JPET Fast Forward. Published on January 13, 2017 as DOI: 10.1124/jpet.116.239152
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Interactions between zinc and allosteric modulators of the glycine receptor

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Interactions of zinc with modulators of the glycine receptor

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Number of Text pages: 27

Number of Tables: 0

Number of Figures: 8

Number of References: 32

Words in Abstract: 237

Words in Introduction: 657

Words in Discussion: 1454

Abbreviations: ANOVA, analysis of variance; GlyR, glycine receptor; EC, effective concentration; MAC, minimum alveolar concentration; MBS, Modified Barth's Saline; TCE, 1,1,1-trichloroethane; TCY, trichloroethylene; Tri, tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wildtype

Recommended Section Assignment: Neuropharmacology

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Abstract

The glycine receptor is a pentameric ligand-gated ion channel involved in fast inhibitory neurotransmission in the central nervous system. Zinc is an allosteric modulator of glycine receptor function, enhancing the effects of glycine at nM to low μM concentrations, and inhibiting its effects at higher concentrations. Low nM concentrations of contaminating zinc in electrophysiological buffers are capable of synergistically enhancing receptor modulation by other compounds such as ethanol. This suggests that, unless accounted for, previous studies of glycine receptor modulation were measuring the effects of modulator plus co-modulation by zinc on receptor function. Since zinc is present *in vivo* at a variety of concentrations, it will influence glycine receptor modulation by other pharmacological agents. We investigated the utility of previously-described "zinc-enhancement insensitive" α1 glycine receptor mutants D80A, D80G, and W170S to probe for interactions between zinc and other allosteric modulators at the glycine receptor. Interestingly, we found that only the W170S mutation conferred complete abolishment of zinc enhancement across a variety of agonist and zinc concentrations. Using α1 W170S receptors, we established that in addition to ethanol, zinc also interacts with inhalants, but not volatile anesthetics, to synergistically enhance channel function. Additionally, we determined that this interaction is abolished at higher zinc concentrations when receptor-enhancing binding sites are saturated, suggesting a mechanism by which modulators such as ethanol and inhalants are capable of increasing receptor affinity for zinc in addition to enhancing channel function on their own.

Introduction

Glycine receptors (GlyRs) are members of the eukaryotic Cys-loop receptor superfamily of ligand-gated ion channels. Responsible for the majority of fast inhibitory neurotransmission in the brainstem and spinal cord, GlyRs are also found in many higher brain regions, including the hippocampus, nucleus accumbens, and frontal cortex (Baer et al., 2009; Jonsson et al., 2009, 2012; Molander and Söderpalm, 2005). Endogenous ligands, such as glycine and taurine, bind at intersubunit sites on the extracellular surface of the receptor, resulting in conformational changes that lead to channel opening. A variety of agents act as positive allosteric modulators of GlyR function, including ethanol, volatile anesthetics, and inhaled drugs of abuse (Mihic et al., 1997; Beckstead et al., 2000; Welsh et al., 2009). In addition to these exogenous compounds, GlyRs are also modulated by endogenous agents, such as the divalent metal cation zinc (Bloomenthal et al., 1994; Laube et al., 1995). Rapidly-exchangeable zinc (referred to as "free" zinc) is ubiquitous in the central nervous system, found tonically at nanomolar concentrations in cerebrospinal fluid (CSF) (Frederickson et al., 2006a), and released phasically from zinc-containing synapses (Frederickson et al., 2006b; Qian and Noebels, 2005; Vogt et al., 2000). Zinc biphasically modulates GlyR function, enhancing currents at concentrations between ~10 nM - 10 μM while inhibiting GlyR function at higher concentrations (Bloomenthal et al., 1994; Laube et al., 1995). This is thought to be the result of at least two distinct zinc-binding sites on the receptor: a high affinity GlyR-enhancing site, and a low affinity inhibitory site (Harvey et al., 1999; Miller et al., 2005; Nevin et al., 2003). While tonic free zinc is found at concentrations on the lower end of the GlyR-enhancing range (Frederickson et al., 2006a), the extracellular concentrations of zinc achieved after synaptic release are still somewhat controversial. Many studies report that local zinc concentrations remain within the GlyR-enhancing range (<10 μM) after synaptic release, while other studies have reported concentrations of up to 100 μM zinc (Frederickson et al., 2006b; Qian and Noebels, 2005; Vogt et al., 2000).

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In addition to being present *in vivo*, zinc is a contaminant commonly found in many types of labware and reagents, at nanomolar concentrations capable of affecting GlyR function (Cornelison and Mihic, 2014; Kay, 2004). Additionally, chelation of contaminating zinc results in a significant decrease in GlyR enhancement produced by a variety of modulators, including ethanol (McCracken et al., 2010, 2013a,b) and recently-discovered peptides (Cornelison et al., 2016). However, the mechanism by which zinc interacts with these other allosteric modulators at the GlyR is unknown. Further, these studies have relied on the use of relatively high (2.5 - 10 mM) concentrations of the zinc-chelator tricine that could conceivably affect channel function in ways apart from its ability to chelate zinc. For example, it is possible that tricine could act itself as a negative allosteric modulator of GlyR function and that the reduction in GlyR currents seen in the presence of tricine could be due to this effect in addition to its chelation of zinc. Additionally, the strong hydrogen bonding potential of tricine could effectively "chelate" hydrophilic allosteric modulators of the GlyR, such as ethanol, thereby seemingly decreasing their effects.

Several single point mutations of the α1 GlyR have been reported to confer insensitivity to enhancement by zinc, including substitutions of the aspartic acid residue at position 80 to alanine (D80A) (Hirzel et al., 2006; Lynch et al., 1998; McCracken et al., 2013a) or glycine (D80G) (Laube et al., 2000), as well as the mutation of tryptophan at position 170 to serine (W170S) (Zhou et al., 2013). To unambiguously rule out the possibility that tricine could modulate GlyR function beyond its ability to chelate zinc, we investigated tricine's actions on these "zinc-insensitive" receptors. We also characterized the effects of higher (10 μM) concentrations of zinc on the activity of other GlyR modulators and present a possible mechanism by which zinc interacts with some of these modulators to enhance their activity at the GlyR.

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Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for isoflurane which was obtained from Marsam Pharmaceutical Inc. (Cherry Hill, NJ) and tricaine which was obtained from Western Chemical, Inc. (Ferndale, WA).

Generation of point mutations

The α1 D80A GlyR mutant cDNA was a gift from the lab of Dr. R. Adron Harris (McCracken et al., 2013a). The α1 D80G and W170S point mutations were generated via site-directed mutagenesis with the QuickChange II mutagenesis kit (Agilent Technologies, Santa Clara, CA) and commercially engineered mutagenesis primers (Integrated DNA Technology, San Diego, CA) using wildtype (WT) α1 GlyR cDNA in a modified pBK-cytomegalovirus vector (Mihic et al., 1997) as a template. Successful mutagenesis was verified via Sanger sequencing using AB 3730 and AB 3730XL DNA analyzers (Thermo-Fisher Scientific, Waltham, MA) at the University of Texas at Austin DNA Sequencing Facility. The GlyR α1 cDNAs were completely sequenced to ensure the absence of any unwanted mutations.

Oocyte isolation and cDNA injection

Female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI) and housed at 19^{o} C on a 12 h light/dark cycle. Frogs were anesthetized with tricaine and portions of their ovaries were removed surgically in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations. Individual oocyte isolation, cDNA injection, and incubation were performed as previously described (Cornelison et al., 2016).

Two-electrode voltage-clamp electrophysiology

Glycine and modulators were diluted in, and all electrophysiological recordings were performed in, modified Barth's saline (MBS) buffer [89 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$ • 7 H_2O, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.5], MBS + 2.5 mM tricine, or MBS + 10 μM $ZnCl_2$. Recordings were performed 1-5 days post cDNA injection as previously described (Cornelison et al., 2016). In all experiments, GlyRs were pre-incubated with modulators for 30 s before co-application with glycine. When maximally-effective concentrations of glycine were used, applications lasted for 10 s and were followed by 15 min (WT and W170s GlyRs) or 20 min (D80A and D80G GlyRs) washouts to allow for full receptor re-sensitization. When concentrations of glycine giving 5-10% of a maximally effective concentration of glycine (EC_{5-10}) were used, applications lasted 45 s followed by a 5 minute washout. EC_{5-10} glycine concentrations were determined separately in each oocyte based on the current produced by an initial application of a saturating concentration of glycine; this was repeated for each buffer tested (i.e. MBS, MBS + 2.5 mM tricine, and MBS + 10 μM $ZnCl_2$), Every EC_{5-10} glycine + modulator co-application was flanked by an EC_{5-10} glycine control for comparison. Maximally-effective glycine responses were checked again at the end of every experiment to account for drift in glycine responses over time. Applications in which control EC_{5-10} glycine responses drifted above EC_{10} or below EC_5 were not used. For zinc concentration-response curves, the effects of 0.03-10 μM $ZnCl_2$ on EC_{5-10} glycine currents were determined. Washout durations after zinc co-application had to be increased from 5 min to 10-15 min when concentrations > 300 nM $ZnCl_2$ were used, in order to allow for complete zinc washout to occur and for return to baseline EC_{5-10} currents. For glycine concentration-response curves, 0.03-100 mM glycine (in MBS + 2.5 mM tricine, with or without 2.5 μM $ZnCl_2$), were applied for 10-45 s as required to reach stable peak currents. Loss of volatile compounds through tubing and evaporation from bath was previously measured (Beckstead et al., 2000; Mihic et al., 1994; Yamakura et al., 1999). All concentrations reported are the bath

concentrations to which the oocytes were exposed. All data were collected from oocytes obtained from at least two different frogs.

Data analysis

Peak currents were measured and used in data analysis. For each oocyte, currents observed in the presence of glycine plus modulators were compared with currents generated by glycine alone and expressed as the mean ± S.E.M of the percent change in glycine-mediated current. Significant differences were determined using the Students t-test, analysis of variance (ANOVA), two-way ANOVA, and Tukey post-hoc tests, as indicated. All statistical testing was performed using SigmaPlot 11.0 (Systat Software, San Jose, CA).

Results

Characterization of α1 WT, D80A, D80G, and W170S GlyR sensitivity to enhancing concentrations of zinc.

In order to verify previously-published data reporting that D80A, D80G, or W170S mutations of the α1 GlyR confer insensitivity to enhancing concentrations of zinc, mutant and WT homomeric receptors were expressed in *Xenopus laevis* oocytes. The effects of 100 nM $ZnCl_2$ and 2.5 mM tricine were determined on currents generated by a concentration of glycine that yielded approximately 5-10% of a maximally-effective glycine response (EC_{5-10}). Surprisingly, in addition to WT receptors, D80A and D80G GlyRs showed robust sensitivity to both contaminating and exogenously-added zinc (Fig. 1). Figs. 1A and 1B show that tricine chelation of contaminating zinc, previously reported to measure around 45 nM (Cornelison and Mihic, 2014), resulted in a similar decrease in EC_{5-10} glycine currents in WT, D80A, and D80G receptors while not affecting W170S GlyRs. Figs 1A and 1C show that the co-application of 100 nM $ZnCl_2$ caused a similar enhancement of EC_{5-10} glycine currents in WT and D80A GlyRs that was significantly reduced in D80G and abolished in W170S GlyRs.

Due to the unexpected findings of robust enhancement by low nanomolar concentrations of zinc in α1 GlyR D80 mutants previously reported as being zinc-insensitive, we performed a more complete characterization of zinc enhancement in both WT and mutant receptors. We first examined zinc-sensitivity of EC_{5-10} glycine currents across a wide range of GlyR-enhancing concentrations. Figure 2 illustrates the difference in GlyR enhancement of WT, D80A, D80G, and W170S GlyRs by co-application of EC_{5-10} glycine with 30 nM to 10 μM $ZnCl_2$. A two-way ANOVA revealed a significant effect of receptor [$F(3,119) = 76.68$, $p < 0.001$], a significant effect of zinc concentration [$F(5,119) = 14.367$, $p < 0.001$], and a significant interaction between receptor and zinc concentration [$F(15,119) = 2.40$, $p < 0.01$]. WT, D80A, and D80G receptors

displayed bimodal zinc-response curves where zinc-enhancement increased in a concentration-dependent manner until peak potentiation was reached at 1 μM. This was followed by a concentration-dependent decrease in zinc enhancement that reflects an increase in zinc binding to the low-affinity inhibitory sites, resulting in competition between zinc-enhancement and inhibition. In contrast, W170S GlyRs showed no zinc enhancement from 30 nM to 1 μM $ZnCl_2$, with inhibition seen at higher concentrations, consistent with previous results (Zhou et al., 2013). When comparing WT to D80 mutant GlyRs, all three receptors displayed similar levels of enhancement at the low and high ends of the curve. However, D80G GlyRs exhibited a shallower concentration-response curve than WT receptors, with significantly decreased peak 1 μM zinc enhancement ($p < 0.05$). D80A GlyRs appeared to have a steeper curve with higher peak zinc-enhancement, but this was not statistically different from WT GlyRs ($p = .120$).

We next investigated how zinc enhancement of receptor function changes with increasing agonist concentration. We first generated glycine concentration-response curves in the presence of 2.5 mM tricine to account for the effects of contaminating zinc on agonist EC. We then plotted glycine EC values (triangles, right ordinate) and potentiation of corresponding currents by application of 2.5 μM $ZnCl_2$ in the presence of 2.5 mM tricine (circles, left ordinate) against glycine concentration (Fig. 3). Zinc potentiation significantly decreased with increasing glycine concentration for WT, D80A, and D80G receptors while W170S GlyRs showed minimal zinc potentiation that did not vary with agonist concentration. When zinc potentiation of WT and D80 mutant GlyRs was plotted against glycine EC (Fig. 4), there appeared to be differences between WT and mutant receptors in the magnitude of zinc enhancement at lower glycine ECs ($< EC_{40}$). In an attempt to quantitate this, we partitioned the data into zinc effects at low ($EC_{0\text{-}20}$) and intermediate-low ($EC_{20\text{-}40}$) glycine. One-way ANOVAs revealed significant differences between receptors at both low [$F(2,26) = 5.812$, $p < 0.01$] and intermediate-low EC [$F(2,9) = 5.886$, $p < 0.05$]. Tukey multiple comparisons post-hoc tests showed that, compared to WT

receptors, zinc-enhancement of D80G receptors was significantly decreased only at intermediate-low (EC_{20-40}) glycine (+38.70 ± 11.64% change for D80G compared to +92.07 ± 8.91% change for WT, $p < 0.01$). There were no significant differences between D80A receptors and WT controls at either glycine concentration.

Effects of zinc chelation on ethanol enhancement of WT and W170S GlyRs.

We next assessed the effects of ethanol on W170S compared to WT GlyRs in the presence and absence of low nanomolar concentrations of zinc. To do this we determined differences in ethanol enhancement of EC_{5-10} glycine currents for both WT and W170S receptors in normal MBS buffer (containing low nanomolar contaminating zinc) and in buffer in which contaminating zinc was chelated by tricine (Fig. 5). Consistent with McCracken et al. (2010), zinc-chelation by tricine significantly reduced enhancement of EC_{5-10} glycine currents by 50 and 200 mM ethanol in WT GlyRs. W170S GlyRs showed significantly decreased EtOH modulation compared to WT receptors in standard MBS and this was not further decreased by tricine. Additionally, there was no difference between the magnitudes of EtOH enhancement of WT and W170S GlyRs in the presence of tricine. Taken together, these data support the idea that α1 W170S GlyRs allow for the study of allosteric modulation of GlyRs in the absence of co-modulation by zinc, despite zinc's continued presence as a contaminant in the assays.

Volatile anesthetic and inhalant sensitivity of WT and W170S GlyRs under low nanomolar zinc conditions.

We next used W170S GlyRs to probe for the involvement of low nanomolar concentrations of zinc on GlyR enhancement by volatile anesthetics and inhalants. The enhancement of EC_{5-10} glycine currents in WT and W170S GlyRs by several volatile anesthetics and inhalants were determined in standard MBS containing low nanomolar zinc. There were no differences in GlyR enhancement of EC_{5-10} glycine currents between WT and W170S receptors

by approximately minimum alveolar concentrations (MAC) of the volatile anesthetics isoflurane, chloroform, or halothane (Fig. 6A). In contrast, the inhalants 1,1,1-trichloroethane (TCE), toluene and trichloroethylene (TCY) showed a significantly decreased ability to enhance EC_{5-10} glycine currents in W170S compared to WT receptors (Fig. 6B), suggesting an interaction between zinc and these inhalants to enhance GlyR function.

Allosteric modulator sensitivity in WT and W170S GlyRs under low micromolar zinc conditions.

Previous studies investigating the interaction between zinc and other allosteric modulators at the GlyR have focused on zinc concentrations ≤ 1 μM, in which only the high affinity GlyR-enhancing zinc site would be occupied. However, synaptically-released zinc may result in higher local concentrations which could occupy both the high affinity enhancing and low affinity inhibitory zinc binding sites. We therefore measured the effects of adding 10 μM $ZnCl_2$ to our buffer on the modulation of EC_{5-10} glycine currents in WT and W170S GlyRs by ethanol (Fig. 7) as well as TCE and halothane (Fig. 8). Figure 7A shows sample tracings of ethanol's effects on EC_{5-10} glycine currents from WT and W170S GlyRs in normal MBS and MBS + 10 μM $ZnCl_2$. Enhancement of EC_{5-10} glycine currents by 50 and 200 mM ethanol were significantly decreased in WT GlyRs when 10 μM $ZnCl_2$ was added to the buffer. However, ethanol modulation of W170S GlyRs was already significantly decreased compared to WT receptors in standard MBS and was not further affected by the addition of zinc. Additionally, there were no significant differences between ethanol modulation of WT and W170S GlyRs in the presence of 10 μM $ZnCl_2$. A similar pattern was seen for TCE, in which the addition of 10 μM $ZnCl_2$ significantly decreased TCE enhancement for WT but not W170S GlyRs with no significant differences between WT and W170S receptors in MBS + 10 μM $ZnCl_2$. (Fig. 8). There were no significant differences in halothane modulation of WT and W170S GlyRs, regardless of the concentration of zinc present.

Discussion

Apart from the ability of nanomolar concentrations of zinc to allosterically enhance GlyR function, low concentrations of zinc also interact with other allosteric modulators of the GlyR, yielding effects greater than those produced by zinc or modulator applied alone (Cornelison et al., 2016; McCracken et al., 2010, 2013a,b). This is thought to be due to the removal of "free zinc" from buffers via chelation by agents such as tricine. However, this does not discount the possibility that chelating agents such as tricine, especially at mM concentrations, might have effects on receptor activation or allosteric modulation in ways distinct from their abilities to chelate zinc. Testing for the effects of allosteric modulators on GlyR mutants resistant to the enhancing actions of zinc would alleviate this concern. We first used homomeric α1 D80A and D80G mutant GlyRs previously reported to be insensitive to the enhancing effects of zinc (Hirzel et al., 2006; Laube et al., 2000; Lynch et al., 1998; McCracken et al., 2013a). However, in our hands these two mutants are not truly insensitive to enhancing concentrations of zinc (Fig.1). We next screened the α1 W170S GlyR and found that both tricine and 100nM $ZnCl_2$ had negligible effects on W170S GlyR currents (Fig. 1), confirming this mutant's reported lack of zinc enhancement and showing that tricine does not appear to act as a modulator of the GlyR beyond its ability to chelate zinc.

The discrepancies between our data and previously-published reports on the zinc-insensitive nature of the α1 GlyR D80 mutants prompted a more thorough characterization of their sensitivities to enhancing concentrations of zinc. We first tested the effects of a variety of GlyR-enhancing zinc concentrations on submaximal glycine currents in WT and mutant receptors (Fig. 2). While WT and D80A receptors showed similar responses across the zinc concentrations tested, D80G receptors exhibited a much shallower zinc concentration-response curve with significantly lower peak enhancement. We previously determined that our

electrophysiological buffers (MBS) contain approximately 45 nM concentrations of contaminating zinc (Cornelison and Mihic, 2014), a concentration similar to what can be found tonically in CSF (Frederickson et al., 2006a). However, other studies have reported 200-800 nM concentrations of zinc in various electrophysiological solutions, suggesting that this could be a significant confound in the published studies in the GlyR field (Thio and Zhang, 2006; Wilkins and Smart, 2002; Zheng et al., 1998). For example, if buffers used in previous studies contained concentrations of zinc sufficient to result in peak enhancement of D80G GlyR function (Fig. 2), then added zinc might appear to have no effect on the mutant receptors while still being capable of enhancing WT receptor function. However this would not explain why we saw at least as great an effect of zinc in D80A as in WT α1 GlyRs (Fig. 2).

Allosteric modulators of ligand-gated ion channels typically show the greatest effects at low agonist concentrations, with decreases in percent enhancement occurring as the agonist concentration is increased (Farley and Mihic, 2015). Since previous studies of α1 D80 mutant GlyRs typically assayed zinc-sensitivity using higher glycine concentrations than reported here ($\geq EC_{25}$), we hypothesized that perhaps D80 mutants might have a steeper rate of decline in zinc-enhancement with increasing agonist concentrations than WT receptors, thereby appearing insensitive to zinc at currents generated by higher concentrations of glycine. We found that D80A, D80G, and WT receptors all showed a decrease in zinc enhancement with increasing agonist concentration (Fig. 3). However, D80G receptors had significantly reduced zinc potentiation compared to WT receptors when activated by glycine concentrations ranging from EC_{20-40} but not EC_{0-20} (Fig. 4). These data suggest that a steeper decline in zinc-enhancement with increasing agonist concentration, coupled with the possible presence of higher levels of contaminating zinc than in the buffers used here, might account for previous reports of abolished zinc enhancement in D80G GlyRs. However, this would not explain why D80A GlyRs show such marked zinc enhancement in our hands.

The GlyR residues that are involved in the high affinity binding of zinc remain unclear. Our data suggests that D80 is not part of a high affinity zinc-binding site, as robust enhancement of receptor function by zinc is seen in both D80A and D80G GlyRs expressed in *Xenopus laevis* oocytes. Other studies of D80 GlyR mutants are also contradictory as to whether D80 is a zinc binding residue. Lynch et al. (1998) found that 1 μM zinc significantly increased the apparent affinities of glycine and taurine in D80A mutant receptors, consistent with a mechanism of positive allosteric modulation. Functionally, however, glycine-mediated currents in D80A GlyRs were insensitive to enhancement by zinc while taurine-mediated currents were still enhanced. Lynch et al. (1988) concluded that, rather than acting as a zinc-binding site, D80 may instead be involved in allosterically linking zinc binding to channel gating. While our results could possibly account for the apparent lack of zinc enhancement in previous reports of D80G GlyRs, we were unable to do the same for the D80A receptor. One speculative possibility is that D80A mutations differentially affect zinc enhancement of receptors expressed in different cell types, as previous studies characterizing D80A receptors were conducted in mammalian cells while we utilized *Xenopus laevis* oocytes.

Another putative high-affinity zinc binding site associated with GlyR enhancement involves residues E192, D194, and H215 (Miller et al., 2005). W170 is in very close proximity to these residues and mutation of W170 to serine is thought to disrupt this binding pocket, thereby preventing enhancement by zinc (Zhou et al., 2013). Since we were able to successfully confirm the zinc-insensitive nature of this mutant, we employed it as a tool to screen for interactions between zinc and other modulators of GlyR function. We first investigated the effects of ethanol on W170S receptors compared to the effects of tricine on ethanol modulation of WT GlyRs (Fig. 5). W170S receptors exhibited a significantly decreased response to ethanol compared to WT receptors in the presence of contaminating concentrations of zinc, and this ethanol effect was not further decreased by tricine. This suggests that tricine has no effect on

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ethanol modulation of GlyRs apart from its effects on zinc chelation and demonstrates that the W170S receptor serves as an appropriate model to investigate modulator function in the absence of co-modulation by zinc. We next used the W170S receptor to probe for zinc interactions with other GlyR modulators, finding significantly reduced enhancement produced by inhalants in W170S GlyRs, suggesting that these modulators also interact with zinc to synergistically enhance receptor function (Fig. 6). However, this was not true for GlyR enhancement by halothane, chloroform and isoflurane, which had similar effects in WT and W170S receptors. This suggests that there are some mechanistic differences in how these volatile anesthetics enhance GlyR function, compared to ethanol and inhaled drugs of abuse.

Previous studies investigating zinc's interactions with other GlyR modulators used zinc concentrations ≤ 1 μM. However, much higher concentrations of zinc may be present after synaptic release. We therefore investigated the effects of 10 μM $ZnCl_2$ on ethanol modulation (Fig. 7). Interestingly, we saw that enhancement by ethanol was significantly decreased in WT receptors in the presence of 10 μM zinc compared to standard MBS. One possible explanation is that occupation of the inhibitory zinc-binding site inhibits the ability of ethanol to modulate the channel. However, when we repeated this experiment with W170S receptors, which only exhibit zinc inhibition, 10 μM zinc had no effect on ethanol modulation. This suggests that saturation of the zinc-enhancing site, not occupation of the inhibitory site, results in the decrease in ethanol's enhancement seen in WT receptors. While this seems counter-intuitive with the findings that lower concentrations of zinc act synergistically with ethanol to enhance GlyR function, these data fit nicely with a mechanism by which ethanol, in addition to modulating the channel on its own, also increases the affinity of zinc at the GlyR-enhancing site. The lower degree of ethanol enhancement seen in the presence of tricine reflects the ability of ethanol itself to enhance GlyR function. In the presence of lower concentrations of zinc, ethanol's enhancement appears larger because in addition to directly modulating channel function, it increases the affinity of zinc

binding to the GlyR-enhancing site, thereby increasing enhancement by zinc as well. However, in the presence of higher concentrations of zinc, the GlyR-enhancing zinc site is already saturated and once again the effects that are seen are those produced by ethanol alone. The similar results observed with TCE, suggest that inhalants interact with zinc by the same mechanism. However, halothane enhancement was unaffected by zinc concentration in WT and W170S receptors, providing further support that volatile anesthetic modulation of GlyRs is not affected by the presence of zinc and that not all of these allosteric modulators act by exactly the same mechanisms.

Authorship Contributions

Participated in research design: Cornelison and Mihic

Conducted experiments: Cornelison, Daszkowski, and Pflanz

Performed data analysis: Cornelison and Mihic

Wrote or contributed to the writing of the manuscript: Cornelison, Daszkowski, and Mihic

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Footnotes

This research was supported by the National Institutes of Health National Institute on Alcohol Abuse & Alcoholism [Grant 5P01AA020683].

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Figure Legends

Figure 1. Effects of low nanomolar concentrations of zinc on α1 homomeric WT, D80A, D80G, and W170S glycine receptors. (A) Sample tracings showing the effects of 100 nM $ZnCl_2$ or 2.5 mM of the zinc-chelator tricine on EC_{5-10} glycine responses in WT or mutant α1 glycine receptors. (B) Summary graph of the effects of 2.5 mM tricine on EC_{5-10} glycine currents in WT and mutant glycine receptors. Tricine had a significantly decreased effect on W170S compared to WT GlyRs. Data are shown as the mean - S.E.M. of 3-4 oocytes. One-way ANOVA followed by Tukey's post-hoc: *, $P < 0.05$. (C) Summary graph of the effects of 100 nM $ZnCl_2$ on EC_{5-10} glycine currents in WT and mutant glycine receptors. 100 nM $ZnCl_2$ had a significantly decreased effect on D80G and W170S compared to WT GlyRs. Data are shown as the mean + S.E.M of 9-16 oocytes. One-way ANOVA followed by Tukey's post-hoc tests: *, $P < 0.05$. ***, $P < 0.001$.

Figure 2. Effects of enhancing concentrations of $ZnCl_2$ on α1 homomeric WT, D80A, D80G, and W170S glycine receptors activated by EC_{5-10} glycine. Data are shown as the mean ± S.E.M. of 4-8 oocytes. A two-way ANOVA showed a significant effect of receptor [$F(3,119) = 76.68$, $p < 0.001$], a significant effect of zinc concentration [$F(5,119) = 14.367$, $P < 0.001$], and a significant interaction between receptor and zinc concentration [$F(15,119) = 2.402$, $p < 0.005$]. WT, D80A, and D80G GlyRs show bimodal concentration-response curves with peak enhancement at 1 μM $ZnCl_2$. Tukey's post-hoc tests revealed peak zinc enhancement was significantly decreased in D80G compared to WT GlyRs ($p < 0.05$) and while peak enhancement appeared to be increased in D80A compared to WT GlyRs, this was not significant ($p > 0.11$). W170S GlyR currents were unaffected by $ZnCl_2$ up to a concentration of 1 μM and inhibited by higher concentrations.

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Figure 3. Glycine dependence of zinc enhancement of GlyR function. WT (A), D80A (B), D80G (C), and W170S (D) GlyRs were tested for their sensitivities to the enhancing effects of zinc at a variety of glycine concentrations. Glycine concentration response-curves were generated (triangles) in the presence of 2.5 mM tricine and ordinate values are shown on the right axis of each panel. At each glycine concentration tested, the percent enhancement produced by 2.5 μM $ZnCl_2$ (in the presence of 2.5 mM tricine) was also determined (circles) and ordinate values are shown on the left axes. Horizontal dotted lines corresponds to 0% zinc enhancement for reference. One-way ANOVAs showed that enhancement by $ZnCl_2$ decreased with increasing glycine concentration for WT [$F(5,30) = 15.435$, $p < 0.001$, n = 5-7], D80A [$F(5,21) = 24.153$, $p < 0.001$, n = 4-5], and D80G [$F(7,31) = 7.785$, $p < 0.001$, n = 4-7], but not W170S [$F(6,28) = 0.472$, $p = 0.823$, n = 3-6] GlyRs. Data are shown as the mean ± S.E.M. of the indicated number of oocytes. Error bars sometimes fell within symbols.

Figure 4. Zinc enhancement of WT and D80 mutant GlyRs decreases as a function of glycine EC. Zinc-enhancement of WT (grey diamonds), D80A (filled circles), and D80G (hollow circles) GlyRs was plotted against glycine EC. One-way ANOVAs revealed significant differences among receptors at both EC_{0-20} glycine [$F(2,26) = 5.812$, $p < 0.01$] and EC_{20-40} glycine [$F(2,9) = 5.886$, $p < 0.05$]. Tukey's multiple comparisons post-hoc tests showed that, compared to WT receptors, zinc-enhancement of D80G receptors was significantly lower at intermediate-low (EC_{20-40}) glycine concentrations (+38.70 ± 11.64% change for D80G, compared to +92.07 ± 8.91% change for WT, $p < 0.01$), but not EC_{0-20} glycine, suggesting a steeper decline in zinc-mediated enhancement of D80G GlyRs with increasing glycine concentration. There were no significant differences in zinc-enhancement of D80A and WT receptors.

Figure 5. Zinc chelation has differential effects on ethanol enhancement of wildtype and W170S GlyRs. (A) Sample tracings showing the effects of zinc chelation on EtOH modulation of GlyRs activated by EC_{5-10} glycine. The effects of 50 and 200 mM EtOH on EC_{5-10} glycine currents

were first determined in standard MBS known to contain low nM concentrations of contaminating zinc, and then determined in MBS containing 2.5 mM of the zinc-chelator tricine. (B) Summary graph of the data presented in panel A. Zinc chelation significantly decreased the effects of 50 and 200 mM EtOH in WT GlyRs. Ethanol enhancement of W170S GlyRs in normal MBS was lower than in WT receptors and was not further decreased by the addition of tricine. There was also no difference in EtOH modulation between WT and W170S glycine receptors in the presence of tricine. Data are shown as the mean + S.E.M. of 4-7 oocytes. Two-way ANOVA followed by Tukey's post-hoc tests: **, P < 0.01. ***, P < 0.001.

Figure 6. Co-modulation by low nanomolar zinc affects allosteric modulation of glycine receptors by inhalants but not volatile anesthetics. (A) Summary graph of the effects of approximately minimum alveolar concentrations of the volatile anesthetics isoflurane, chloroform and halothane on WT and W170S glycine receptors activated by EC_{5-10} glycine. No effect of receptor was seen. Data shown as the mean + S.E.M. of 4-5 oocytes. (B) Summary graph of the effects of the inhalants TCE, toluene and TCY on WT and W170S glycine receptors activated by EC_{5-10} glycine. Data are shown as the mean + S.E.M. of 5 oocytes. Student's t-test *, P < 0.05. **, P < 0.01.

Figure 7. Micromolar concentrations of zinc, which may be found after synaptic release, inhibit EtOH enhancement of α1 WT but not W170S GlyRs. (A) Sample tracings showing the effect of 10 μM of added $ZnCl_2$ on EtOH modulation of α1 WT and W170S GlyRs activated by EC_{5-10} glycine. The effects of 50 and 200 mM EtOH on EC_{5-10} glycine currents were first determined in standard MBS buffer, known to contain low nM concentrations of contaminating zinc, and then determined in MBS containing 10 μM $ZnCl_2$. EC_{5-10} glycine concentrations were independently determined in the absence (leftmost 4 tracings) and presence (rightmost 4 tracings) of 10 μM zinc, so that the two sets of tracings would produce similar currents when glycine was applied in

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the absence and presence of 10 μM zinc; i.e., in the four rightmost tracings in WT receptors, a lower glycine concentration was used to compensate for the enhancing effects of 10 μM zinc. (B) Summary graph of the data presented in panel A. The addition of 10 μM $ZnCl_2$ to the buffer significantly decreased the effects of 50 and 200 mM EtOH in WT GlyRs. Ethanol enhancement of W170S GlyRs in normal MBS was decreased compared to wildtype receptors but was not further decreased by added $ZnCl_2$. There was also no difference in EtOH modulation between WT and W170S glycine receptors in the presence of added $ZnCl_2$. Data are shown as the mean + S.E.M. of 5-6 oocytes. Two-way ANOVA followed by Tukey's post-hoc tests: **, $P < 0.01$. ***, $P < 0.001$.

Figure 8. Micromolar concentrations of zinc inhibit TCE but not halothane enhancement of α1 WT but not W170S GlyRs. The addition of 10 μM $ZnCl_2$ significantly decreased the effects of 0.56 mM TCE on WT GlyR function. TCE enhancement of W170S GlyRs in normal MBS was decreased compared to wildtype receptors and was not further decreased by added $ZnCl_2$. There was no difference in TCE modulation between WT and W170S glycine receptors in the presence of added $ZnCl_2$. There were no significant differences in halothane modulation of WT or W170S GlyRs, regardless of the concentration of zinc present. Data are shown as the mean + S.E.M. of 4 oocytes. Two-way ANOVA followed by Tukey's post-hoc tests: **, $P < 0.01$. ***, $P < 0.001$.

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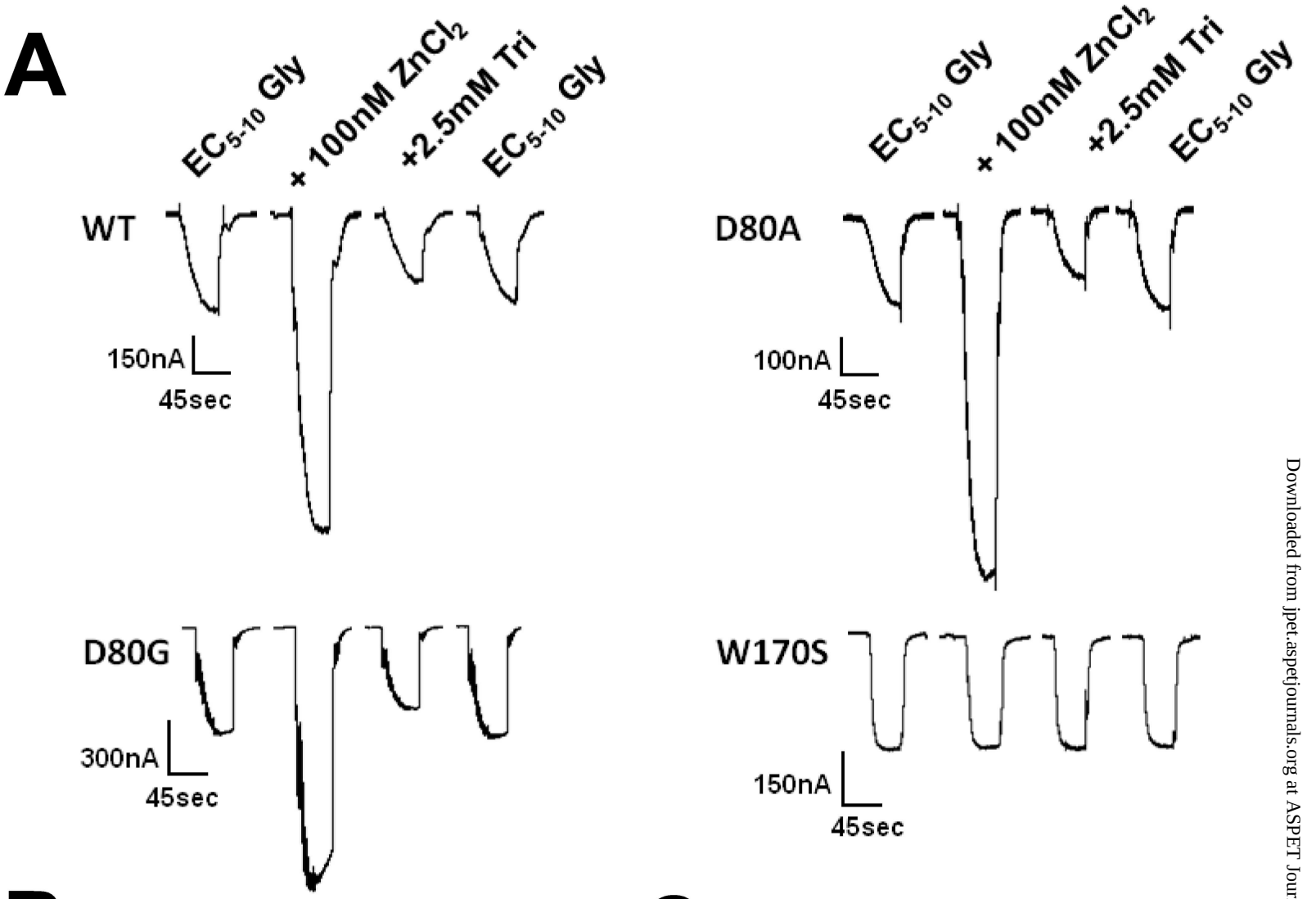

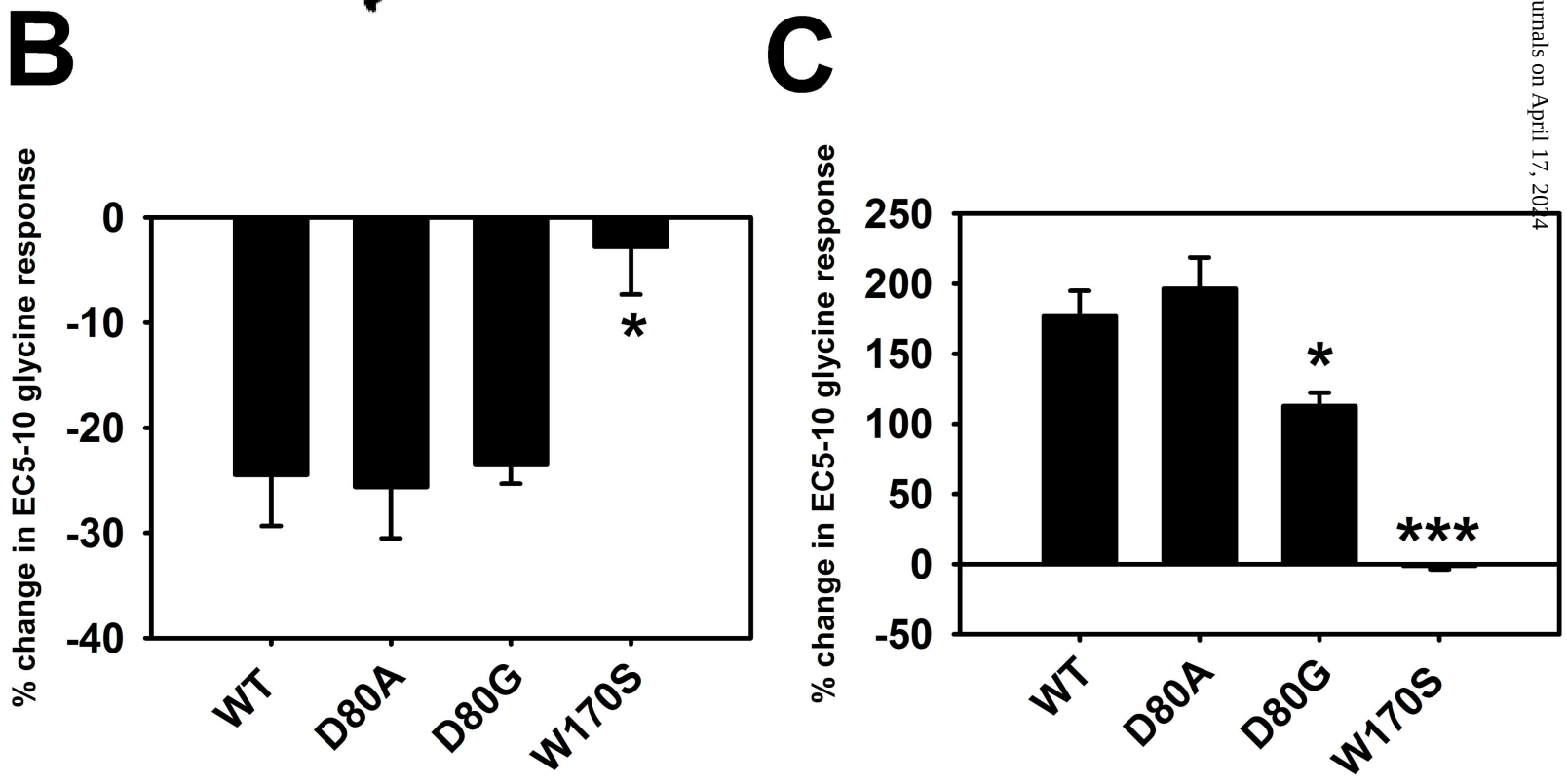

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Figure 2

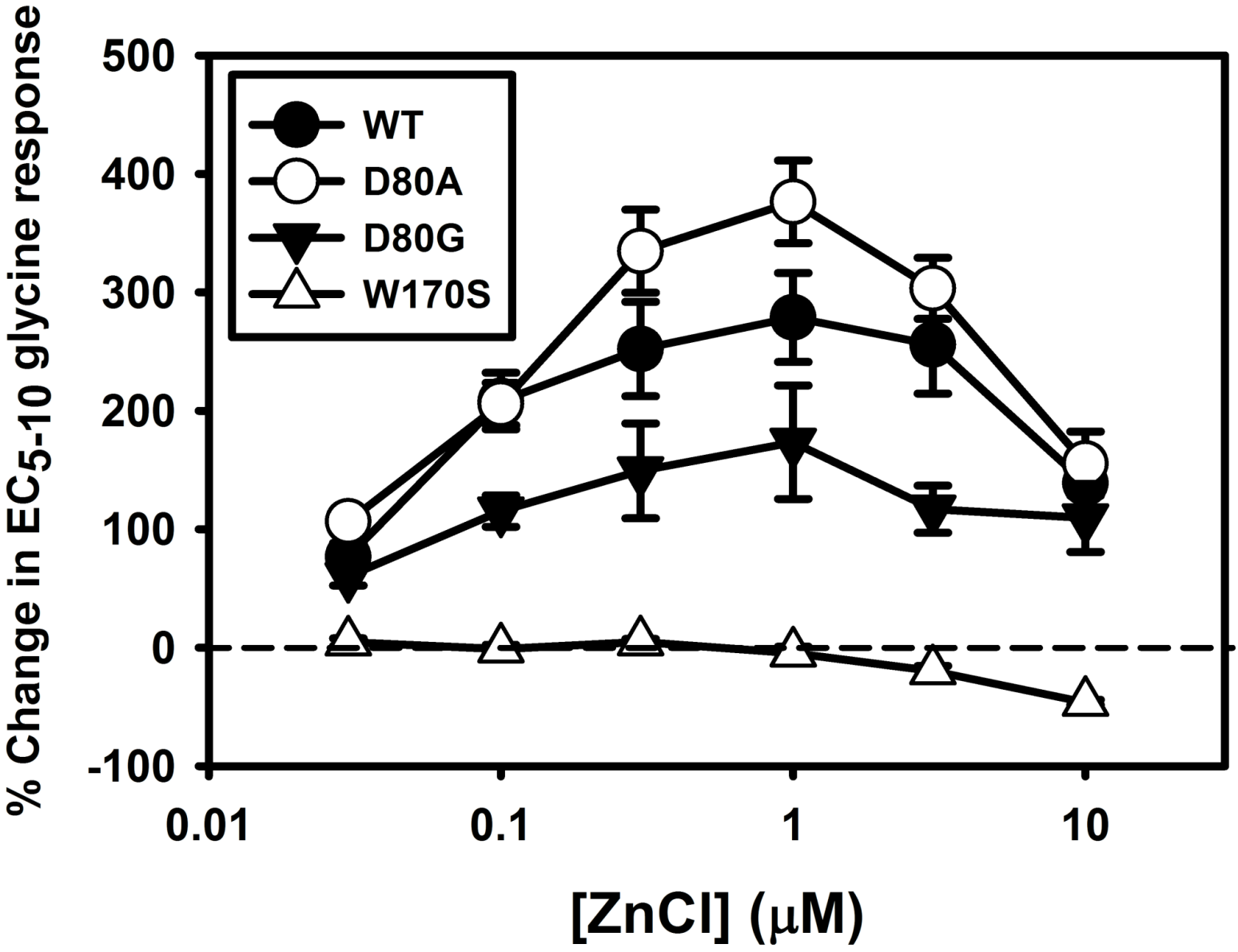

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Figure 3

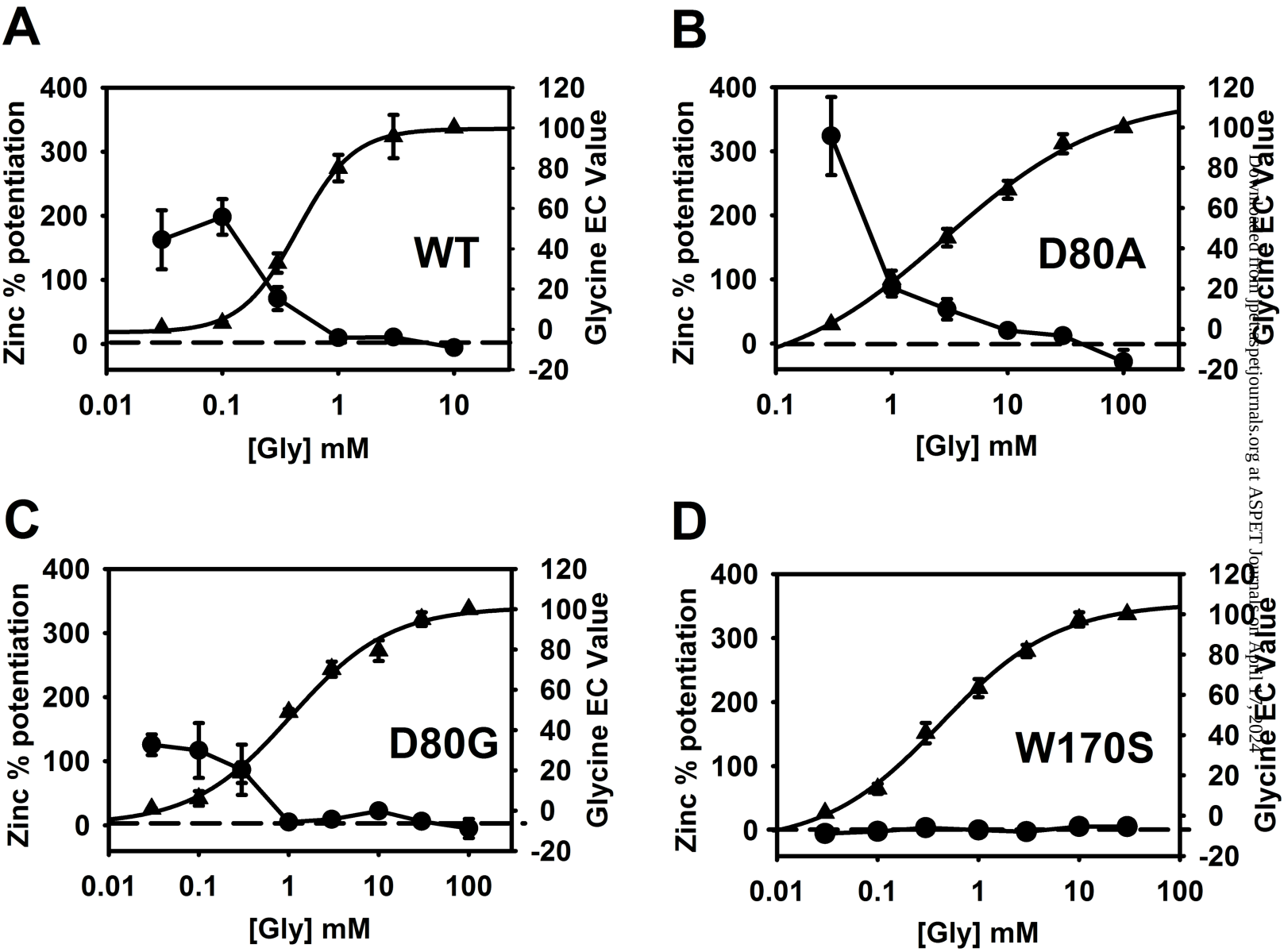

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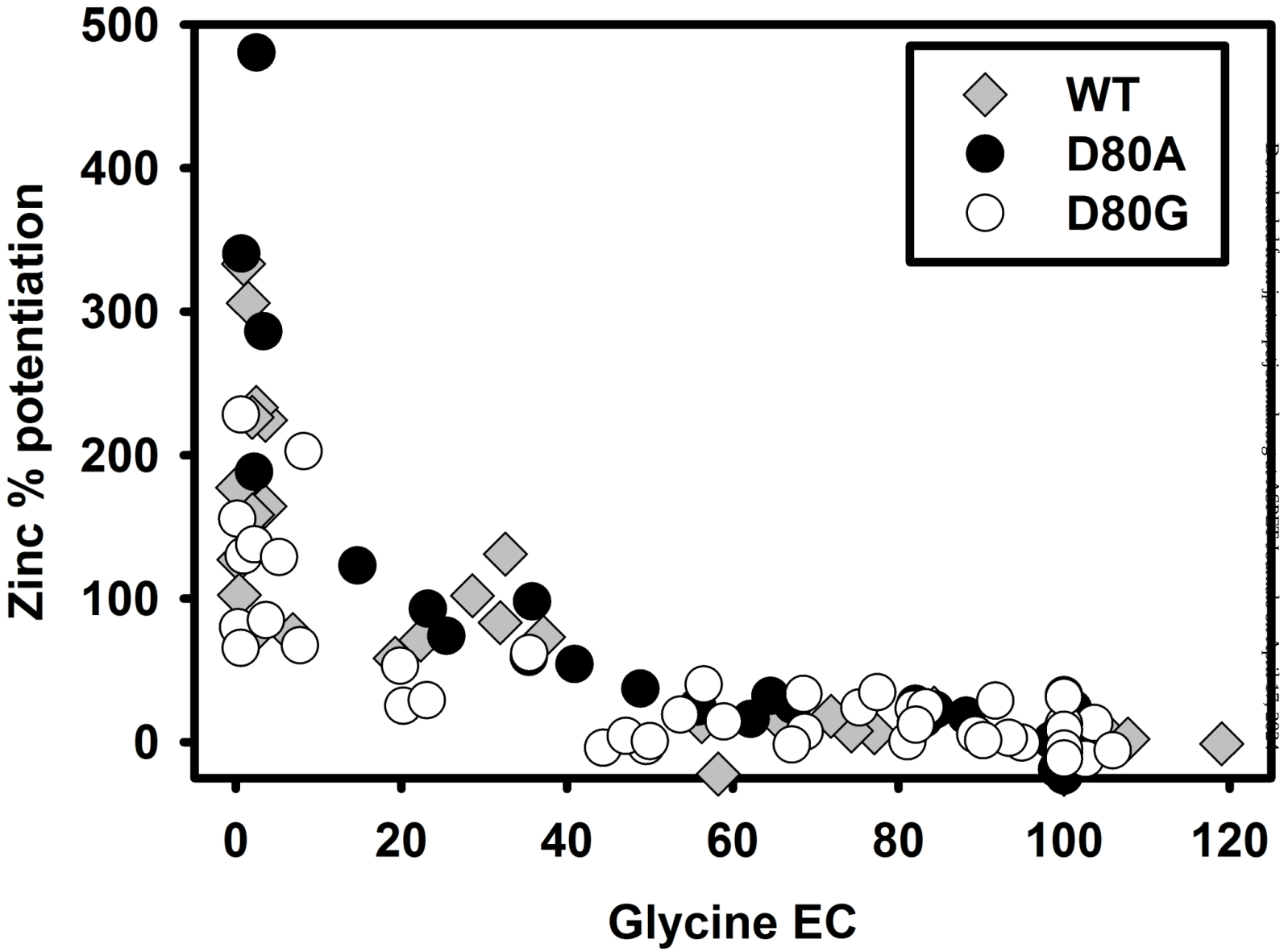
Figure 4



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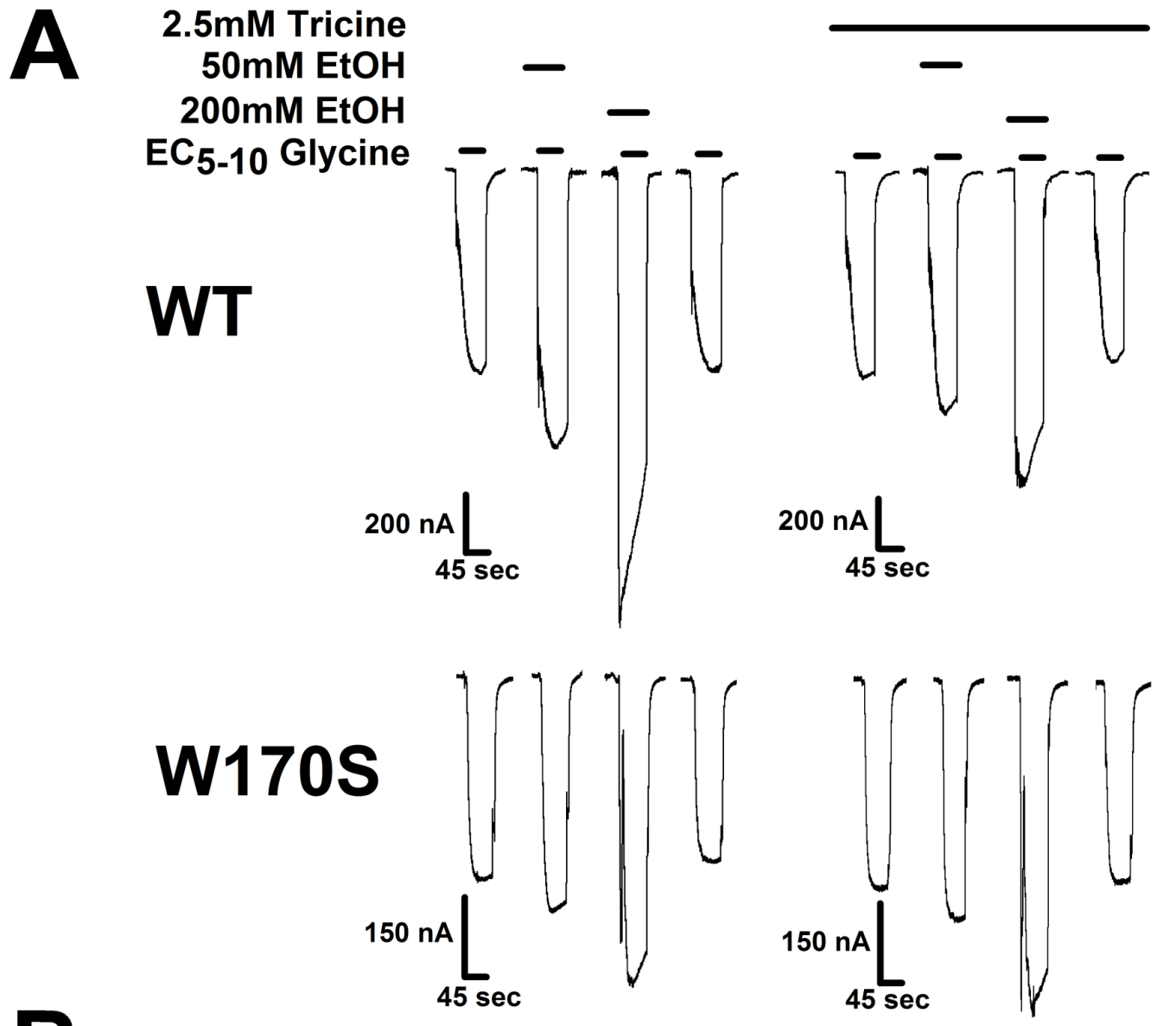

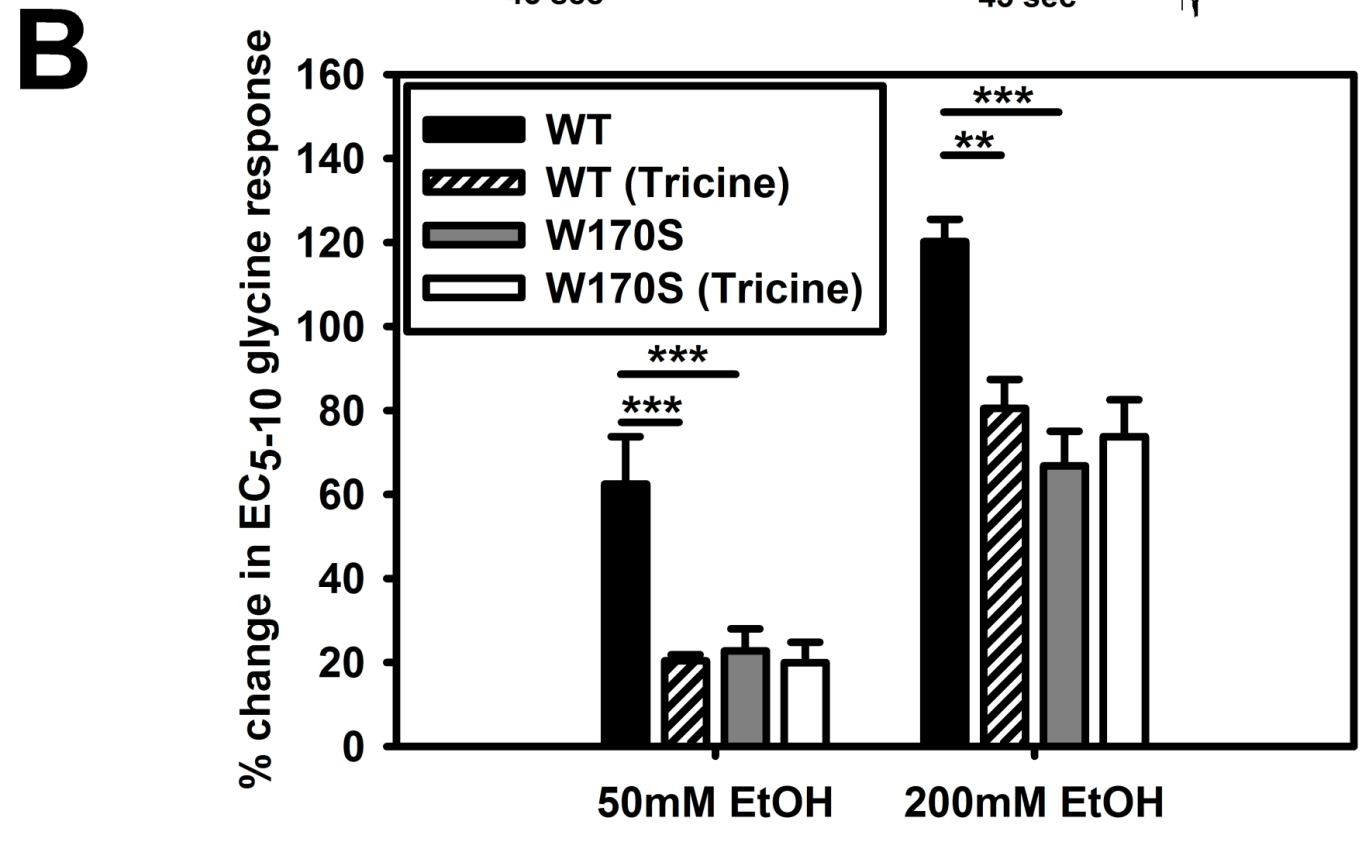

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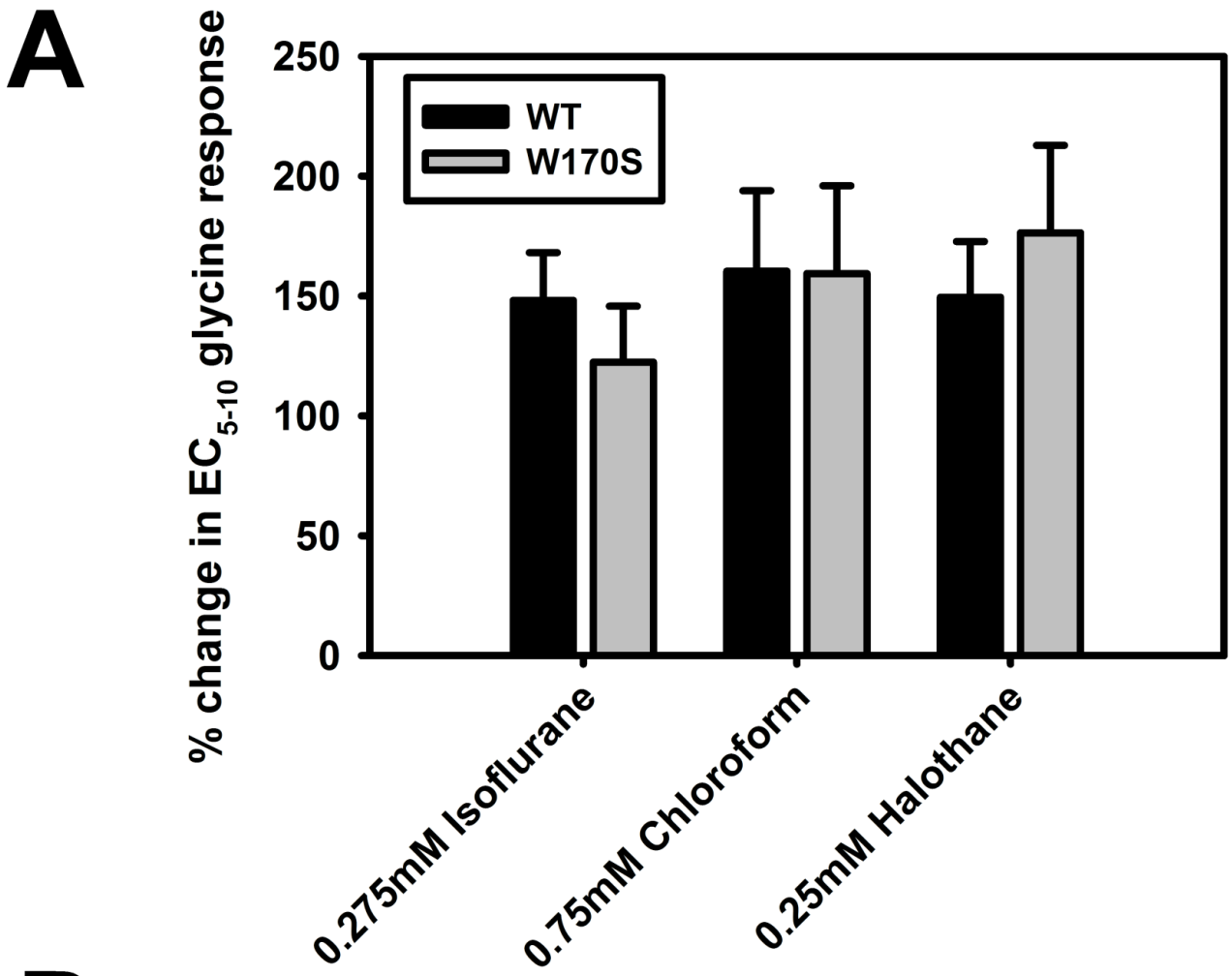

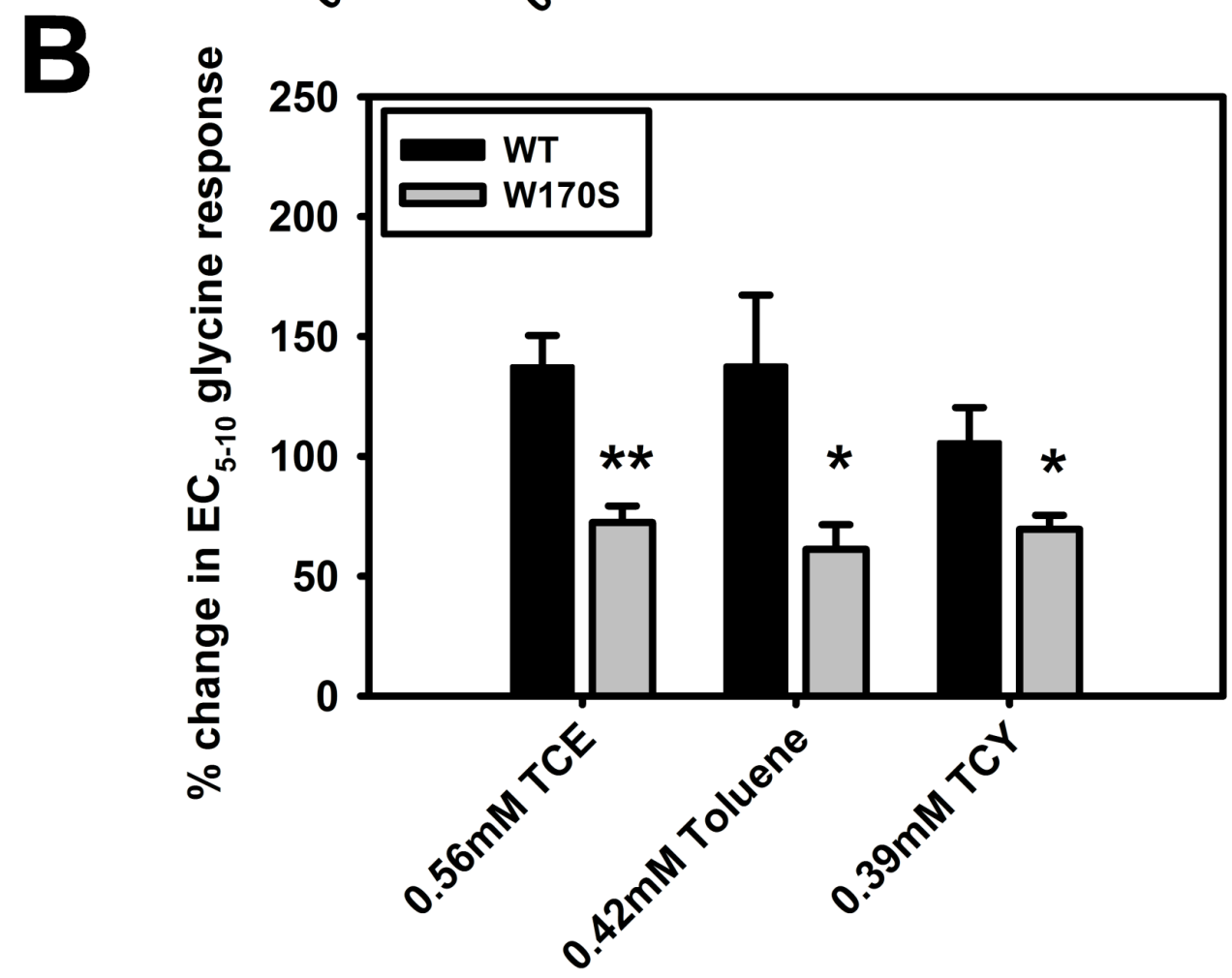

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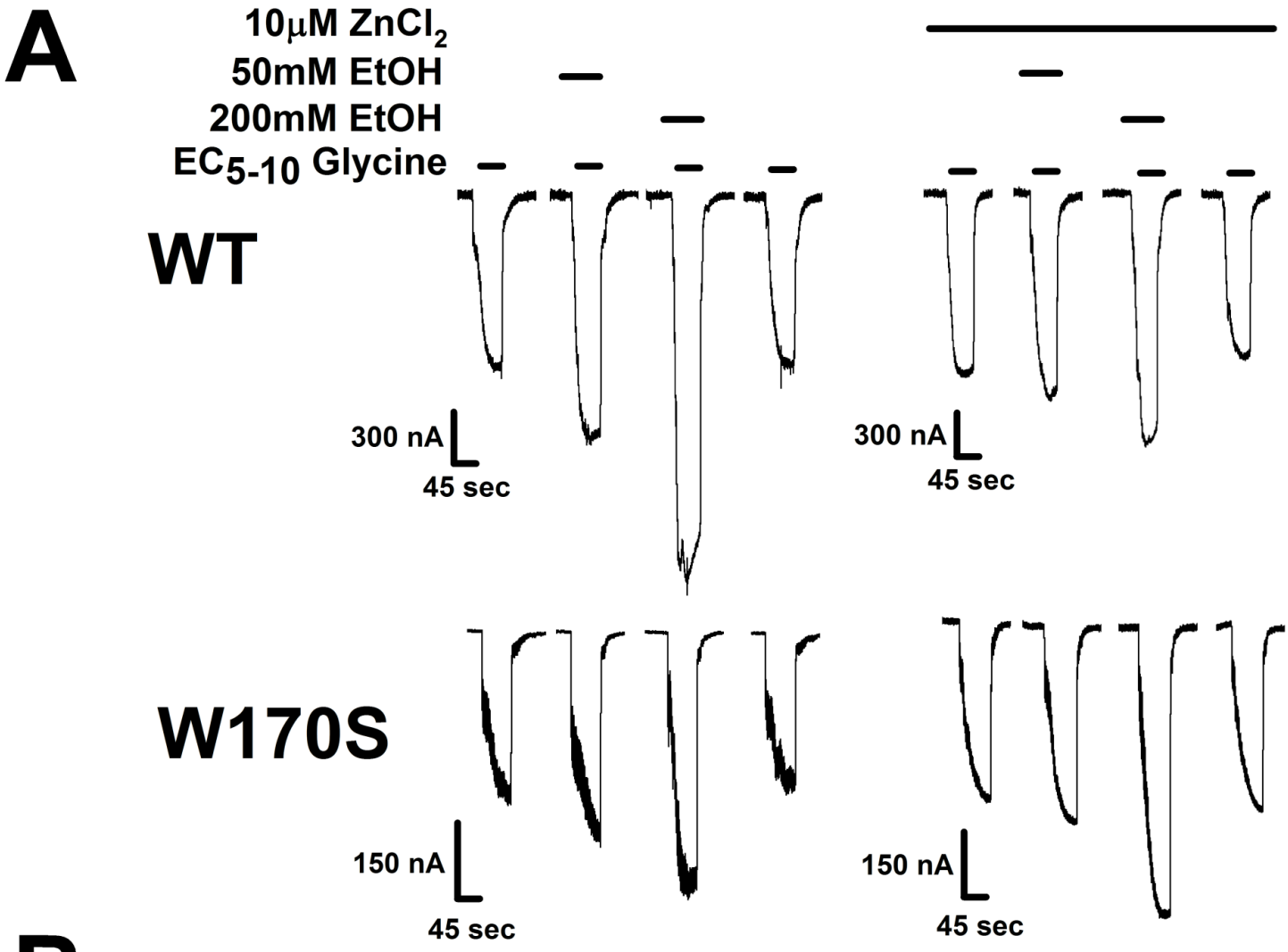

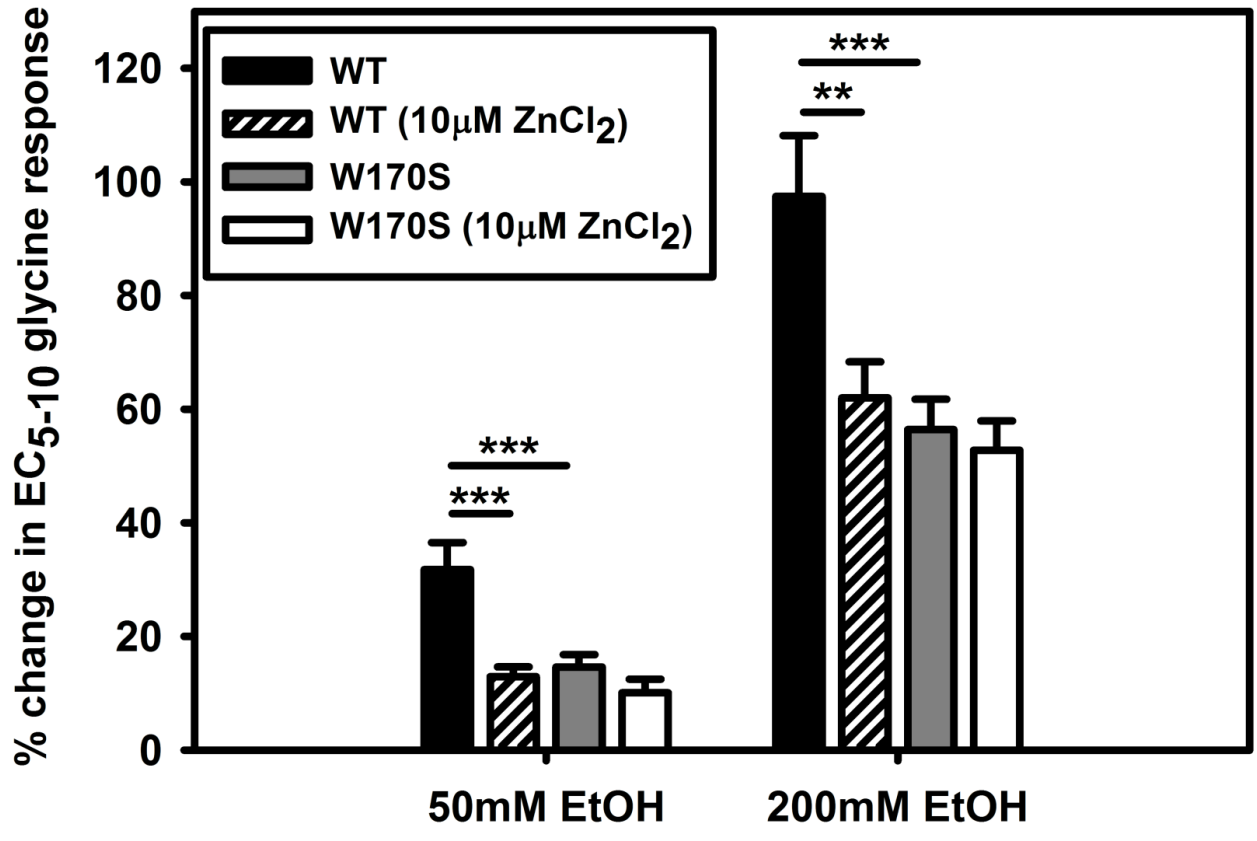

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Figure 8