peptides were tested electrophysiologically in the presence of the Zn-chelating agent tricine. Tricine blocked the enhancing effects of all peptides tested and, interestingly, blocked the inhibitory effect of D7.1-115. In order to determine the peptide effects on GlyR were not purely the result of Zn contamination within the peptides, two peptides with different degrees of activity at the GlyR underwent ICP-MS analysis. D7.2-122 caused a 78.38 ± 10.11 % potentiation of EC5-10 glycine currents while D7.2-123 caused a -7.59 ± 2.55 % potentiation of EC5-10 glycine
currents. While both peptides had very different effects at the GlyR, they had almost the same levels of contaminating Zn, indicating that Zn contamination is likely not the cause of the different peptide effects. We therefore hypothesize that either A) Zn interacts with some peptides to alter their conformations in such a way as to allow them to affect GlyR function and this is prevented by chelation; or B) Zn is acting as a co-agonist with glycine at the GlyR and only receptors activated by both glycine and Zn exhibit significant peptide effects. It is interesting to note that concentrations of ethanol that potentiate EC$_{50}$ glycine responses on $\alpha$1-containing GlyR at about the same degree as the peptides used here (~20mM-50mM EtOH) are also blocked in the presence of tricine, and that it is not until the concentration of alcohol is increased that a potentiating effect can again be seen (McCracken et al., 2010). Therefore, it seems more likely that Zn is acting as a co-agonist with glycine at GlyR and is required for effective modulation of the channel by certain compounds, such as peptides and alcohol.
6.0| Conclusion

Zn is a potent and bi-phasic modulator of the GlyR, enhancing $\alpha_1$–containing GlyR activity at concentrations in the low nM to 10$\mu$M range and inhibiting $\alpha_1$–containing GlyR activity at higher concentrations (Bloomenthal et al., 1994; Laube et al., 1995; Harvey et al., 1999). Here, we demonstrate that Zn is a common contaminant in buffers used in electrophysiological solutions at levels sufficient to affect GlyR functioning. While present in small quantities in ultrapure di-H$_2$O, most contamination appears to come from reagents used to prepare the buffers or from plastic-ware, such as pipets, used to prepare the solutions. Further, we established that physiologically-relevant concentrations of Zn decrease GlyR responses to drugs of abuse when activated by saturating levels of taurine but not glycine. This could be physiologically relevant due to speculation that taurine is likely to be an endogenous GlyR ligand in brain regions affected by these compounds. The presence of low concentrations of Zn in these regions results in GlyR responses that are not the result of a simple summation of responses to single modulators, and this needs to be taken into consideration when investigating the role of the GlyR in vivo. Finally, we utilized phage display to identify novel peptide modulators of the GlyR that are Zn-dependent. This finding may indicate that Zn plays a role in making GlyRs more amenable to allosteric modulation by various compounds. Zn bound at the GlyR may make the receptor more amenable to the conformational changes brought about by interaction with allosteric modulators to alter channel function (i.e. increase modulator efficacies). Alternatively, Zn may push the receptor into a state that increases modulator affinity. Ultimately, future studies should take contaminating Zn into account when investigating the activity of agonists and modulators on GlyR.
References


Wilkins, M.E., Smart, T.G., 2002. Redox modulation of GABA\textsubscript{A} receptors obscured by Zn2+ complexation. *Neuropharmacol* 43, 938-944.


