Activation and modulation of recombinant glycine and $GABA_A$ receptors by 4-halogenated analogues of propofol

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Short title: Actions of halogenated propofol analogues

Abstract

Background and Purpose. Glycine receptors are important players in pain perception and movement disorders, and therefore an important therapeutic target. Glycine receptors can be modulated by the intravenous anesthetic propofol (2,6-diisopropylphenol); however, the drug is more potent, by at least one order of magnitude, on $GABA_A$ receptors. It has been proposed that halogenation of the propofol molecule generates compounds with selective enhancement of glycinergic modulatory properties.

Experimental Approach. We synthesized 4-bromopropofol, 4-chloropropofol, and 4-fluoropropofol. The direct activating and modulatory effects of these drugs and propofol were compared on recombinant rat glycine and human $GABA_A$ receptors expressed in oocytes. Behavioral effects of the compounds were compared in the tadpole loss-of-righting assay.

Key Results. The concentration-response curves for potentiation of homomeric α1, α2, and α3 glycine receptors were shifted to lower drug concentrations by 2-10-fold for the halogenated compounds. Direct activation by all compounds was minimal with all subtypes of the glycine receptor. The four compounds were essentially equally potent modulators of the α1β3γ2L $GABA_A$ receptor with EC_{50}s between 4 and 7 μM. The EC_{50}s for loss-of-righting in *Xenopus* tadpoles, a proxy for loss of consciousness and considered to be mediated by actions on $GABA_A$ receptors, ranged from 0.35 to 0.87 μM.

Conclusions and Implications. We confirm that halogenation of propofol more strongly affects modulation of homomeric glycine receptors than α1β3γ2L $GABA_A$ receptors. However, the effective concentrations of all tested halogenated compounds remained lower for $GABA_A$ receptors. We infer that 4-bromo-, 4-chloro, or 4-fluoropropofol are not selective homomeric glycine receptor modulators.

TARGETS
Ligand-gated ion channels[a]
$GABA_A$ receptor
$GABA_A$ receptor α1 subunit
$GABA_A$ receptor β3 subunit
$GABA_A$ receptor γ2 subunit
Glycine receptor
Glycine receptor α1 subunit
Glycine receptor α2 subunit
Glycine receptor α3 subunit

LIGANDS
GABA
Glycine
Propofol

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a]Alexander *et al.*, 2015).

Abbreviations

4-BP, 4-bromopropofol; 4-CP, 4-chloropropofol; 4-FP, 4-fluoropropofol

Introduction

Glycine receptors are members of the Cys-loop receptor family (Lynch, 2004; Lynch, 2009). The Cl^--permeable channels mediate inhibitory neurotransmission in the retina, brainstem, and spinal cord. Functional receptors are formed as homopentamers of one of four α subunits or heteropentamers consisting of α and β subunits. Subunit expression is region-specific and regulated developmentally. For example, prenatal glycinergic transmission is largely mediated by α2 homomers, whose expression is reduced sharply following birth (Malosio *et al.,* 1991). Receptors containing the α1 subunit are dominant in adult retina and spinal cord. Interference with α1 expression, or introduction of mutations disrupting receptor function, can result in the hyperekplexia phenotype (Bode *et al.,* 2014; Schaefer *et al.,* 2013). Glycine receptors containing the α3 subunit are expressed in the retina and in nociceptive sensory neurons in the spinal cord dorsal horn where they may mediate pain associated with chronic peripheral inflammation (Harvey *et al.*, 2004; Haverkamp *et al.,* 2003). Thus, glycine receptors can be an important potential target in a variety of physiological processes.

Recent studies have reported that halogenated analogues of propofol act as potent activators and modulators of the glycine receptor. In mouse ventral horn neurons, submicromolar concentrations of 4-bromopropofol (4-BP) elicit tonic current that can be blocked by strychnine, but not bicuculline (Eckle *et al.*, 2014). 4-Chloropropofol (4-CP) reportedly potentiates currents from heterologously expressed α1 receptor with the concentration producing half-maximal effect below 1 nM (de la Roche *et al.*, 2012). These results led to the proposal that these, and other novel halogenated analogues of propofol, may be clinically useful in treating conditions mediated by glycine receptor functional deficiencies such as hyperekplexia, or employed as a starting point in the development of non-sedative analgesics.

Propofol and its analogues can also activate and potentiate the γ-aminobutyric acid type A ($GABA_A$) receptor (Hales *et al.*, 1991; Krasowski *et al.*, 2001). These actions likely underlie the loss-of-righting reflex and other anesthetic endpoints following application of propofol (Jurd *et al.*, 2003). Previous work on halogenated analogues of propofol has demonstrated that bromine substitution at the ortho-position reduces anesthetic and GABAergic activity of the analogues, while compounds with halogen-substitutions para to the phenolic hydroxyl group remain strong GABAergic modulators (Krasowski *et al.,* 2001; Trapani *et al.,* 1998).

To provide a direct comparison of activation and modulation of glycine and $GABA_AR$ by this class of compounds and to test the potential for receptor selectivity, we compared direct activation and modulation of recombinant α1, α2 and α3 homomeric glycine receptors, and α1β3γ2L $GABA_A$ receptors by propofol, 4-BP, 4-CP, and 4-fluoropropofol (4-FP). We show that these compounds are weak activators of the glycine receptor, with maximal observed responses at approximately 20% of the response to saturating glycine. All halogenated analogues were more potent than propofol at potentiating responses to a low concentration of glycine, with the EC_{50}s for potentiation left-shifted by up to an order of magnitude. 4-BP and 4-CP, but not 4-FP, were also more potent than propofol at modulating the α1β3γ2L $GABA_A$ receptor. The EC_{50}s for loss-of-righting, a proxy for loss of consciousness in humans (Franks, 2008), were measured in *Xenopus* tadpoles and found to be 0.35-0.87 μM for propofol and its analogues. We infer that while halogenation of the propofol molecule does alter target selectivity, biasing it towards modulation of glycine receptors, 4-halogenated analogues of propofol do not attain sufficient selectivity for use as selective glycinergic modulators.

Methods

Molecular biology and expression of receptors

The glycine and $GABA_A$ receptors were expressed in *Xenopus* oocytes. The cDNAs in the pcDNA3 (glycine α3, $GABA_A$ α1, β3, γ2L), pcDNA3.1 (glycine α2), or pGEMHE vector (glycine α1) were linearized by digestion with Xba I, NheI, Hind III, or BglII (NEB Labs, Ipswich, MA). The cRNAs were produced using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with a total of 18-33 ng cRNA in a final volume of 20-60 nl, and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM Na pyruvate, 5 mM HEPES; pH 7.4) at 16 ^oC. The ratio of $GABA_A$ receptor cRNAs used for injection was 5:1:5 (α1:β3:γ2L). Electrophysiological measurements were conducted within 1-3 days after injection.

Electrophysiology

Electrophysiological experiments were conducted using the standard two-electrode voltage clamp approach. Voltage and current electrodes were regular patch-clamp electrodes that when filled with 3 M KCl had resistances of less than 1 MΩ. The oocytes were clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT) was perfused continuously at approximately 5 ml min^{-1}. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES; pH 7.4) was perfused between all test applications. Solutions were gravity-applied from 30-ml glass syringes with glass luer slips via Teflon tubing to reduce adsorption, and were switched manually. A typical experiment consisted of recording of a 10 s baseline, followed by a 20-60 s drug application, and then a bath application (up to 10 min) until full recovery. Duration of drug application depended on the nature of drug and its concentration, and was aimed at reaching a saturated peak response. The current responses were amplified with an Axoclamp 900A (Molecular Devices, Sunnyvale, CA) or OC-725C amplifier (Warner Instruments, Hamden, CT), filtered at 40 Hz, digitized with a Digidata 1320 or 1200 series digitizer (Molecular Devices) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were subsequently analyzed with Clampfit (Molecular Devices) to determine the maximal amplitude of current response.

The maximal final DMSO concentration in drug solutions was 0.25%. Control experiments

showed that 0.5% DMSO was without effect on holding current (<0.1% of the response to saturating transmitter). Coapplication of 0.5% DMSO with an EC_{50} concentration of a transmitter had no effect (glycine α1, $GABA_A$ α1β3γ2L) or a small effect (<10%; glycine α2, α3) on the peak response.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Activation concentration-response curves were fitted for data from each individual cell with the following equation:

$$Y=Y_{max}*([drug]^{nH}/([drug]^{nH}+EC_{50}{}^{nH})) \quad (1)$$

where EC_{50} is the concentration of drug producing a half-maximal effect, n_H characterizes the slope of relationship, and Y_{max} is the high concentration asymptote. Fitting was conducted using the NFIT software (The University of Texas Medical Branch at Galveston, Galveston, TX). Parameters of the fit are reported as mean ± S.E.M. The concentration-response data are presented as response relative to Y_{max}.

Potentiation concentration-response relationships were determined by exposing cells to a low concentration of agonist (EC_{4-14} of glycine or GABA), in the absence and presence of several concentrations of a modulator (4-BP, 4-CP, 4-FP, or propofol). Control applications to saturating glycine or GABA were typically done after every 2-3 test applications, to verify overall stability of responses. There were no apparent changes in potentiation parameters for data obtained within this range of EC values. The concentration-response curves for potentiation are presented as response relative to the peak response in the presence of saturating agonist. The curves were fitted for data from each individual cell with the following equation:

$$Y=Y_{min}+(Y_{max}-Y_{min})*([drug]^{nH}/([drug]^{nH}+EC_{50}{}^{nH})) \quad (2)$$

where EC_{50} is the concentration of drug producing a half-maximal effect, n_H characterizes the

slope of relationship, and Y_{min} and Y_{max} are the low and high concentration asymptotes, respectively.

Statistical analysis was done using ANOVA with Bonferroni correction (Stata/IC 12.1, StataCorp, College Station, TX).

Behavioural assays

Predetermined concentrations of 4-BP, 4-CP, 4-FP, or propofol were added to beakers containing 100 ml of oxygenated Tadpole Ringer's solution (5.8 mM NaCl, 67 µM KCl, 34 µM $Ca(NO_3)_2$, 83 µM $MgSO_4$, 419 µM Tris-HCl, 80 µM Tris-Base; pH 7.5). Ten randomly chosen tadpoles were distributed into each beaker and allowed to equilibrate in the Tadpole Ringer's solution for 3 hrs. In the end of the equilibration period, the loss-of-righting reflex (LRR) was measured by turning the tadpole over using a hooked glass rod. LRR was defined as the inability of a tadpole to right itself within 5 seconds on its back in three consecutive trials. The operator was blinded to the concentration of compound in the beaker.

Compliance with requirements for studies using animals

Oocytes from the African clawed frog (*Xenopus laevis*) were used for expression of glycine and $GABA_A$ receptors. Oocytes are a widely used and standard expression system for recombinant receptor-channels. Their use permits studies of a defined population of receptors to provide specific and reliable pharmacological information. Frogs were purchased from Xenopus 1 (Dexter, MI), and were housed and cared for in a Washington University Animal Care Facility under the supervision of the Washington University Division of Comparative Medicine. Harvesting of oocytes was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Oocytes were harvested from mature female frogs under tricaine anaesthesia. Oocytes were harvested twice per frog. Frogs were euthanized under tricaine anaesthesia by rapid decapitation. The protocol is approved by the Animal Studies Committee of Washington University in St. Louis (Approval No. 20140150).

Early prelimb-bud stage tadpoles from *Xenopus laevis* were used in behavioural assays. The use of tadpoles is justified because there are no cell lines or computer modeling systems that

replace the use of animals for behavioural studies. Tadpoles (purchased from Nasco, Fort Atkinson, WI) were kept in continuously aerated Tadpole Ringer's solution in aquaria and used within 6 days of receipt. The aquaria are inspected and approved by the Washington University Animal Care Committee. Behavioural assays using *Xenopus laevis* tadpoles were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. We have complied with the ARRIVE guidelines for work involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010; McGrath *et al.*, 2015). A total of 370 tadpoles were used in the experiments described here. Tadpoles were euthanized after each experiment by addition of lethal concentrations of tricaine. The protocol for tadpole use is approved by the Animal Studies Committee of Washington University in St. Louis (Approval No. 20150076).

Chemical syntheses

4-Chloropropofol was synthesized according to the literature procedure (Wheatley *et al.*, 1958). The synthesis of 4-bromopropofol was adapted from the literature procedure (Wheatley *et al.*, 1958) as follows. To a stirred, orange solution of tetrabutylammonium tribromide (4.82 g, 10 mmole) in chloroform (20 ml) was added 2,6-diisopropylphenol (1.85 ml, 1.78 g, 10 mmole) at room temperature. After 30 min the chloroform was evaporated from the almost colourless solution and the residue was treated with water (30 ml) and diethyl ether (30 ml). The mixture was shaken and the two layers were separated. The organic layer was washed with water (3x) and dried ($MgSO_4$). Filtration and concentration gave an orange oil which was chromatographed on silica gel using hexane : ethyl acetate (19 : 1) as eluent to give the product as a pale yellow oil (1.59 g, 62%).

4-Fluoropropofol was synthesized as follows.

4-Nitro-2,6-diisopropylanisole (Chastrette *et al.*, 1986; Meek *et al.*, 1968): 2,6-Diisopropylanisole (760 mg, 4 mmole) was dissolved in chloroform (5 ml) and treated with potassium nitrate (456 mg, 4.56 mmole) and trifluoroacetic anhydride (2 ml). The flask was loosely stoppered and the mixture stirred at room temperature for 5 days. The mixture was filtered, the solid washed with chloroform (1 x 5 ml) and the combined filtrate and washings were concentrated to a light brown oil (1.41 g). This was chromatographed on silica gel using hexane : ethyl acetate (19 : 1) as eluent to give the

product as a yellow oil (910 mg, 96%).

4-Amino-2,6-diisopropylanisole (Ponomarev *et al.*, 1964): The 4-nitro derivative (910 mg, 3.84 mmole) was dissolved in ethanol (10 ml) and treated with tin dichloride dihydrate (2.25 g, 10 mmole). The mixture was stirred and heated at 90^{o}C for 4 h. The reaction mixture was cooled to room temperature and then ice/water (50 ml) was added followed by solid sodium hydroxide until the pH of the mixture was >13. Dichloromethane (50 ml) was added and the mixture was vacuum filtered. The resultant two phases were separated and the aqueous layer was extracted with dichloromethane (2 x 20 ml). The combined organic layers were dried (Na_2SO_4), filtered and concentrated to an orange-brown oil (900 mg). This was chromatographed on silica gel using hexane : ethyl acetate (3 : 2) as eluent to give the product as a red oil (564 mg, 71%).

4-Fluoro-2,6-diisopropylanisole (Kriuchkova *et al.*, 1960): The 4-amino derivative (564 mg, 2.72 mmole) was dissolved in dry THF (3 ml), stirred, cooled in an ice/acetone bath and treated with boron trifluoride etherate (0.65 ml, 5.2 mmole) and then t-butyl nitrite (0.36 ml, 313 mg, 3.05 mmole). The dark brown solution was allowed to warm to room temperature over 10 min then diluted with perfluorodecalin (3 ml). The resultant two-phase mixture was subjected to rotary evaporation to remove most of the THF and tBuOH (failure to do this or substituting a hydrocarbon solvent for perfluorodecalin results in some 2,6-diisopropylanisole which is very difficult to remove from its fluorinated analogue) and then stirred and heated to 115^{o}C over about 20 min. At 60 – 65^{o}C some gas evolution occurred. Once 115^{o}C had been attained, the flask was removed from the oil bath and cooled to room temperature. Acetonitrile (50 ml) was added. The lower, colourless, perfluorodecalin layer was removed and the acetonitrile evaporated to give a very dark brown, viscous liquid (1.367 g). This was chromatographed on silica gel using hexane : ethyl acetate (19 : 1) as eluent to give the product as a yellow oil (255 mg, 45%).

4-Fluoropropofol (Picard *et al.*, 1996). The 4-fluoroanisole (255 mg, 1.22 mmole) was dissolved in dichloromethane (15 ml) and cooled in an ice bath. To the stirred solution was added a solution of boron tribromide in dichloromethane (1 M, 5 ml, 5 mmole) and the resultant solution stirred at 0^{o}C for 45 min and then at room temperature for 3 h. The reaction was cooled in an ice bath and treated with water (15 ml) in one go and then the mixture was poured into diethyl ether (50 ml). The layers were shaken and separated and the organic layer was washed with saturated, aqueous sodium bicarbonate (1x) and water (1x) and dried ($MgSO_4$). The drying agent was filtered off and the filtrate was concentrated to a light brown oil (250 mg). This was chromatographed on silica gel

using hexane : ethyl acetate (19 : 1) as eluent to give the product as an off-white solid, m.p. 41 – 43^{o}C, (178 mg, 74%).

Materials

Rat α1, α2 and α3 homomeric glycine receptors and human α1β3γ2L $GABA_A$ receptors were expressed in *Xenopus* oocytes. The glycine α1 clone was obtained from Dr. J. Lynch (The University of Queensland), α2 from Dr. H. Akagi (Gunma University), and α3 from Dr. H. Betz (Max-Planck-Institute for Brain Research). The $GABA_A$ receptor subunit clones were provided by Drs. G. White (Neurogen Corporation) and D. Weiss (University of Texas Health Science Center, San Antonio). Inorganic salts used in buffers, glycine, and GABA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of glycine and GABA were made in bath solution at 500 mM. The stock solutions were stored in aliquots at -20^{o}C, and diluted as needed on the day of experiment. Stock solutions of propofol (200 mM) or its analogues (100-200 mM) were made in DMSO. Dilutions to working concentrations were made on the day of experiment.

Results

Direct activation of glycine and $GABA_A$ receptors by propofol and its 4-halogenated analogues

Oocytes expressing glycine or $GABA_A$ receptors responded with inward current to applications of propofol or its halogenated analogues. In general, the peak currents in response to applications of 4-halogenated analogues were small compared to responses to saturating concentrations of transmitter. Application of 100 μM 4-BP (the highest concentration tested) resulted in peak currents that were 12 ± 3% (mean ± S.E.M.; n = 5 cells), 6 ± 1% (n = 4), or 2 ± 0.1% (n = 4) of the response to saturating glycine in cells expressing α1, α2, or α3 homomeric glycine receptors, respectively. In oocytes expressing α1β3γ2L $GABA_A$ receptors, 100 μM 4-BP elicited a response that was 27 ± 2% (n = 4) of the response to saturating (1 mM) GABA.

To attempt to gain insight into whether the failure of these drugs to efficiently activate glycine receptors results from impaired binding (low-affinity) or gating (low-efficacy) we recorded current responses at lower drug concentrations. Relative to peak responses to saturating glycine, the mean responses to 0.2 μM and 10 μM 4-BP were 0.03 ± 0.02% (n = 5) and 0.12 ± 0.04% (n = 5) in α1, 0.01 ± 0.004% (n = 4) and 0.23 ± 0.12% (n = 4) in α2, and 0.22 ± 0.15% (n = 5) and 0.32 ± 0.18% (n = 6) in α3 glycine receptors. The responses to 0.2 μM and 10 μM 4-BP in α1β3γ2L $GABA_A$ receptors were 0.02 ± 0.01% (n = 4) and 6.2 ± 0.6% (n = 8) of the peak response to saturating GABA.

Application of 100 μM 4-CP elicited peak responses that were 14 ± 3% (n = 5), 21 ± 6% (n = 5), or 6 ± 1% (n = 5) of the response to saturating glycine in oocytes expressing α1, α2, or α3 glycine receptors, respectively. The α1β3γ2L $GABA_A$ receptors responded to application of 100 μM 4-CP with a peak response that was 50 ± 8% (n = 5) of the response to saturating GABA.

The 4-fluorinated analogue of propofol was the weakest activator. Exposure to 100 μM 4-FP resulted in peak responses that were 1.6 ± 0.2% (n = 4), 0.4 ± 0.1% (n = 4), or 0.3 ± 0.2% (n = 5) of the response to saturating glycine in oocytes expressing α1, α2, or α3 glycine receptors, respectively. In oocytes expressing α1β3γ2L $GABA_A$ receptors, application of 100 μM 4-FP elicited a peak response that was 33 ± 3% (n = 5) of the response to 1 mM GABA. Sample current traces are shown in Figure 1A.

We also tested the effect of the parent compound, propofol. In agreement with previous data (Feng *et al.*, 2004), the α1β3γ2L $GABA_A$ receptors were efficaciously activated by 100 μM

propofol, which produced a response that was 90 ± 3% (n = 7) of the response to saturating GABA. In contrast, in glycine receptors, propofol was an ineffective agonist. At 100 μM, propofol elicited responses that were 0.8 ± 0.3% (n = 4), 1 ± 0.3% (n = 4), or 0.2 ± 0.01% (n = 4) of the response to saturating glycine in α1, α2, or α3 receptors. A summary of direct activation data showing the ratios of responses to propofol and its analogues compared to saturating glycine or GABA is given in Figure 1B.

Potentiation of glycine receptors by propofol and its 4-halogenated analogues

We first determined the concentration-response relationships for receptor activation by glycine. Cells expressing each of the α subunits were exposed to 7-8 concentrations of glycine. The raw current amplitudes were fitted with the Hill equation for each cell individually. The EC_{50}s of the glycine activation curves were 79 ± 14 μM (n = 5), 64 ± 3 μM (n = 5), and 398 ± 52 μM (n = 5) in oocytes expressing α1, α2, and α3 receptors, respectively. These values are similar to those in prior reports (Downie *et al.*, 1996; Mascia *et al.*, 1996; Pistis *et al.*, 1997; Zhang *et al.*, 2008).

We next investigated the effect of propofol or its 4-halogenated analogues on responses elicited by glycine. Coapplication of 4-BP with a low concentration of glycine potentiated current responses (Figure 2). Homomeric α1 glycine receptors were activated by 17.5-22 μM glycine ($<EC_{15}$) and additionally exposed to 1-500 μM 4-BP. The test responses were normalized to control responses to a saturating concentration (0.5 mM) of glycine that was applied after every 2-3 test responses and at the end of recordings from each cell, to confirm overall stability of responses. The data show that 4-BP potentiates glycine-activated α1 receptors with an EC_{50} of 42 μM (n = 6). In an analogous experimental protocol, the EC_{50} for potentiation of α1 glycine receptors by 4-CP was 32 μM (n = 5), by 4-FP 78 μM (n = 5), and for potentiation by propofol 138 μM (n = 6). Thus, the 4-halogenated compounds are more potent than propofol at potentiating α1 glycine receptors. The EC_{50}s, maximal current levels in the presence of potentiator, and the Hill coefficients are given in Table 1.

The α2 subunit-containing glycine receptors were activated by 30-35 μM glycine ($<EC_{15}$) in the presence of 1-200 μM 4-BP, 1-200 μM 4-CP, 5-500 μM 4-FP, or 5-1000 μM propofol. The test responses were normalized to control responses to a saturating concentration (0.5 mM) of glycine. The half-maximal concentration for potentiation by 4-BP was 15 μM (n = 5). 4-CP potentiated α2

receptors with an EC_{50} of 23 μM (n = 5), and 4-FP potentiated the receptors with an EC_{50} of 62 ± 6 μM (n = 5). Potentiation in the presence of propofol yielded an EC_{50} of 95 μM (n = 5). The data and the results of statistical analyses are provided in Table 1.

The α3 receptors were activated by 85-100 μM glycine (<EC_{15}). Coapplication of 4-BP with glycine enhanced current responses. The half-maximal concentration of 4-BP for potentiation was 11 μM (n = 5). 4-CP and 4-FP produced EC_{50}s of 21 μM (n = 5) and 42 μM (n = 5), respectively. The EC_{50} for potentiation by propofol was 112 μM (n = 5). Sample traces are shown in Figure 2, and the data are summarized in Figure 3 and Table 1.

Potentiation of α1β3γ2L $GABA_A$ receptors by propofol and its 4-halogenated analogues

We tested potentiation of α1β3γ2L $GABA_A$ receptors by propofol and its halogenated analogues. The experiments were conducted using the experimental protocol described above for glycine receptors. A low concentration (1-3 μM) of GABA was applied in the presence of 0.2-50 μM 4-BP, 0.1-50 μM 4-CP, 0.2-50 μM 4-FP or 0.2-100 μM propofol. The current amplitudes were normalized to the response to saturating (1 mM) GABA.

For propofol, recordings from five cells produced an EC_{50} of 7 μM. Potentiation data obtained in the presence of the halogenated analogues of propofol yielded EC_{50}s of 4 μM (n = 7), 5 μM (n = 5), and 7 μM (n = 5) for 4-BP, 4-CP, and 4-FP, respectively. The concentration-response data are summarized in Figure 3 and Table 1.

Propofol and its 4-halogenated analogues elicit loss-of-righting in Xenopus tadpoles

$GABA_A$ receptor-mediated activity underlies the ability of propofol to induce or maintain sedation (for review, see (Franks, 2008)). To gain insight into the sedative actions of the halogenated analogues, we examined the tadpole loss-of-righting reflex (LRR). This anaesthetic endpoint correlates very well with anaesthesia in mammals, including humans (Franks et al., 1993), and is considered a surrogate for loss of consciousness models (Franks, 2008).

The tadpoles were incubated in beakers containing 0.1-10 μM of 4-BP, 0.06-3 μM 4-CP, 0.03-10 μM 4-FP, or 0.03-10 μM propofol. The righting reflex (see Methods) was estimated at the end

of a 3-hour exposure period. The number of tadpoles with LRR was plotted as a fraction of total and the EC_{50}s estimated using the Waud method (Waud, 1972).

All compounds were potent sedatives. The EC_{50}s were 0.44 ± 0.06 μM, 0.35 ± 0.03 μM, 0.87 ± 0.10 μM, and 0.52 ± 0.08 μM for 4-BP, 4-CP, 4-FP, and propofol. The concentration-response data are shown in Figure 4.

Discussion and conclusions

The intravenous anesthetic propofol directly activates and potentiates currents from both glycine and $GABA_A$ receptors (Pistis *et al.*, 1997). The sensitivities of the two receptors to the drug are, however, quite different, and it is considered that propofol's actions at clinical doses are mediated by $GABA_A$ rather than glycine receptors (Rudolph *et al.*, 2004). Previous work has indicated that para-halogenation of the propofol molecule may produce compounds with increased potency on glycine receptors. The 4-chloro substituted analogue was proposed to enhance currents elicited from heterologously-expressed α1 homomeric glycine receptors at subnanomolar concentrations (de la Roche *et al.*, 2012). Nanomolar concentrations of 4-BP, but not propofol, elicited strychnine-sensitive inward currents in spinal slices and reduced the discharge rate of ventral horn neurons (Eckle *et al.*, 2014). In contrast, halogenation of propofol may have little effect on its ability to activate or modulate $GABA_A$ receptors. Compounds with chloro-, bromo-, or iodogroups at the para position have been shown to directly activate and potentiate the α1β1γ2 $GABA_A$ receptor with essentially the same half-maximal concentrations and maximal effects as the parent compound propofol (Trapani *et al.*, 1998). In tadpoles, 4-iodopropofol and propofol produce loss-of-righting with similar EC_{50}s (Lingamaneni *et al.*, 2001). Based on these findings, it has been proposed that halogenated propofol analogues may be considered selective glycinergic modulators.

Here, we have compared direct activation and modulation of recombinant glycine and $GABA_A$ receptors by propofol and its 4-halogenated analogues 4-BP, 4-CP, and 4-FP. We employed α1, α2, and α3 homomeric glycine receptors. This choice was based on the previous study that implicated spinal cord receptors producing tonic, rather than synaptic, currents in the actions of 4-BP (Eckle *et al.*, 2014). Homomeric α subunit containing glycine receptors are likely located presynaptically where they regulate the release of transmitter or extrasynaptically where they mediate tonic glycinergic currents (Grudzinska *et al.*, 2005; Takahashi *et al.*, 1992; Turecek *et al.*, 2002) because synaptic localization requires a presence of the β subunit (Meyer *et al.*, 1995). The α4 subunit, that is expressed in the chick brain and mouse retina, was not studied here. We also note that inclusion of the β subunit is not expected to change receptor sensitivity to propofol or its halogenated analogues (Haeseler *et al.*, 2005). We also employed the α1β3γ2L $GABA_A$ receptor. This subtype, along with one where β2 replaces β3, is the major target for propofol to produce

various anesthetic endpoints (Jurd *et al.*, 2003; Reynolds *et al.*, 2003).

The key finding is that halogenation of propofol at the para position leads to a 2-10-fold reduction in the EC_{50}s for potentiation of glycine receptors. The greatest effect was observed with α3 receptors for which addition of the bromide group to propofol resulted in a leftward shift from 112 to 11 μM in the midpoints of the concentration-response curves (Table 1). The changes in EC_{50}s were not accompanied by a significant or systematic increase in relative current levels from maximally-potentiated receptors. We infer that halogenation at the para position increases affinity of the compound rather than its efficacy on glycine receptors.

Propofol and its 4-halogenated analogues were weak direct activators of glycine receptors. Comparison of currents obtained in the presence of 0.2, 10, and 100 μM 4-BP suggests that the small responses are due to low affinity of the compound rather than low efficacy.

Halogenation of propofol weakly affected modulation of $GABA_A$ receptors. In oocytes expressing α1β3γ2L $GABA_A$ receptors, the potentiation curves for 4-BP and 4-CP were leftshifted by less than 2-fold compared to propofol, whereas the concentration-response relationships for 4-FP and propofol were indistinguishable. Halogenation of propofol also had small effect on the EC_{50}s for loss-of-righting in *Xenopus* tadpoles. The EC_{50}s for 4-BP and 4-CP were slightly lower while that for 4-FP was higher than the EC_{50} for propofol. Loss-of-righting is considered to be mediated by actions on the $GABA_A$ receptor (Belelli *et al.*, 2003; Reith *et al.*, 1999) and there is good correlation between loss-of-righting in tadpoles and inhibition of binding of *t*-butylbicyclophosphorothionate or modulation of $GABA_A$ receptor function (Akk *et al.*, 2007; Krasowski *et al.*, 2001). Of note, the nominal values for EC_{50}s of potentiation by all tested analogues were lower for $GABA_A$ than glycine receptors. Thus, we infer that 4-BP, 4-CP or 4-FP cannot plausibly be used as selective glycine receptor modulators.

This inference is not in agreement with conclusions made in a prior study that found significant effects on the discharge rate of mouse ventral horn neurons and strychnine-sensitive tonic currents in organotypic spinal cultures by 50-200 nM 4-BP (Eckle *et al.*, 2014). In our experiments on recombinant homomeric glycine receptors, 200 nM 4-BP was essentially inert in the absence of glycine (relative to saturating glycine mean responses ranged from 0.01-0.22%), while potentiation of glycine-elicited currents was reliably observed at micromolar concentrations. Since Eckle and coworkers (2014) did not provide a full concentration-response relationship for their observed effects, precise comparison of our results is difficult. The increase in tonic current in the presence

of 200 nM 4-BP may have represented the low-concentration tail of the potentiation curve. However, using the data in Table 1, we calculate that potentiation by 200 nM 4-BP is minimal, ranging from 0.2 to 4.1% for currents elicited by EC_5 glycine.

Our data contradict a previous study that reported a subnanomolar EC_{50} for potentiation of glycine α1 receptors by 4-CP (de la Roche et al., 2012). We observed an EC_{50} of ~30 μM for potentiation of α1 receptors by 4-CP. The cause for discrepancy is unclear to us. We note that other phenol analogues with the 4-chloro substituent, 3-methyl-4-chlorophenol and 3,5-dimethyl-4-chlorophenol also show potentiation EC_{50}s in the micromolar range (Haeseler et al., 2005).

In sum, our findings indicate that halogenation at the para position generates compounds more capable of potentiating glycine receptors than the parent compound propofol. The effect manifests as a reduction in the EC_{50}s of the concentration-response curves for potentiation. However, we emphasize that 4-halogenated analogues of propofol remain more potent as potentiators of the α1β3γ2L $GABA_A$ than homomeric glycine receptors. It may be argued that these compounds are less desirable as selective, clinical agents because halogenation at the para position leads to reduction in $GABA_A$-selectivity that characterizes the parent compound propofol.

Author contributions

NPF, ASE, and GA conceived the study and designed the experiments; CJE and EHS synthesized the halogenated analogues of propofol; AG, DS, and BDM conducted the experiments; AG, DS, BDM, CJE, and GA analyzed the data; CJE, EHS, NPF, ASE, and GA wrote the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interests.

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Figure legends

Figure 1. Direct activation of glycine and $GABA_A$ receptors.

(A) Sample currents from oocytes expressing α1, α2, or α3 glycine, or α1β3γ2L $GABA_A$ receptors. The receptors were activated by 100 μM 4-BP, 4-CP, or 4-FP (left trace in each pair) or a saturating concentration of glycine (1 mM, 0.5 mM, or 2 mM for α1, α2, and α3, respectively) or GABA (1 mM). In each pair, the traces for propofol analogue and glycine (or GABA) are from the same cell. (B) Summary of direct activation of glycine and $GABA_A$ receptors by saturating concentrations of propofol and its analogues. The responses (mean ± S.E.M.) are normalized to responses to saturating concentrations of the transmitter from the same sets of cells.

Figure 2. Potentiation of glycine and $GABA_A$ receptors.

Sample currents from oocytes expressing α1, α2, or α3 glycine, or α1β3γ2L $GABA_A$ receptors. The receptors were activated by a low concentration ($<EC_{15}$) of transmitter alone or in the presence of two concentrations of 4-BP (A), 4-CP (B), or 4-FP (C). The concentrations of potentiators were selected to show minimal and near-maximal potentiating effects. Full concentration-response relationships are shown in Figure 3 and fitting results summarized in Table 1.

Figure 3. Concentration-response relationships for glycine and $GABA_A$ receptors.

Oocytes expressing glycine α1, α2, or α3 subunit, or $GABA_A$ α1β3γ2L subunits were activated by a low concentration of transmitter ($<EC_{15}$) in the presence of 4-BP, 4-CP, 4-FP, or propofol. The data points (mean ± S.E.M. from five to seven cells for each receptor-drug combination) show responses normalized to peak current in the presence of saturating transmitter in the same cell. The curves show predicted concentration-response relationships based on data in Table 1.

Figure 4. Tadpole behavioural data.

Exposure to 4-BP (A), 4-CP (B), 4-FP (C), or propofol (D) causes loss-of-righting (LRR) in *Xenopus* tadpoles. The data are plotted as quantal dose-response relationships with each tadpole represented as one circle. Ten tadpoles were used at each concentration. The ordinate depicts the response as no effect (0) or LRR (1). The EC_{50}s (and slopes) were 0.44 ± 0.06 μM (3.32 ± 0.93), 0.35 ± 0.03 μM (6.01 ± 1.69), 0.87 ± 0.10 μM (4.36 ± 1.30), and 0.52 ± 0.08 μM (2.10 ± 0.43) for 4-BP, 4-CP, 4-FP, and propofol.

Table 1. Summary of concentration-response data for potentiation of glycine and $GABA_A$ receptors.

Parameter	Gly α1	Gly α2	Gly α3	GABA α1β3γ2L
4-BP: EC_{50}	42 ± 5 μM ns,ns,***	15 ± 2 μM ns,***,***	11 ± 1 μM ns,*,***	4 ± 1 μM ns,*,**
n_H	1.3 ± 0.2	1.3 ± 0.3	2.1 ± 0.3	2.5 ± 0.6
I_{max}	0.63 ± 0.06 ns,ns,**	0.62 ± 0.07 ns,ns,ns	0.50 ± 0.07 ns,ns,ns	0.68 ± 0.06 **,ns,ns
4-CP: EC_{50}	32 ± 1 μM ns,ns,***	23 ± 1 μM ns,**,***	21 ± 1 μM ns,ns,***	5 ± 1 μM ns,*,*
n_H	1.3 ± 0.2	1.5 ± 0.1	2.1 ± 0.3	1.3 ± 0.2
I_{max}	0.81 ± 0.04 ns,ns,ns	0.62 ± 0.03 ns,ns,ns	0.56 ± 0.05 ns,ns,ns	0.84 ± 0.05 **,ns,*
4-FP: EC_{50}	78 ± 3 μM ns,ns,**	62 ± 6 μM ***,**,**	42 ± 7 μM *,ns,***	7 ± 1 μM *,*,ns
n_H	2.1 ± 0.3	1.5 ± 0.2	1.9 ± 0.2	3.6 ± 0.2
I_{max}	0.69 ± 0.02 ns,ns,ns	0.59 ± 0.05 ns,ns,ns	0.70 ± 0.06 ns,ns,ns	0.70 ± 0.02 ns,ns,ns
Propofol: EC_{50}	138 ± 20 μM ***,***,**	95 ± 9 μM ***,***,**	112 ± 9 μM ***,***,***	7 ± 1 μM **,*,ns
n_H	1.2 ± 0.2	1.7 ± 0.2	1.6 ± 0.1	2.1 ± 0.3
I_{max}	0.88 ± 0.04 **,ns,ns	0.69 ± 0.08 ns,ns,ns	0.63 ± 0.03 ns,ns,ns	0.66 ± 0.05 ns,**,ns

The table summarizes the results from fitting the concentration-response curves for potentiation for glycine and $GABA_A$ receptors expressed in *Xenopus* oocytes in the presence of 4-BP, 4-CP, 4-FP,

or propofol. EC_{50} is the concentration producing a half-maximal response, n_H is the Hill coefficient, and I_{max} is the fitted high-concentration asymptote relative to the response to saturating concentration of transmitter. The data are presented as mean ± S.E.M. for 5 to 7 cells. Statistical analysis was conducted by comparing EC_{50} and I_{max} values for a given receptor between 4-BP, 4-CP, 4-FP, and propofol, using ANOVA with Bonferroni correction (Stata/IC 12.1, StataCorp, College Station, TX). The symbols underneath the values apply to comparison of the parameter for a given drug to same parameter for the other three drugs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Figure 1.

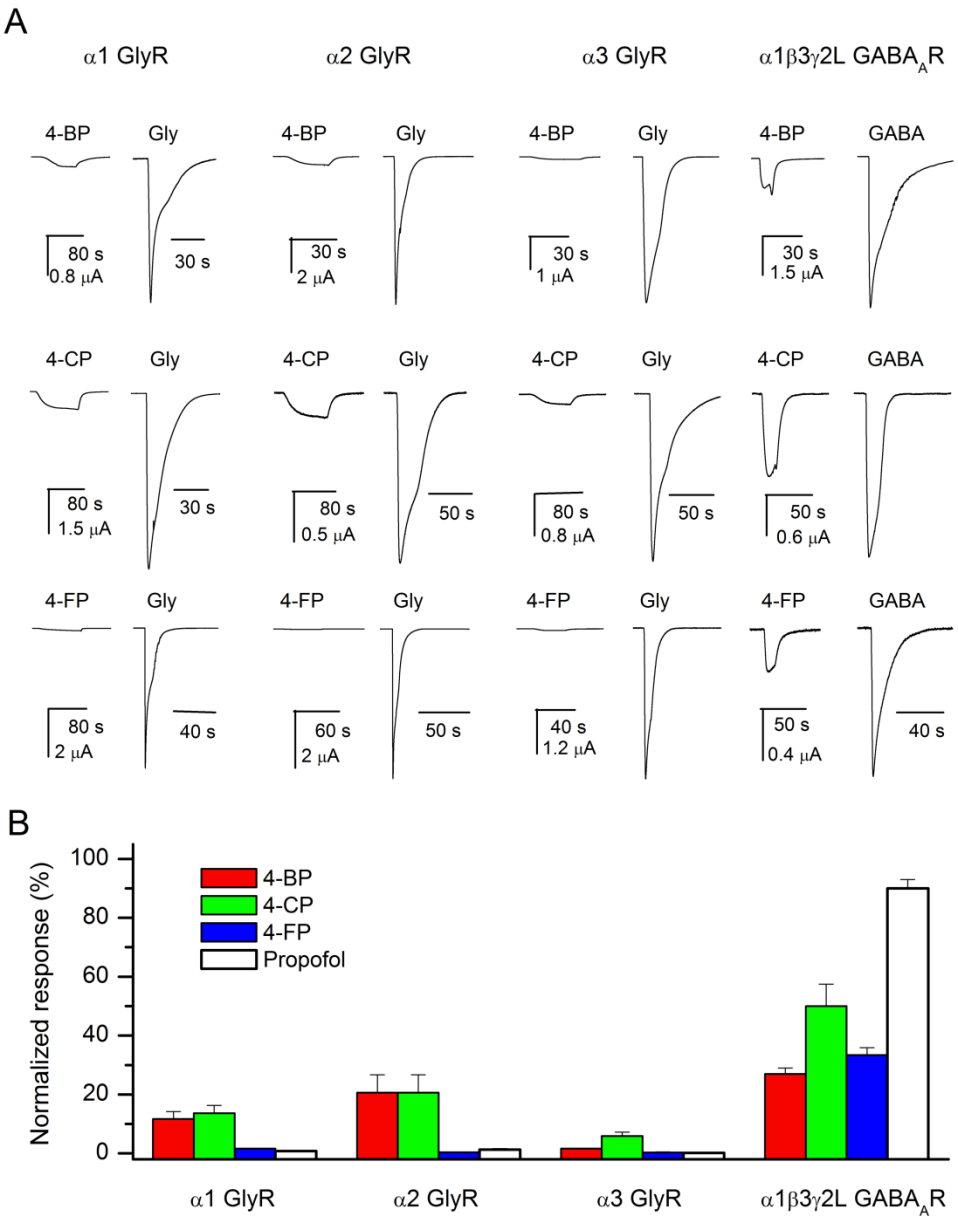
A
α1 GlyR
α2 GlyR
α3 GlyR
α1β3γ2L GABA$_A$R
4-BP
Gly
GABA
4-CP
4-FP
80 s
0.8 μA
30 s
2 μA
1 μA
1.5 μA
80 s
1.5 μA
80 s
0.5 μA
50 s
80 s
0.8 μA
50 s
50 s
0.6 μA
80 s
2 μA
40 s
60 s
2 μA
50 s
40 s
1.2 μA
50 s
0.4 μA
B
Normalized response (%)
100
80
60
40
20
0
4-BP
4-CP
4-FP
Propofol
α1 GlyR
α2 GlyR
α3 GlyR
α1β3γ2L GABA$_A$R

Figure 2.

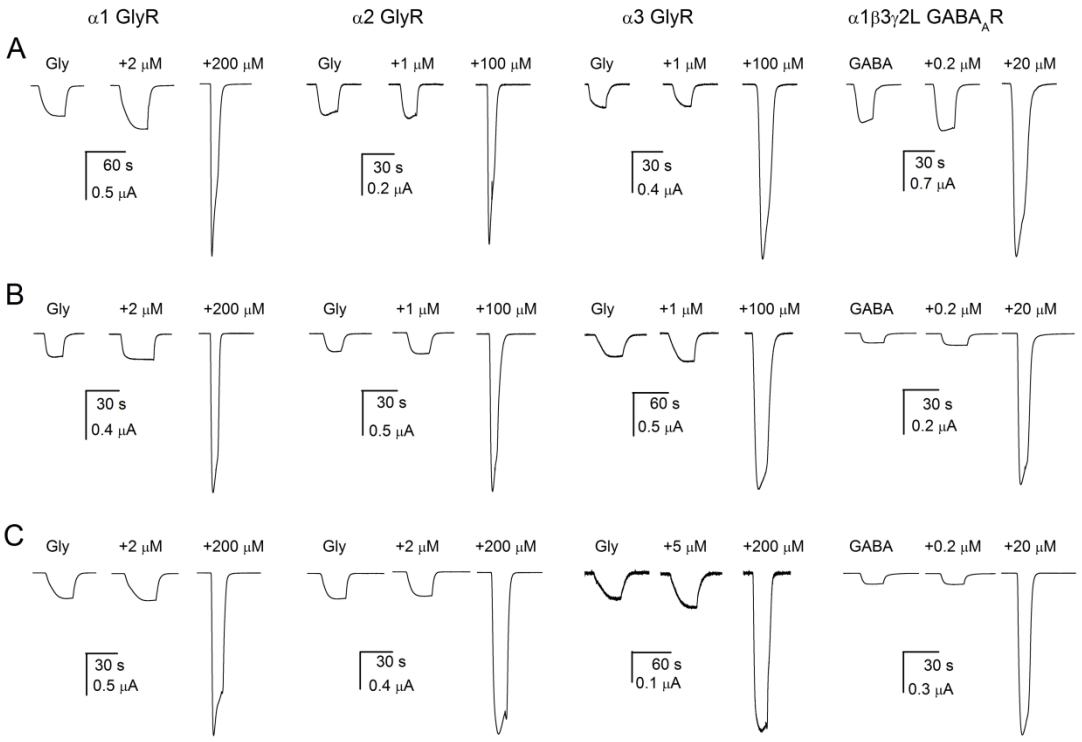
α1 GlyR
α2 GlyR
α3 GlyR
α1β3γ2L GABA$_A$R
A
Gly
+2 μM
+200 μM
60 s
0.5 μA
Gly
+1 μM
+100 μM
30 s
0.2 μA
Gly
+1 μM
+100 μM
30 s
0.4 μA
GABA
+0.2 μM
+20 μM
30 s
0.7 μA
B
Gly
+2 μM
+200 μM
30 s
0.4 μA
Gly
+1 μM
+100 μM
30 s
0.5 μA
Gly
+1 μM
+100 μM
60 s
0.5 μA
GABA
+0.2 μM
+20 μM
30 s
0.2 μA
C
Gly
+2 μM
+200 μM
30 s
0.5 μA
Gly
+2 μM
+200 μM
30 s
0.4 μA
Gly
+5 μM
+200 μM
60 s
0.1 μA
GABA
+0.2 μM
+20 μM
30 s
0.3 μA

Figure 3.

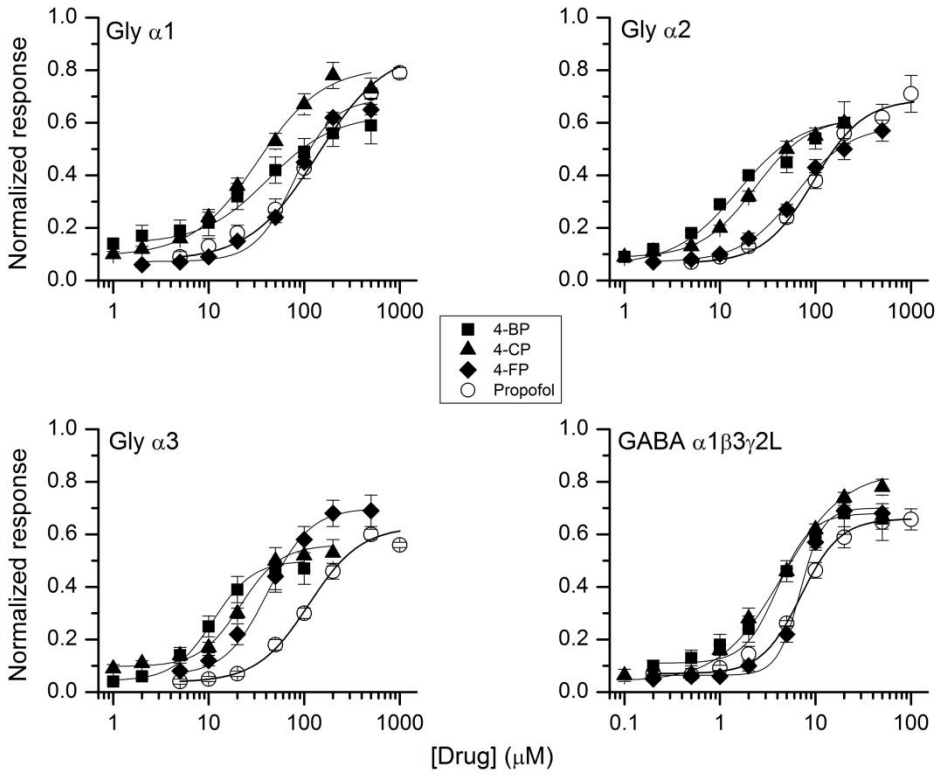
Gly α1
Gly α2
Gly α3
GABA α1β3γ2L
Normalized response
[Drug] (μM)
4-BP
4-CP
4-FP
Propofol

Figure 4.