

RESEARCH PAPER

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

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BACKGROUND AND PURPOSE

Glycine receptors are important players in pain perception and movement disorders and therefore important therapeutic targets. Glycine receptors can be modulated by the intravenous anaesthetic 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. Behavioural effects of the compounds were compared in the tadpole loss-of-righting assay.

KEY RESULTS

Concentration–response curves for potentiation of homomeric $\alpha 1$, $\alpha 2$ and $\alpha 3$ 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 $\alpha 1\beta 3\gamma 2L$ GABA_A receptor with EC₅₀ between 4 and 7 μM . The EC₅₀ 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 $\alpha 1\beta 3\gamma 2L$ GABA_A receptors. However, the effective concentrations of all tested halogenated compounds remained lower for GABA_A receptors. We infer that 4-bromopropofol, 4-chloropropofol and 4-fluoropropofol are not selective homomeric glycine receptor modulators.

Abbreviations

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

Tables of Links

TARGETS
Ligand-gated ion channels
GABA _A receptor
GABA _A receptor α 1 subunit
GABA _A receptor β 3 subunit
GABA _A receptor γ 2 subunit
Glycine receptor
Glycine receptor α 1 subunit
Glycine receptor α 2 subunit
Glycine receptor α 3 subunit

LIGANDS
GABA
Glycine
Propofol

These Tables 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 (Alexander *et al.*, 2015).

Introduction

Glycine receptors are members of the Cys-loop receptor family (Lynch, 2004; Lynch, 2009). These 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 α 2 homomers, whose expression is reduced sharply following birth (Malosio *et al.*, 1991). Receptors containing the α 1 subunit are dominant in adult retina and spinal cord. Interference with α 1 expression, or introduction of mutations disrupting receptor function, can result in the hyperekplexia phenotype (Schaefer *et al.*, 2013; Bode and Lynch, 2014). Glycine receptors containing the α 3 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 (Haverkamp *et al.*, 2003; Harvey *et al.*, 2004). Thus, glycine receptors can be important potential targets 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 α 1 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 functional deficiencies of glycine receptors, 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 GABA_A receptor (Hales and Lambert, 1991; Krasowski

et al., 2001). These actions likely underlie the loss-of-righting reflex (LRR) and other anaesthetic 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 anaesthetic and GABAergic activity of the analogues, while compounds with halogen substituents 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_A receptors by this class of compounds and to test the potential for receptor selectivity, we compared direct activation and modulation of recombinant α 1, α 2 and α 3 homomeric glycine receptors, and α 1 β 3 γ 2L 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 EC₅₀ 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 α 1 β 3 γ 2L GABA_A receptor. The EC₅₀ for the LRR, a proxy for loss of consciousness in humans (Franks, 2008), were measured in *Xenopus* tadpoles and found to be 0.35–0.87 μ M 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

Chemical syntheses

4-Chloropropofol was synthesized according to the published procedure (Wheatley and Holdrege, 1958).

The synthesis of 4-BP was adapted from the published procedure (Wheatley and Holdrege, 1958) as follows. 2,6-Diisopropylphenol (1.85 mL, 1.78 g, 10 mmol) was added to a stirred, orange solution of tetrabutylammonium tribromide (4.82 g, 10 mmol) in chloroform (20 mL), 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 (3×) and dried (MgSO₄). 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 (Meek *et al.*, 1968; Chastrette *et al.*, 1986): 2,6-Diisopropylanisole (760 mg, 4 mmol) was dissolved in chloroform (5 mL) and treated with potassium nitrate (456 mg, 4.56 mmol) and trifluoroacetic anhydride (2 mL). The flask was loosely stoppered, and the mixture was stirred at room temperature for 5 days. The mixture was filtered; the solid washed with chloroform (1 × 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 and Burmistrov, 1964): The 4-nitro derivative (910 mg, 3.84 mmol) was dissolved in ethanol (10 mL) and treated with tin dichloride dihydrate (2.25 g, 10 mmol). The mixture was stirred and heated at 90°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 × 20 mL). The combined organic layers were dried (Na₂SO₄), 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 and Zavgorodnii, 1960): The 4-amino derivative (564 mg, 2.72 mmol) 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 mmol) and then *t*-butyl nitrite (0.36 mL, 313 mg, 3.05 mmol). 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 *t*-BuOH (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°C over about 20 min. At 60–65°C, some gas evolution occurred. Once 115°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 mmol) was dissolved in dichloromethane (15 mL) and cooled in an ice bath. The stirred solution was added to a solution of boron tribromide in dichloromethane (1 M, 5 mL, 5 mmole) and the resultant solution stirred at 0°C for 45 min and then at room temperature for 3 h. The reaction mixture was cooled in an ice bath, then water (15 mL) was added and the whole 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 (1×) and water (1×) and dried (MgSO₄). The drying agent was filtered 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°C, (178 mg, 74%).

Animals

All animal care and experimental protocols complied with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Studies Committee of Washington University in St. Louis (Approval no. 20140 150 and no. 20150076). Frogs and tadpoles (from the African clawed frog, *Xenopus laevis*) were housed and cared for in a Washington University Animal Care Facility under the supervision of the Washington University Division of Comparative Medicine. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Frogs were purchased from Xenopus 1 (Dexter, MI) and oocytes were harvested from mature female frogs under tricaine anaesthesia, twice per frog. Frogs were killed under tricaine anaesthesia by rapid decapitation. Early prelimb-bud stage tadpoles from *Xenopus laevis* were used in behavioural assays. 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. A total of 370 tadpoles were used in the experiments described here. Tadpoles were killed after each experiment by addition of lethal concentrations of tricaine.

Frog oocytes 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. The use of tadpoles is justified because there are no cell lines or computer modelling systems that replace the use of animals for these behavioural studies.

Molecular biology and expression of receptors

The glycine and GABA_A receptors were expressed in *Xenopus* oocytes. The cDNAs in the pcDNA3 (glycine α 3, GABA_A α 1,

$\beta 3$, $\gamma 2L$), pcDNA3.1 (glycine $\alpha 2$) or pGEMHE vector (glycine $\alpha 1$) 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°C. The ratio of GABA_A receptor cRNAs used for injection was 5:1:5 ($\alpha 1$: $\beta 3$: $\gamma 2L$). 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 M Ω . 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 900 A (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 analysed 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). Co-application of 0.5% DMSO with an EC₅₀ concentration of a transmitter had no effect (glycine $\alpha 1$, GABA_A $\alpha 1\beta 3\gamma 2L$) or a small effect (<10%; glycine $\alpha 2$, $\alpha 3$) on the peak response.

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 (NO₃)₂, 83 μM MgSO₄, 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 h. In the end of the equilibration period, the 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 s on its back in three consecutive trials. The operator was blinded to the concentration of compound in the beaker.

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:

$$Y = Y_{\max} * \left([\text{drug}]^{n_H} / ([\text{drug}]^{n_H} + \text{EC}_{50}^{n_H}) \right), \quad (1)$$

where EC₅₀ 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 \pm SEM. The concentration–response data are presented as response relative to Y_{\max} .

Potential of 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:

$$Y = Y_{\min} + (Y_{\max} - Y_{\min}) * \left([\text{drug}]^{n_H} / ([\text{drug}]^{n_H} + \text{EC}_{50}^{n_H}) \right), \quad (2)$$

where EC₅₀ 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 carried out with one-way ANOVA and Bonferroni's correction (Stata/IC 12.1, StataCorp, College Station, TX). $P < 0.05$ was chosen to indicate significant differences between group means.

Materials

Rat $\alpha 1$, $\alpha 2$ and $\alpha 3$ homomeric glycine receptors and human $\alpha 1\beta 3\gamma 2L$ GABA_A receptors were expressed in *Xenopus* oocytes. The glycine $\alpha 1$ clone was obtained from Dr J. Lynch (The University of Queensland), $\alpha 2$ from Dr H. Akagi (Gunma University) and $\alpha 3$ 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°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 with responses to saturating concentrations of transmitter. Application of 100 μ M 4-BP (the highest

concentration tested) resulted in peak currents that were $12 \pm 3\%$ (mean \pm SEM; $n = 5$ cells), $6 \pm 1\%$ ($n = 4$) or $2 \pm 0.1\%$ ($n = 4$) of the response to saturating glycine in cells expressing $\alpha 1$, $\alpha 2$ or $\alpha 3$ homomeric glycine receptors respectively. In oocytes expressing $\alpha 1\beta 3\gamma 2L$ GABA_A receptors, 100 μ M 4-BP elicited a response that was $27 \pm 2\%$ ($n = 4$) 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 μ M and 10 μ M 4-BP were $0.03 \pm 0.02\%$ ($n = 5$) and $0.12 \pm 0.04\%$ ($n = 5$) in $\alpha 1$, $0.01 \pm 0.004\%$ ($n = 4$) and $0.23 \pm 0.12\%$ ($n = 4$) in $\alpha 2$ and $0.22 \pm 0.15\%$ ($n = 5$) and $0.32 \pm 0.18\%$ ($n = 6$) in $\alpha 3$ glycine receptors. The responses

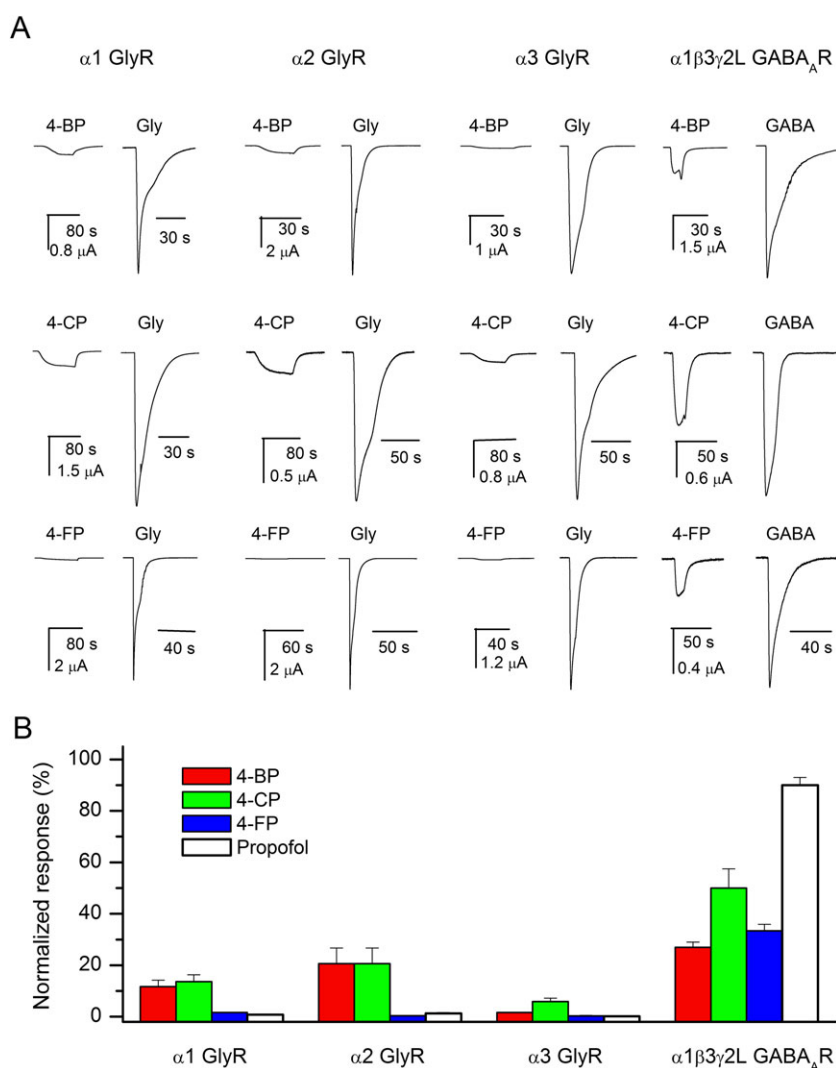


Figure 1

Direct activation of glycine and GABA_A receptors. (A) Sample currents from oocytes expressing $\alpha 1$, $\alpha 2$ or $\alpha 3$ glycine (GlyR) or $\alpha 1\beta 3\gamma 2L$ GABA_A receptors (GABA_AR). The receptors were activated by 100 μ M 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 $\alpha 1$, $\alpha 2$ and $\alpha 3$ 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 \pm SEM) are normalized to responses to saturating concentrations of the transmitter from the same sets of cells.

to 0.2 μM and 10 μM 4-BP in $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors were $0.02 \pm 0.01\%$ ($n = 4$) and $6.2 \pm 0.6\%$ ($n = 8$) of the peak response to saturating GABA.

Application of 100 μM 4-CP elicited peak responses that were $14 \pm 3\%$ ($n = 5$), $21 \pm 6\%$ ($n = 5$) or $6 \pm 1\%$ ($n = 5$) of the response to saturating glycine in oocytes expressing $\alpha 1$, $\alpha 2$ or $\alpha 3$ glycine receptors respectively. The $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors responded to application of 100 μM 4-CP with a peak response that was $50 \pm 8\%$ ($n = 5$) 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 $1.6 \pm 0.2\%$ ($n = 4$), $0.4 \pm 0.1\%$ ($n = 4$) or $0.3 \pm 0.2\%$ ($n = 5$) of the response to saturating glycine in oocytes expressing $\alpha 1$, $\alpha 2$ or $\alpha 3$ glycine receptors respectively. In oocytes expressing $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors, application of 100 μM 4-FP elicited a peak response that was $33 \pm 3\%$ ($n = 5$) 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 and Macdonald, 2004), the $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors were efficaciously activated by 100 μM propofol, which produced a response that was $90 \pm 3\%$ ($n = 7$) of the response to saturating GABA. In contrast, in glycine receptors, propofol was an ineffective agonist. At 100 μM , propofol elicited responses that were $0.8 \pm 0.3\%$ ($n = 4$), $1 \pm 0.3\%$ ($n = 4$) or $0.2 \pm 0.01\%$ ($n = 4$) of the response to saturating glycine in $\alpha 1$, $\alpha 2$ or $\alpha 3$ receptors. A summary of direct activation data showing the ratios of responses to propofol and its analogues compared with 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 EC₅₀ of the glycine activation curves were $79 \pm 14 \mu\text{M}$ ($n = 5$), $64 \pm 3 \mu\text{M}$ ($n = 5$) and $398 \pm 52 \mu\text{M}$ ($n = 5$) in oocytes expressing $\alpha 1$, $\alpha 2$ and $\alpha 3$ receptors respectively. These values are similar to those already reported (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. Co-application of 4-BP, with a low concentration of glycine, potentiated current responses (Figure 2). Homomeric $\alpha 1$ glycine receptors were activated by 17.5–22 μM glycine (<EC₁₅) 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 showed that 4-BP potentiated glycine-activated $\alpha 1$ receptors with an EC₅₀ of 42 μM ($n = 6$). In an analogous experimental protocol, the EC₅₀ for potentiation of $\alpha 1$ 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 were more potent than propofol at potentiating $\alpha 1$ glycine receptors. The EC₅₀, maximal current levels in the presence of potentiator, and the Hill coefficients are given in Table 1.

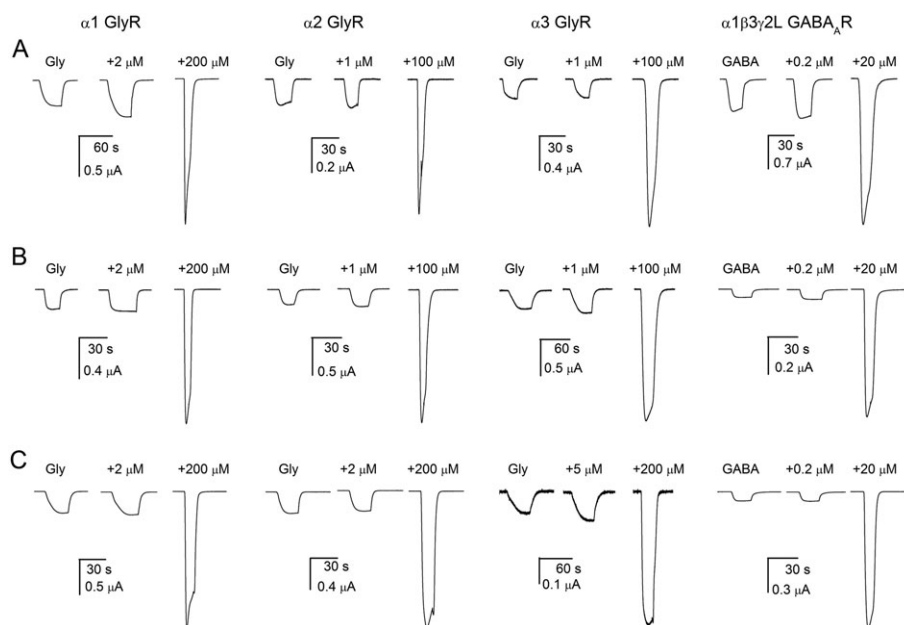


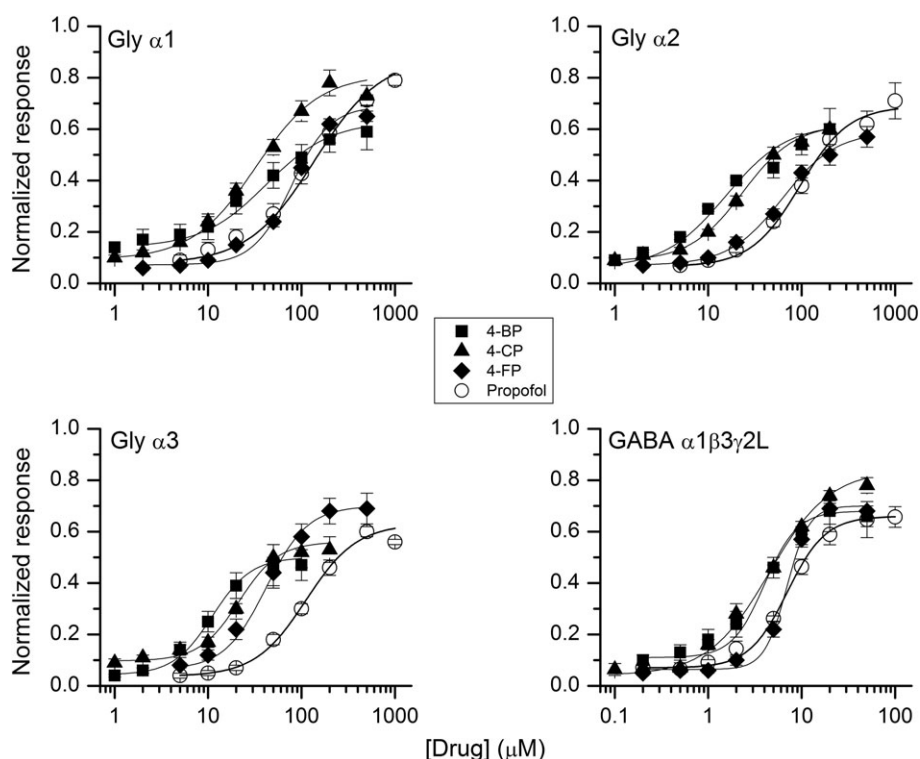
Figure 2

Potentiation of glycine and GABA_A receptors. Sample currents from oocytes expressing $\alpha 1$, $\alpha 2$ or $\alpha 3$ glycine (GlyR) or $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors (GABA_AR). The receptors were activated by a low concentration (<EC₁₅) 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.

Table 1Summary of concentration–response data for potentiation of glycine and GABA_A receptors

Parameter	Gly $\alpha 1$	Gly $\alpha 2$	Gly $\alpha 3$	GABA $\alpha 1\beta 3\gamma 2L$
4-BP : EC ₅₀	42 ± 5 μ M *	15 ± 2 μ M ^{†*}	11 ± 1 μ M ^{†*}	4 ± 1 μ M ^{†*}
n_H	1.3 ± 0.2	1.3 ± 0.3	2.1 ± 0.3	2.5 ± 0.6
I_{max}	0.63 ± 0.06 *	0.62 ± 0.07	0.50 ± 0.07	0.68 ± 0.06 [†]
4-CP : EC ₅₀	32 ± 1 μ M *	23 ± 1 μ M ^{†*}	21 ± 1 μ M *	5 ± 1 μ M ^{†*}
n_H	1.3 ± 0.2	1.5 ± 0.1	2.1 ± 0.3	1.3 ± 0.2
I_{max}	0.81 ± 0.04	0.62 ± 0.03	0.56 ± 0.05	0.84 ± 0.05 ^{†*}
4-FP : EC ₅₀	78 ± 3 μ M *	62 ± 6 μ M ^{†*}	42 ± 7 μ M ^{†*}	7 ± 1 μ M ^{†*}
n_H	2.1 ± 0.3	1.5 ± 0.2	1.9 ± 0.2	3.6 ± 0.2
I_{max}	0.69 ± 0.02	0.59 ± 0.05	0.70 ± 0.06	0.70 ± 0.02
Propofol : EC ₅₀	138 ± 20 μ M ^{†*}	95 ± 9 μ M ^{†*}	112 ± 9 μ M ^{†*}	7 ± 1 μ M ^{†*}
n_H	1.2 ± 0.2	1.7 ± 0.2	1.6 ± 0.1	2.1 ± 0.3
I_{max}	0.88 ± 0.04 [#]	0.69 ± 0.08	0.63 ± 0.03	0.66 ± 0.05 [†]

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. EC₅₀ 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 ± SEM for five to seven cells. EC₅₀ and I_{max} values for a given receptor were compared between 4-BP, 4-CP, 4-FP and propofol. The symbols next to the values denote significant differences ($P < 0.05$) from the corresponding values for 4-BP (#), 4-CP (†), 4-FP (‡) or propofol (*); one-way ANOVA with Bonferroni correction (Stata/IC 12.1, StataCorp, College Station, TX).

**Figure 3**

Concentration–response relationships for glycine and GABA_A receptors. Oocytes expressing glycine $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunit or GABA_A $\alpha 1\beta 3\gamma 2L$ 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 ± SEM 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.

The $\alpha 2$ 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 $\alpha 2$ receptors with an EC_{50} of 23 μM ($n = 5$), and 4-FP potentiated the receptors with an EC_{50} of $62 \pm 6 \mu\text{M}$ ($n = 5$). 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 $\alpha 3$ 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 $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors by propofol and its 4-halogenated analogues

We tested potentiation of $\alpha 1\beta 3\gamma 2\text{L}$ 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 the righting reflex (LRR) in *Xenopus* tadpoles

GABA_A receptor-mediated activity underlies the ability of propofol to induce or maintain sedation (see Franks, 2008). To gain insight into the sedative actions of the halogenated analogues, we examined the tadpole LRR. This anaesthetic endpoint correlates very well with anaesthesia in mammals, including humans (Franks and Lieb, 1993) and is considered as 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 LRR was measured at the end of a 3 h exposure period. The number of tadpoles with LRR was plotted as a fraction of total and the EC_{50} estimated using the method described by Waud (1972).

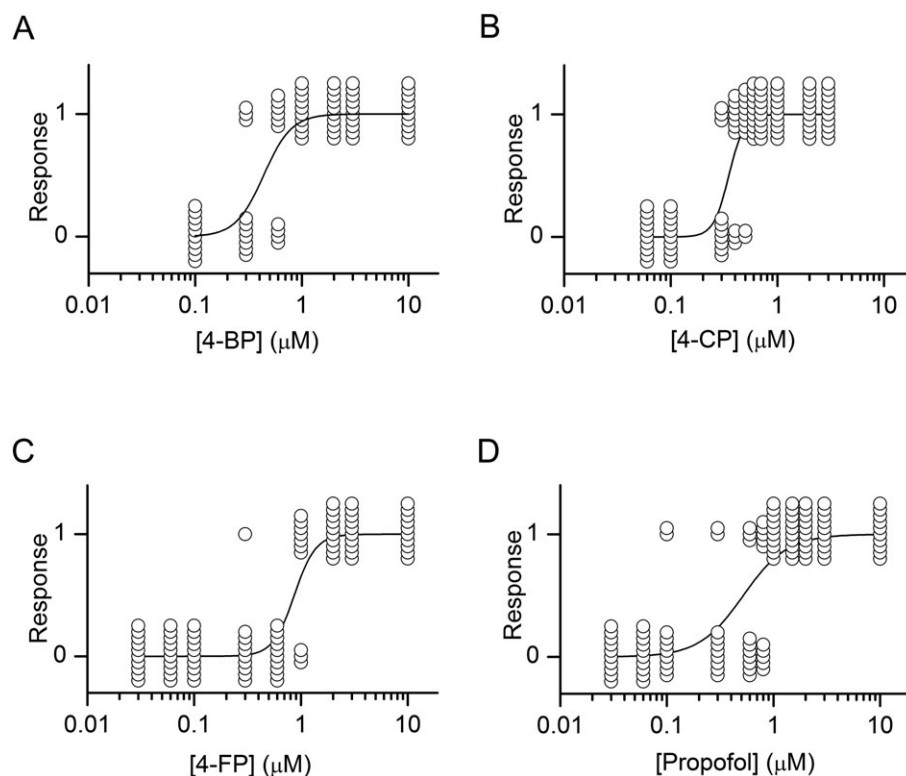


Figure 4

Tadpole behavioural data. Exposure to 4-BP (A), 4-CP (B), 4-FP (C) or propofol (D) causes loss-of-righting 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} (and slopes) were $0.44 \pm 0.06 \mu\text{M}$ (3.32 ± 0.93), $0.35 \pm 0.03 \mu\text{M}$ (6.01 ± 1.69), $0.87 \pm 0.10 \mu\text{M}$ (4.36 ± 1.30) and $0.52 \pm 0.08 \mu\text{M}$ (2.10 ± 0.43) for 4-BP, 4-CP, 4-FP and propofol respectively.

All compounds were potent sedatives. The EC_{50} were $0.44 \pm 0.06 \mu\text{M}$, $0.35 \pm 0.03 \mu\text{M}$, $0.87 \pm 0.10 \mu\text{M}$ and $0.52 \pm 0.08 \mu\text{M}$ for 4-BP, 4-CP, 4-FP and propofol respectively. The concentration–response data are shown in Figure 4.

Discussion and conclusions

The intravenous anaesthetic 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 and Antkowiak, 2004). Previous work has indicated that parahalogenation (at position 4) of the propofol molecule may produce compounds with increased potency on glycine receptors. The 4-chloro substituted analogue was reported to enhance currents elicited from heterologously expressed $\alpha 1$ 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 iodo- substituents at the para position of propofol have been shown to directly activate and potentiate the $\alpha 1\beta 1\gamma 2$ $GABA_A$ 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 induce LRR with similar EC_{50} (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 studied these actions on $\alpha 1$, $\alpha 2$ and $\alpha 3$ 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 to be located presynaptically where they regulate the release of transmitter or extrasynaptically where they mediate tonic glycinergic currents (Takahashi *et al.*, 1992; Turecek and Trussell, 2002; Grudzinska *et al.*, 2005) because synaptic localization requires a presence of the β subunit (Meyer *et al.*, 1995). The $\alpha 4$ 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 used the $\alpha 1\beta 3\gamma 2$ $GABA_A$ receptor. This subtype, along with one where $\beta 2$ replaces $\beta 3$, is the major target for propofol to produce various anaesthetic 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} for potentiation of glycine receptors. The greatest effect was observed with $\alpha 3$ 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} 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 $\alpha 1\beta 3\gamma 2$ $GABA_A$ receptors, the potentiation curves for 4-BP and 4-CP were shifted to the left by less than two-fold compared with propofol, whereas the concentration–response relationships for 4-FP and propofol were indistinguishable. Halogenation of propofol also had small effect on the EC_{50} for LRR in *Xenopus* tadpoles. The EC_{50} for 4-BP and 4-CP were slightly lower while that for 4-FP was higher than the EC_{50} for propofol. The LRR is considered to be mediated by actions on the $GABA_A$ receptor (Reith and Sillar, 1999; Belelli *et al.*, 2003), and there is good correlation between LRR in tadpoles and inhibition of binding of *t*-butylbicyclopophosphorothionate or modulation of $GABA_A$ receptor function (Krasowski *et al.*, 2001; Akk *et al.*, 2007). Of note, the numerical values for EC_{50} 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 an earlier 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. As Eckle *et al.* (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 also contradict another study reporting a subnanomolar EC_{50} for potentiation of glycine $\alpha 1$ receptors by 4-CP (de la Roche *et al.*, 2012). We found an EC_{50} of approximately 30 μM for potentiation of $\alpha 1$ receptors by 4-CP. The cause for discrepancy is unclear to us. We note that phenol analogues with the 4-chloro substituent, 3-methyl-4-chlorophenol and 3,5-dimethyl-4-chlorophenol also show potentiation EC_{50} in the micromolar range (Haeseler *et al.*, 2005).

In summary, our findings indicate that halogenation of propofol at the para position generates compounds more capable of potentiating glycine receptors than the parent compound, propofol. The effect is manifested as a reduction in the EC_{50} of the concentration–response curves for potentiation. However, we emphasize that 4-halogenated analogues of propofol remain more potent as potentiators of the

$\alpha 1\beta 3\gamma 2L$ GABA_A, than of 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 selectivity for the GABA_A receptors that characterizes the parent compound propofol.

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Author contributions

N.P.F., A.S.E. and G.A. conceived the study and designed the experiments; C.J.E. and E.H.S. synthesized the halogenated analogues of propofol; A.G., D.S. and B.D.M. conducted the experiments; A.G., D.S., B.D.M., C.J.E. and G.A. analysed the data; and C.J.E., E.H.S., N.P.F., A.S.E. and G.A. wrote the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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