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RESEARCH PAPER

A photoswitchable GABA receptor channel blocker

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Funding information

Agència de Gestió d'Ajuts Universitaris i de Recerca, Grant/Award Number: 2017-SGR-

Background and Purpose: Anion-selective Cys-loop receptors (GABA and glycine receptors) provide the main inhibitory drive in the CNS. Both types of receptor operate via chloride-selective ion channels, though with different kinetics, pharmacological profiles, and localization. Disequilibrium in their function leads to a variety of disorders, which are often treated with allosteric modulators. The few available GABA and glycine receptor channel blockers effectively suppress inhibitory currents in neurons, but their systemic administration is highly toxic. With the aim of developing an efficient light-controllable modulator of GABA receptors, we constructed azobenzene-nitrazepam (Azo-NZ1), which is composed of a nitrazepam moiety merged to an azobenzene photoisomerizable group.

Experimental Approach: The experiments were carried out on cultured cells expressing Cys-loop receptors of known subunit composition and in brain slices using patch-clamp. Site-directed mutagenesis and molecular modelling approaches were applied to evaluate the mechanism of action of Azo-NZ1.

Abbreviations: Azo-NZ, azobenzene-nitrazepam; TM, transmembrane domain; V_h , holding membrane potential

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1442; Ministerio de Economía y Competitividad, Grant/Award Number: CTQ2016-80066R; Barcelona Supercomputing Center, Grant/Award Numbers: BCV-2016-3-0005 and BCV-2016-2-0002; MINECO; Ramón Areces Foundation; Fundaluce Foundation; Human Brain Project WAVESCALES; FEDER; AGAUR/Generalitat de Catalunya, Grant/Award Number: 2017-SGR-1442; Algorish; Russian Science Foundation, Grant/Award Number: 18-15-00313; ERA SynBIO, Grant/Award Number: PCIN-2015-163-C02-01

Key Results: At visible light, being in *trans*-configuration, Azo-NZ1 blocked heteromeric $\alpha 1/\beta 2/\gamma 2$ GABA_A receptors, $\rho 2$ GABA_A (GABA_C), and $\alpha 2$ glycine receptors, whereas switching the compound into *cis*-state by UV illumination restored the activity. Azo-NZ1 successfully photomodulated GABAergic currents recorded from dentate gyrus neurons. We demonstrated that in *trans*-configuration, Azo-NZ1 blocks the Cl⁻-selective ion pore of GABA receptors interacting mainly with the 2' level of the TM2 region.

Conclusions and Implications: Azo-NZ1 is a soluble light-driven Cl⁻-channel blocker, which allows photo-modulation of the activity induced by anion-selective Cys-loop receptors. Azo-NZ1 is able to control GABAergic postsynaptic currents and provides new opportunities to study inhibitory neurotransmission using patterned illumination.

1 | INTRODUCTION

The major inhibitory neurotransmission in the mammalian CNS is provided by GABA_A, GABA_A ρ (also known as GABA_C ρ receptor; see Alexander et al., 2015), and glycine receptors (Langosch, Becker, & Betz, 1990). In different combinations, these receptors are widely expressed throughout the nervous system and are involved in physiological functions such as cognition, learning, memory, and motor control. Disequilibrium in the functioning of GABA and glycine (Gly) receptors leads to important disorders including epilepsy, anxiety, depression, misbalance of blood pressure, sleep disorders, schizophrenia, and hyperekplexia (Johnston, Chebib, Hanrahan, & Mewett, 2003; Tan, Rudolph, & Lüscher, 2011; Sieghart, 2015; Lynch, 2004).

The activity of GABA_A receptors can be successfully regulated with allosteric modulators like benzodiazepines, including flumazenil and diazepam. They are among the most popular pharmacological drugs and are commonly prescribed to treat insomnia, anxiety, and convulsions. Benzodiazepines can potentiate GABA receptors through interaction with different sites in the receptor displaying nano- and micromolar affinities (Sieghart, 2015). Therefore, benzodiazepine derivatives are convenient scaffolds to develop new modulators of GABA receptor function.

Light-switchable ligands have become a useful tool to specifically control ionic channels, cellular functions, the activity of neuronal circuits, and behaviour (Gorostiza & Isacoff, 2008; Kramer, Mouro, & Adesnik, 2013). The pharmacological activity of ligands can be regulated with photochromic groups—molecules that change their configuration upon illumination with different wavelengths. Azobenzene undergoes a rapid *trans*–*cis* photoisomerization and has been widely used for this purpose. In the dark, or under visible light irradiation, azobenzene is in the elongated *trans*-configuration, while upon illumination with UV light (360–380 nm), it isomerizes to its *cis*-state and becomes shorter by several angstroms (Gorostiza & Isacoff, 2008; Yu & Ikeda, 2004).

With the aim of regulating the activity of GABA receptors with light, we constructed a series of azobenzene derivatives of nitrazepam and

What is already known

- Pharmacological activity of ligands can be regulated with azobenzene photochromic group, which changes its configuration upon illumination with light of different wavelength.

What this study adds

- Azobenzene-nitrazepam based photochromic compound Azo-NZ1 is a light controllable channel blocker of heteromeric GABA_A, homomeric GABA_C $\rho 2$ receptors, and $\alpha 2$ GlyRs.
- Azo-NZ1 modulates in a light-dependent manner synaptic GABAergic currents in dentate gyrus of hippocampal brain slices.

What is the clinical significance

- Azo-NZ1 modulates in a light-dependent manner synaptic GABAergic currents in dentate gyrus of hippocampal brain slices.

characterized their properties. Herein, we describe the interaction of one of these compounds, azobenzene-nitrazepam-1 (Azo-NZ1), with several subtypes of anion-selective Cys-loop receptors (GABA_A, GABA_C and glycine receptors). Azo-NZ1 successfully photocontrolled GABAergic currents in cultured cells and in brain slices, but in contrast to the intended design to obtain an allosteric ligand of the classical diazepam site, we found that the azobenzene sulfonyl group introduced alters the binding properties of the compound, enabling light-controlled blocking of the chloride-selective ion pore. Thus, Azo-NZ1 reveals a class of photochromic ligand with a structure capable of blocking the anion-selective channel pore of several Cys-loop receptors.

2 | METHODS

2.1 | Synthesis of compounds

7-Aminonitrazepam (Guandalini et al., 2008; Severino et al., 2008) and tetrabutylammonium 4-nitrosobenzenesulfonate (Prievisch & Rück-Braun, 2005) were synthesized according to previously reported procedures. Commercial reagents and starting materials were purchased from Acros Organics, Alfa-Aesar, Fisher Scientific, Sigma Aldrich, or VWR and used without any further purification. Solvents used were of p.a. quality and dried according to common procedures, if necessary. Commercially obtained phosphate buffer (pH = 7.4) was used for investigations of the photochromic properties. Dry nitrogen was used as inert gas atmosphere. A Biotage Isolera flash purification system with UV/Vis detector using Sigma Aldrich MN silica gel 60 M (40–63 μ m, 230–400 mesh) for normal phase or pre-packed Biotage SNAP cartridges (KP C18 HS) for reversed phase chromatography was used for automated flash column chromatography. Reaction monitoring via TLC and determination of R_f values was accomplished on alumina plates coated with silica gel (Merck silica gel 60 F254, 0.2 mm). Melting points were measured with a Stanford Research Systems OptiMelt MPA 100 device and are uncorrected. NMR spectra were measured on Bruker Avance 300 (^1H 300.13 MHz, ^{13}C 75.48 MHz), Bruker Avance 400 (^1H 400.13 MHz, ^{13}C 100.61 MHz), and Bruker Avance III 600 (^1H 600.25 MHz, ^{13}C 150.95 MHz) instruments. The spectra are referenced against the NMR solvent (DMSO- d_6 ; $\delta_{\text{H}} = 2.50$ ppm), and chemical shifts, δ , are reported in ppm. Resonance multiplicity is abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and b (broad). Carbon NMR signals are reported using DEPT 135 and ^1H - ^{13}C HSQC spectra with (+) for primary/tertiary, (–) for secondary, and (q) for quaternary carbons. An Agilent Q-TOF 6540 UHD (ESI-MS) instrument was

used for recording mass spectra. UV/Vis absorption spectroscopy was accomplished using a Varian Cary BIO 50 UV/Vis spectrometer in 10-mm quartz cuvettes. IR spectra were recorded on an Agilent Cary 630 FT-IR spectrometer, and the peak positions are reported in wavenumbers (cm^{-1}). Analytical HPLC measurements were performed on an Agilent 1220 Infinity LC (column: Phenomenex Luna 3 μ m C18(2) 100 Å, 150 $\times$ 2.00 mm; flow: 0.3 $\text{ml}\cdot\text{min}^{-1}$ at 30°C; solvent A: MilliQ water with 0.05% vol TFA; solvent B: MeCN). The ratios in the photostationary states (PSSs) were determined by HPLC measurements with a detection wavelength at the isosbestic points. For determination of the thermal half-lives, the solutions were irradiated until the photostationary state was reached. Then the solutions were left at 25°C, and the recovery of the absorbance of the *trans*-isomer at λ_{max} was measured. Consequently, the thermal half-life was calculated by fitting the data with a single exponential function. An Agilent 1260 system (column: Phenomenex Luna 10 μ m C18(2) 100 Å, 250 $\times$ 21.2 mm; flow: 22.0 $\text{ml}\cdot\text{min}^{-1}$; solvent A: MilliQ water with 0.05% vol TFA; solvent B: MeCN) was used for preparative HPLC purification. Light sources for irradiation: $\lambda = 365$ nm (Herolab handheld lamp UV-6 L, 6 W), $\lambda = 455$ nm (OSRAM Oslon SSL 80 LED, 700 mA, 1.12 W). The power of the light is given based on the specifications supplied by the company when the lamps were purchased. Final compounds for biological testing possess a purity $\geq 95\%$, as determined by HPLC measurements with detection at 220 and 254 nm.

Azo-NZ1 ((E)-4-((2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-7-yl)diazanyl)benzenesulfonic acid) was synthesized according to the following procedure (Figure 1b). Tetrabutylammonium 4-nitrosobenzenesulfonate (236 mg, 0.54 mmol, 1.0 eq.) was added to a solution of 7-aminonitrazepam (68 mg, 0.27 mmol, 1.0 eq.) in acetic acid (2 ml) and CH_2Cl_2 (1 ml). After the mixture had been stirred for 24 hr at room temperature, the solvent was removed in vacuo.

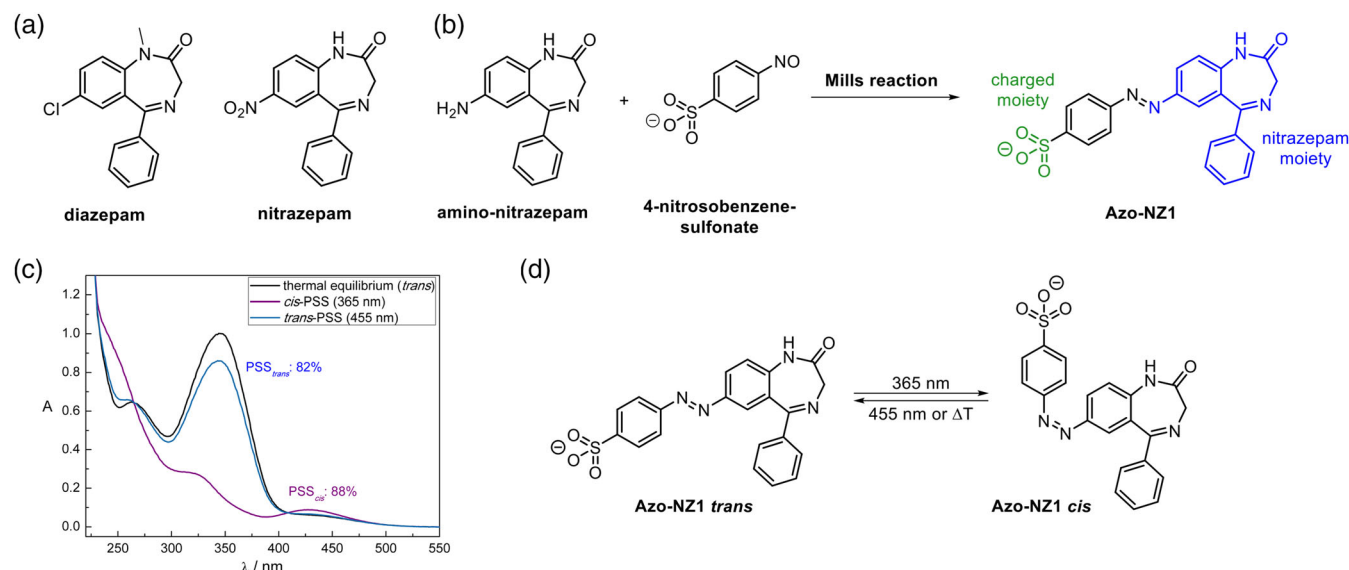


FIGURE 1 Design of Azo-NZ1. (a) Chemical formula of diazepam and nitrazepam. (b) Synthetic approach for the synthesis of Azo-NZ1. (c) UV-Vis spectra of azo-nitrazepam Azo-NZ1 (50 μ M in phosphate buffer +0.1% DMSO) from the *trans*-isomer (black), the PSS at irradiation with UV light of $\lambda = 365$ nm (purple), and the PSS at irradiation with blue light of $\lambda = 455$ nm (blue). (d) Schematic representation of Azo-NZ1 in its *trans*- and *cis*-configurations

Purification by automated flash column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 3–25% MeOH) and subsequent preparative HPLC (2–65% MeCN in 10 min, $t_R = 7.2$ min) yielded Azo-NZ1 (69 mg, 61%) as a yellow solid. R_f 0.03 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1); mp 280°C (decomposition); $^1\text{H-NMR}$ (600 MHz, $\text{DMSO-}d_6$) $\delta = 11.32$ (s, 1H), 8.23 (dd, $J = 8.8$, 2.4 Hz, 1H), 7.81–7.78 (m, 2H), 7.77–7.73 (m, 3H), 7.68 (t, $J = 7.4$ Hz, 1H), 7.64 (dd, $J = 8.3$, 1.3 Hz, 2H), 7.57 (t, $J = 7.7$ Hz, 2H), 7.52 (d, $J = 8.9$ Hz, 1H), 4.34 (s, 2H); $^{13}\text{C-NMR}$ (151 MHz, $\text{DMSO-}d_6$) $\delta = 172.5$ (q), 168.9 (q), 151.4 (q), 151.1 (q), 146.5 (q), 143.0 (q), 135.8 (q), 132.5 (+), 130.7 (+), 128.8 (+), 128.6 (+), 126.8 (+), 126.2 (+), 124.4 (q), 122.9 (+), 122.2 (+), 54.6 (–); IR (neat) $\nu = 3489$, 3135, 2930, 1715, 1614, 1484, 1435, 1387, 1342, 1230, 1163, 1115, 1029, 1006, 846, 742, 697 cm^{-1} ; HRMS (ESI) calculated for $\text{C}_{22}\text{H}_{16}\text{N}_4\text{O}_4\text{S}$ ($\text{M} + \text{H}$) $^+ m/z = 421.0965$; found 421.0964.

2.2 | Cell culture and transfection

The experiments were carried out on cultured CHO cells (CHO-K1, ATCC Cat# CCL-61, RRID:CVCL_0214) obtained from the American Type Tissue Culture Collection (ATCC, Molsheim, France) that were maintained in culture conditions as previously described (Maleeva, Peiretti, Zhorov, & Bregestovski, 2017; Mukhtarov et al., 2013). For electrophysiological analysis, cells were transfected with cDNAs of several Cys-loop receptors. For expression of heteromeric GABA_A receptors, cells were simultaneously transfected with cDNA (concentrations $0.9\text{--}1.2 \mu\text{g}\cdot\mu\text{l}^{-1}$) of $\alpha 1$, $\beta 2$, and $\gamma 2$ (isoform S) subunits. For expression of homomeric GABA_C receptors, cells were transfected with cDNA of $\rho 1$ or $\rho 2$ subunits. For the expression of homomeric glycine receptors and **5-HT3A receptors**, cDNAs of $\alpha 1$, $\alpha 2$, and A subunits, respectively, were used.

One day before transfection, cells were plated on the cover slips (12 mm in diameter) and placed inside 35-mm cell culture dishes with 2 ml of medium. Transfection was performed using the Lipofectamine 3000 protocol (Life Technology, USA). To facilitate identification of transfected cells, a GFP ($0.5 \mu\text{g}\cdot\mu\text{l}^{-1}$) was added to the transfection mixture. Electrophysiological recordings were performed in the fluorescent cells 24–72 hr after transfection.

2.3 | Electrophysiological recordings on CHO cells

Whole-cell recordings were performed at room temperature ($20\text{--}25^\circ\text{C}$) using an EPC-9 amplifier (HEKA Elektronik, Germany). Cells were continuously superfused with external solution containing (mM): NaCl 140, CaCl_2 2, KCl 2.8, MgCl_2 4, HEPES 20, glucose 10; pH 7.4; 320–330 mOsm. Intracellular solution used for filling recording patch pipettes contained (mM): KCl 140, MgCl_2 2, MgATP 2, HEPES10, BAPTA (tetrapotassium salt) 2; pH 7.3; 290 mOsm.

Recording pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus Ltd, USA) and had resistances of 5–10 MOhms. For the rapid replacement of the solutions, the fast application system was used. Three parallel rectangular tubes ($100 \times 100 \mu\text{m}$) were positioned 40–50 μm above the recorded cell. The movement of the tubes was controlled by a computer-driven fast exchange system (SF 77A Perfusion Fast-Step, Warner, USA) allowing a 10–90% solution

exchange in 3–5 ms, as measured by open electrode controls (1/10 external solution/water). Cells with a low input resistance (<150 MOhms) and a rapid run-down ($>30\%$ with repetitive application) were excluded from analysis.

The effect of Azo-NZ1 was examined either using a “long” protocol for application of solutions, at constant holding membrane potentials (V_h) of +30 and -30 mV or using a “ramp” protocol that allowed a linear change of membrane potential from -60 to $+60$ mV (see Figure 3 c). For GABA_C $\rho 2$, $\rho 1$ P2'S , and **$\alpha 2$ glycine receptors**, the controls correspond to the amplitude of the current just before application of Azo-NZ1, whereas for GABA_A receptors, the control corresponds to the amplitude of the current at wash-out, due to the desensitization of this type of receptor.

UV (365 nm) LED (Thorlabs) was placed at the distance of 4–5 cm from the cell that was being recorded. The power of UV light was reaching $0.6 \text{ mW}\cdot\text{mm}^{-2}$ at the level of recording chamber, as determined using an optical power metre (Thorlabs).

2.4 | Electrophysiological recording on brain slices and electrical stimulation

Experiments were performed on white laboratory ICR outbred mice of both genders, kept at 12 hr “day”/“night” cycle. Other details are described previously (Petukhova, Ponomareva, Mukhamedyarov, Maleeva, & Bregestovski, 2018). All animal protocols and experimental procedures were approved by the Local Ethics Committee of Kazan State Medical University (N742.13.11.84 and N1045-72) and by the INSERM Ethics Committee for Animal Experimentation (#30-03102012). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*.

Coronal slices, containing hippocampus, were obtained from 3- to 4-week-old mice. Animals were decapitated under isoflurane anaesthesia. Whole brains were rapidly removed from the skull and immersed for 10 min in ice-cold artificial CSF (aCSF) solution containing (in mM): NaCl 126, KCl 3.5, CaCl_2 2, MgCl_2 1.3, NaHPO_4 1.2, glucose 10, NaHCO_3 25 (pH 7.3–7.4, 290–300 mOsm). The aCSF was continuously oxygenated with 95% O_2 and 5% CO_2 to maintain the physiological pH. Sagittal slices (350 μm) were cut using a tissue slicer (Microm International, Germany) in high K^+ solution, containing (in mM): Kgluconate 120, HEPES-acid 10, Nagluconate 15, EGTA 0.2, NaCl 4 (pH 7.2, 290–300 mOsm). After being cut, slices were placed for 15 min at room temperature in a choline-based solution, containing (in mM): choline chloride 110, KCl 2.5, NaH_2PO_4 1.25, MgCl_2 10, CaCl_2 0.5, NaHCO_3 25, glucose 10, sodium pyruvate 5 (pH 7.3–7.4, 290–300 mOsm). Then, slices were placed in a chamber filled with oxygenated aCSF. Before use, slices were allowed to recover for at least 1 hr at room temperature. Experiments were performed during the period of 1–6 hr after slicing.

During the recordings, slices were placed in a conventional chamber superfused with aCSF ($32\text{--}34^\circ\text{C}$). The inhibitory postsynaptic currents evoked (eIPSCs) were recorded from granule cells of dentate gyrus using the patch-clamp technique in whole-cell configuration at -70 or 0 mV. Patch electrodes were pulled from borosilicate capillary

glass (Sutter Instruments flaming/brown micropipette puller P-97) and filled with intracellular solution containing (in mM): Kgluconate 120, KCl 20, MgCl₂ 2, MgATP 2, HEPES/KOH 10, BAPTA 1; pH 7.3; 290 mOsm. The resistance of electrodes used was 5–7 MΩ. Membrane currents were recorded at 3–10 kHz using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Germany).

In granule cells of dentate gyrus, postsynaptic currents consist of two main populations: glutamatergic and GABAergic. In our experimental conditions, the reversal potentials for glutamate- and GABA-induced currents were about 0 and –50 mV, respectively. To isolate GABAergic synaptic events from glutamatergic ones without using specific pharmacological antagonists, we performed analysis of Azo-NZ1 action on eIPSCs at $V_h = 0$ mV. At this potential, glutamatergic events are not detectable, and GABAergic currents appear as outwardly directed deflections. An application of **bicuculline** at the end of recordings resulted in the complete suppression of these currents, ensuring its pure GABAergic nature.

For the induction of GABAergic responses, stimulation pulses were generated by the DS3 Constant Current Isolated Stimulator (Digitimer, England). Pulses were delivered every 10 s at $V_h = 0$ mV, the amplitudes of control eIPSCs ranged from 50 to 400 pA. Electrodes for stimulation were prepared from double-barrelled borosilicate theta-tubes (OD = 1.5 mm, ID = 1.0 mm, septum = 0.2 mm, from Warner Instruments, USA). They were pulled using the programme similar to those for preparing patch-clamp recording pipettes, polished, and backfilled with aCSF. To deliver an electrical stimulus, two fine silver wires were inserted into the theta-glass.

After a whole-cell configuration had been obtained with a neuron, the stimulation electrode was placed on the granule cell layer in close proximity within the recording cell (distance 100–200 μm) located towards the hilar region. This allowed the stimulation of presynaptic fibres from the presumed GABAergic basket cells, as described by others (Haselmann, Röpke, Werner, Kunze, & Geis, 2015; Korshoej, Holm, Jensen, & Lambert, 2010).

Previous studies demonstrated that azobenzene-based UV-controlled molecules might be successfully used in living tissues for research purposes (Lin et al., 2015; Yue et al., 2012). However, after prolonged exposure, UV and near-UV irradiation can be damaging (Kienzler et al., 2013). To avoid direct illumination of brain slices with UV light, which might modify synaptic function, we illuminated the aCSF with diodes emitting either UV (365 nm) or blue (455 nm) light. The speed of the perfusion was about 20 ml·min^{–1}, and it was taking 18–20 s for the illuminated solution to arrive to the experimental chamber.

Effects of Azo-NZ1 on the amplitudes of eIPSCs were studied using the following protocol: 20–30 eIPSCs were recorded in control condition, then slices were incubated in aCSF + Azo-NZ1 for 5–10 min at visible or blue (455 nm) light (the compound in *trans*-configuration); the aCSF + Azo-DZ1 was illuminated with UV light for 3–5 min (switching the compound into *cis*-configuration); and subsequently illuminated for 3–5 min with blue light. Changes in UV/blue light illumination could be repeated several times on the same neuron before it is washed with control aCSF. Control tests showed that UV illumination of the pure aCSF for 5 min had no effect on eIPSCs.

2.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Electrophysiological recordings were performed using PatchMaster (HEKA Electronic, Germany) software. To plot concentration–response curves, responses to different concentrations of GABA were fitted using a non-linear fitting routine of the Origin 7.5 software (OriginLabs, USA) with the Hill equation:

$$I = I_{\max} / (1 + (EC_{50}/[A])^{n_H}),$$

where I is the normalized current amplitude induced by the agonist at concentration $[A]$, $I_{\max}$ is a maximal current induced at given cell, n_H is the Hill coefficient, and EC_{50} is the concentration at which a half-maximum response was induced.

Ionic current recordings were analysed with Igor Pro 6.02 and Origin 9.0 software. In some cases, data were normalized to perform a comparison between different types of receptor or different agonist concentrations. Data are presented as means ± SEM. Significance of difference was evaluated by using two-sample t test, paired sample Wilcoxon signed rank test, and Mann–Whitney U test. Differences were considered significant at a value of P below .05.

2.6 | Materials

All the drugs were obtained from Tocris or Sigma–Aldrich (France). Stock solutions of Azo-NZ1 (10 mM) and diazepam (50 mM) were prepared using DMSO and then diluted to the final concentration in extracellular solution. Stock solutions of **GABA** (1 M), **glycine** (1 M), **5-HT** (10 mM), APV (40 mM), and bicuculline (10 mM) were prepared using MilliQ water.

2.7 | Modelling

Five different GABA receptor structures were used, all corresponding to open channel states. For GABA_A receptors, we used the homology model of the heteropentameric α1/β2/γ2 receptor from reference (Bergmann, Kongsbak, Sørensen, Sander, & Balle, 2013). Two homology models were generated for the p1 and p2 GABA_A (GABA_C) receptors, using as a template the GluCl receptor in the open channel state (PDB code 3RI5; Hibbs & Gouaux, 2011) and the Swiss-Model server (Biasini et al., 2014). The sequence identity between the GluCl template and the p1 and p2 receptors is 37% and 36.6%, and it is even higher when only the pore-lining helices are considered (54.2% and 58.3%, respectively). Indeed, GluCl has been proposed to be the best template to model the structure of GABA_A p receptors (Naffaa, Chebib, Hibbs, & Hanrahan, 2015). For the S2'G p1 and P2'S p2 mutant receptors, the mutation was introduced in silico using the Molefacture tool (version 1.3) in VMD (version 1.9.2; Humphrey, Dalke, & Schulten, 1996).

While this manuscript was under review, new cryo-electron microscopy structures of GABA_A receptors were published (Lavery et al., 2019; Masiulis et al., 2019). These structures correspond to either closed or desensitized states of the receptor and hence were not used

for our docking analysis. Nonetheless, we superimposed the structural model of the open state used here (Bergmann, Kongsbak, Sørensen, Sander, & Balle, 2013) with the cryo-EM structures of GABA_A receptor in the closed and desensitized states (Figure S9), in order to investigate the possibility of Azo-NZ1 binding to these other two pore conformations. In the closed state, the pore is narrower than in the open state (Figure S9A,B) and thus would hinder binding of the longer and bulkier *trans*-Azo-NZ1, compared to the smaller pore blocker picrotoxinin captured in the cryo-EM structure. For the desensitized state, the pore size is not as different from the open state (Figure S9B,C), and thus, we cannot exclude binding of *trans*-Azo-NZ1. However, we speculate that the non-chloride-conductive desensitized state might be less suitable to bind the chloride-mimic sulphonate group of *trans*-Azo-NZ1. In addition, binding of *trans*-Azo-NZ1 to Ser2' could prevent desensitization, similarly to picrotoxinin (Gielen, Thomas, & Smart, 2015) and thus would be incompatible with a desensitized state.

2.7.1 | Azo-NZ1 ligand

The initial structures of the Azo-NZ1 compound (*cis*- and *trans*-isomers) were created employing Avogadro (version 1.1.1; Hanwell et al., 2012). For each isomer, two 1,4-diazepine ring conformations, M and P, were considered, which differ in orientation (below or above the plane, respectively) of C3 and the phenyl substituent of C5 (Figure S10). For classical benzodiazepines that bind to the canonical allosteric site, the M conformation is the bioactive one (i.e., with higher affinity for receptor; Richter et al., 2012). However, it is not known a priori whether Azo-NZ1 would exhibit similar conformational preferences, since it binds in a distinct site. Therefore, all four ligand structures (*cis*/M, *cis*/P, *trans*/M, and *trans*/P) were optimized using quantum mechanical calculations in order to (a) optimize the manually built ligand structures and (b) estimate the relative stability of the M and P conformers. Calculations were performed with the Gaussian 09 (G09) programme package (Frisch et al., 2013), using density functional theory (Rajagopal & Callaway, 1973), with the B3LYP functional (Stephens, Devlin, Chabalowski, & Frisch, 1994) and the 6-31++G(d,p) basis set. For the *trans*-isomer, the two conformers differ only by 0.03 kcal·mol⁻¹, and thus, their Boltzmann populations are very similar (51.4% and 48.6% for P and M, respectively). In other words, the two conformers can be present at room temperature. In the case of the *cis*-isomer, the P conformer is significantly more stable (by 3.5 kcal·mol⁻¹) than the M one, and thus, it is the predominant conformer (with a Boltzmann population of 99.7%).

2.7.2 | Docking calculations

Autodock Vina (version 1.1.2; Trott & Olson, 2010) was employed for ligand-receptor docking. Given that experimental data indicate that Azo-NZ1 binds inside the pore, we centred our search space around the M2 helices of the pore. A flexible docking approach was used, in which both the receptor and the ligand were considered as flexible. On the one hand, the ligand was allowed to change its geometry (by exploring all the possible ligand torsions) in order to optimize its fit

inside the pore. On the other hand, the side chains of the pore-lining residues at positions 2', 6', 9', 13', and 16' were allowed to move and adapt to the ligand poses. The maximum energy difference between the best and worst binding modes and the exhaustiveness were set to default values (3 and 8 kcal·mol⁻¹, respectively). Instead, the maximum number of modes was increased to 20 in order to increase the docking sampling. This protocol was repeated 10 times [for GABA_A ρ (GABA_C) receptors] and eight times (for GABA_A receptors), starting with different random seeds, so that a total number of 200 and 160 binding modes was obtained for each of the four possible conformers of azo-NZ1 (200 for *cis*/M and *cis*/P, and 160 for *trans*/M and *trans*/P). The docking poses of each conformer were analysed separately. For simplicity, only the *trans*/P and *cis*/P results are discussed in the main text. The results of the corresponding M conformers are almost identical (and in the case of *cis*/M, it has very low population).

We would like to note here that, upon UV light irradiation, *cis*-Azo-NZ1 may either remain in the channel (assuming another binding conformation that unblocks the pore) or exit the pore (reaching the bath solution). The outcome will depend on the mean open time of the channel and the dissociation rate of *cis*-Azo-NZ1. Unfortunately, the dissociation rate is particularly difficult to study either experimentally or computationally. Nevertheless, we expected it to be slow, based on the observations for GABA_A receptors with another pore blocker picrotoxinin (Gielen, Thomas, & Smart, 2015; Korshoej, Holm, Jensen, & Lambert, 2010). Therefore, since with the data at hand we cannot distinguish between the two possibilities put forward above, we decided to perform docking calculations not only with *trans*-Azo-NZ1 but also with the *cis*-isomer, in order to model the situation immediately after UV irradiation. These dockings are aimed at investigating the change in the receptor-ligand binding mode upon *trans*-*cis* isomerization (both in terms of position along the pore and interactions with the pore-lining residues).

2.7.3 | Analysis of the docking results

We used two different approaches to pinpoint the exact binding site of Azo-NZ1. The first approach was to use the number density of the sulphonate group to follow ligand binding. Previous studies have successfully used this type of analysis to identify ligand binding sites in other ion channels (Raju, Barber, LeBard, Klein, & Carnevale, 2013). The underlying assumption is that regions of continuous density (or high occupancy) should represent regions of tighter binding. We computed the number density value, using the Volmap plugin (Cohen, Arkhipov, Braun, & Schulten, 2006) of VMD (Humphrey, Dalke, & Schulten, 1996). For each binding pose, the sulphonate group of Azo-NZ1 was considered a "particle" by replacing each of its atoms with a normalized Gaussian distribution (with width 1.5 times its atomic radius) and additively distributing those Gaussians on a three-dimensional grid (with dimensions 0.5 × 0.5 × 0.5 Å³). The resulting particle density is thus equivalent to the number of binding poses whose sulphonate group is located in a given volume of the pore.

The second approach was based on the analysis of the receptor-ligand interactions. First, the ligand binding poses were clustered using

the quality threshold algorithm implemented in VMD (<https://github.com/luisico/clustering>), and then the representative structure of the most populated cluster(s) was analysed using the Binana algorithm (Durrant & McCammon, 2011). The images of the modelling section were generated with UCSF Chimera (Pettersen et al., 2004) or VMD (Humphrey, Dalke, & Schulten, 1996).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

3 | RESULTS

3.1 | Design of Azo-NZ1

The design of our photochromic ligand was based on the diazepam structure (Figure 1a), a widely used modulator of GABAergic function. Benzodiazepines are capable of binding in several interaction sites in the receptor (Middendorp, Maldifassi, Baur, & Sigel, 2015; Sieghart, 2015), and this offers, in principle, greater flexibility to introduce

relatively bulky substitutions like azobenzene without leading to a loss of functional activity of the compound. This choice was strengthened by the fact that the benzodiazepine nitrazepam, a diazepam derivative (Figure 1a), can be easily functionalized for a subsequent Mills reaction via reduction of its nitro-group (Severino et al., 2008) and thus be directly incorporated as part of the azobenzene (Figure 1b).

Therefore, the nitro-group of nitrazepam was reduced providing an aniline, which was used for further functionalization to set up the azo-bridge in a Mills-type reaction (see Section 2). To increase the solubility of the azo-nitrazepam, sulfanilic acid was used as precursor for the formation of the nitroso compound (Priewisch & Rück-Braun, 2005) providing a negative charge at physiological pH values (Kim, Gao, & Burgess, 2009). This compound showed robust photochromism with a preference for the *trans*-configuration in darkness and under visible light (455 nm), while under UV illumination (365 nm), the *cis*-configuration is favoured (Figures 1c,d and S11).

3.2 | Action of Azo-NZ1 on heteromeric GABA_A $\alpha 1/\beta 2/\gamma 2$ receptors

Analysis of the GABA dose/response curves showed that EC₅₀ for $\alpha 1/\beta 2/\gamma 2$ GABA_A receptors was $8 \pm 3 \mu\text{M}$ (Figure 2a; $n = 6$), which was close to the previously reported values (Fisher, 2004). Diazepam (10 μM) co-applied with non-saturating concentration of GABA

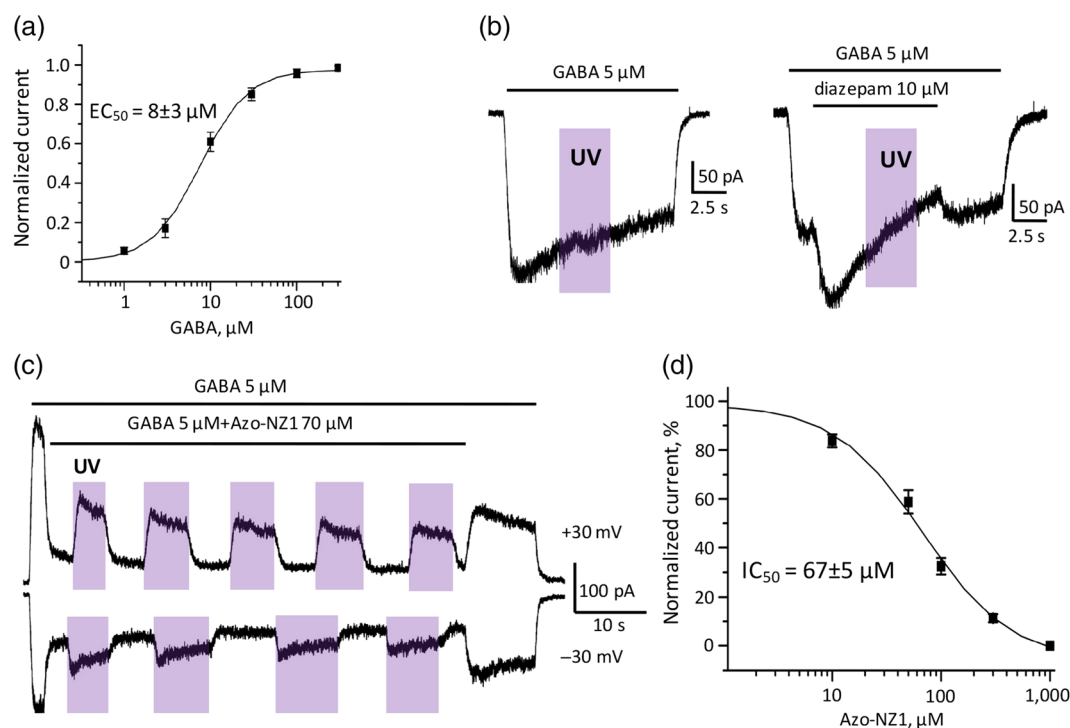


FIGURE 2 Azo-NZ1 modulates activity of GABA_A $\alpha 1/\beta 2/\gamma 2$ receptors in a UV-dependent manner. (a) Cumulative dose/response curve for GABA-induced currents in control conditions ($n = 6$). (b) Traces of currents induced by 5- μM GABA (left panel) and by co-application of 5- μM GABA with 10- μM diazepam (right panel) at $V_h = -30 \text{ mV}$. Durations of GABA and GABA/diazepam applications are indicated by black bars above the traces. Duration of UV illumination is indicated by violet rectangles. Note the absence of the effect of UV illumination on the responses. (c) Effect of 70- μM Azo-NZ1 on the amplitude of currents induced by 5- μM GABA at $V_h = +30 \text{ mV}$ (top trace) and -30 mV (bottom trace). Note the stronger inhibition of currents by Azo-NZ1 at positive potentials. (d) Cumulative dose-response curve for Azo-NZ1 at GABA_A receptors ($n = 6$)

caused an increase in the current amplitude (Figure 2b), which is in accordance with previous observations (Bormann & Clapham, 1985). UV illumination neither modified the currents induced by GABA nor by GABA plus diazepam (Figure 2b).

Ionic currents were evoked by 5 μ M of GABA alone or with addition of Azo-NZ1. The photochromic compound in concentrations 0.5–1 μ M did not produce effect on the GABA-induced currents, while application of Azo-NZ1 at 10 μ M and higher concentrations caused its decreasing. As illustrated in Figure 2c, Azo-NZ1 at 70 μ M produced a prominent decrease of the amplitude of GABA_A-mediated currents under visible light, when the compound was in *trans*-configuration. Illumination with UV light (365 nm) resulted in recovery of currents till values were close to the control. The degree of Azo-NZ1-induced suppression at positive potential was more pronounced: the amplitude of GABA-induced currents decreased by 78% and 51% at +30 mV and –30 mV, respectively (Figure 2c). Analysis of

concentration dependencies of Azo-NZ1 action shows that at –30 mV, the IC₅₀ was 67 ± 5 μ M ($n = 6$; Figure 2d), and thus, further experiments were performed with concentrations of Azo-NZ1 close to IC₅₀.

Using a “ramp” protocol (Figure 3c, inset), we have shown that, under visible light, 50- μ M Azo-NZ1 decreased currents induced by 5- μ M GABA by $76 \pm 5\%$ and $40 \pm 4\%$ at +60 mV and –60 mV, respectively ($n = 5$, $P < .05$), confirming that Azo-NZ1 is more efficient at positive potentials (Figure 3c). The recovery during UV illumination was not complete ($P < .05$), presumably, because of incomplete photoswitching of Azo-NZ1 in *cis*-configuration.

The voltage dependence of the inhibitory action of Azo-NZ1 suggested that the compound might act as an open channel blocker of GABA_A receptors (Maleeva, Peiretti, Zhorov, & Bregestovski, 2017). To test this hypothesis, we compared the action of Azo-NZ1 at different concentrations of the agonist. The amplitude of currents induced

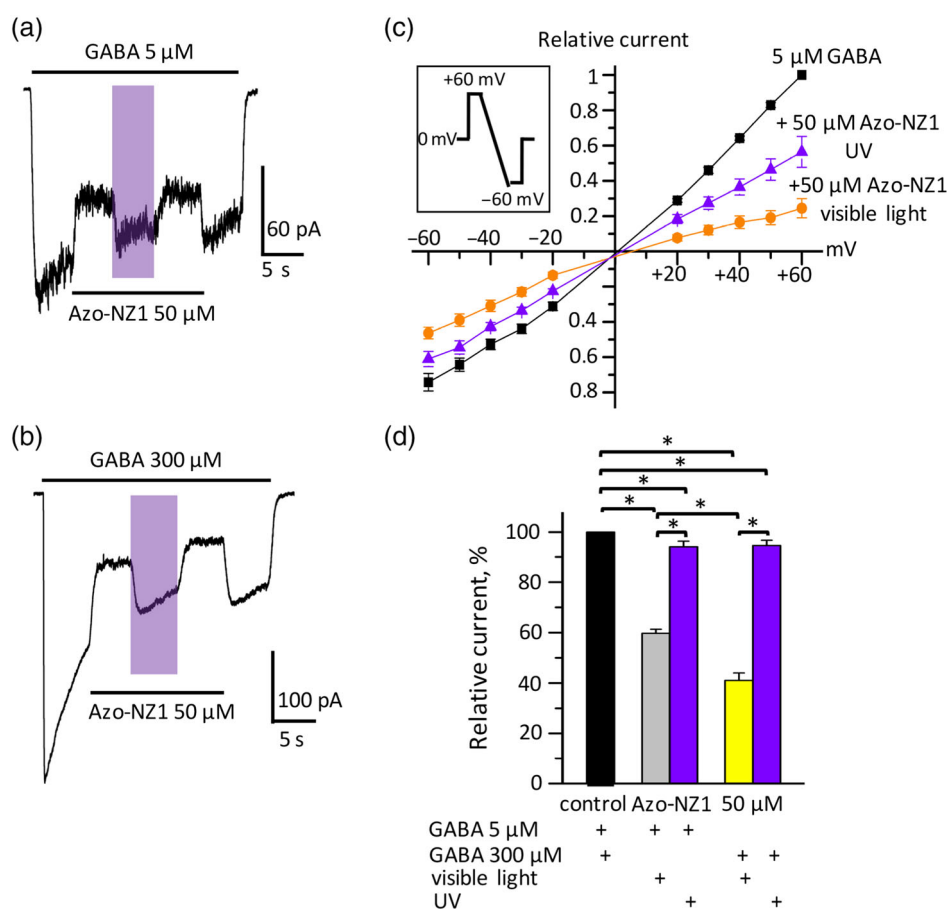


FIGURE 3 Strength of suppression of GABA_A receptors by Azo-NZ1 depends on the membrane potential and becomes stronger at higher agonist concentration. (a) Representative trace of the current induced by application of 5- μ M GABA and by co-application of 5- μ M GABA with 50- μ M Azo-NZ1, $V_{\text{hold}} = -30$ mV. (b) Representative recording of the current induced by saturating concentration of GABA (300 μ M) and by a mixture of 300- μ M GABA with 50- μ M Azo-NZ1, $V_{\text{hold}} = -30$ mV. (c) Cumulative current-voltage relationship curves obtained during application of 5- μ M GABA, a mixture of 5- μ M GABA with 50- μ M Azo-NZ1 at visible light, and GABA + Azo-NZ1 upon UV illumination ($n = 5$). Scheme of the ramp protocol is presented in the inset on the left. (d) Summary of GABA_A receptor-mediated suppression induced by Azo-NZ1. Mean relative amplitude (%) of currents induced by a mixture of Azo-NZ1 with low (5 μ M) and saturating (300 μ M) concentrations of GABA at visible light and upon UV illumination. Grey column: 5- μ M GABA + 50- μ M Azo-NZ1 at visible light; yellow column: 300- μ M GABA + 50- μ M Azo-NZ1 at visible light; violet columns: 5- or 300- μ M GABA + 50- μ M Azo-NZ1 at UV illumination. Data are presented as mean $\pm$ SEM; $V_{\text{hold}} = -30$ mV summary from six to eight cells, * $P < .05$

by GABA concentration below EC_{50} (5 μ M) was decreased by application of Azo-NZ1 (50 μ M) (Figure 3a,d). Azo-NZ1 co-applied with saturating concentration of GABA (300 μ M) produced an even stronger inhibitory effect (Figure 3b,d). Thus, Azo-NZ1 acts as a non-competitive antagonist of GABA_A receptors, and its binding does not coincide with the agonist-binding site. In both cases, UV illumination restores current amplitude, though not completely, close to the control values.

3.3 | Action of Azo-NZ1 on homomeric p1 and p2 GABA_A (GABA_C) receptors

To further investigate the hypothesis that Azo-NZ1 acts as a blocker of the GABA receptor channels without interacting with the diazepam-binding site located at the α/γ subunits interface, we analysed the action of this photochromic ligand on benzodiazepine-insensitive p1 and p2 GABA_A (GABA_C) homomeric receptors. Concentration-dependency analyses showed that the EC_{50} for GABA of p1 and p2 GABA_C receptors were similar (Figure 4a). In accordance with previous observations (Ragozzino et al., 1996), GABA-induced currents

mediated by these receptors do not exhibit desensitization and are characterized by a very long deactivation period after agonist application.

We found that Azo-NZ1 did not alter the activity of human p1 GABA_C receptors (Figure 4b), but it enabled robust photoswitching of p2 receptors (Figure 4d). The IC_{50} for GABA p2 receptors comprised 128 ± 25 μ M ($n = 6$), and thus, all the experiments with p2 receptors were performed at 100 μ M of Azo-NZ1, as this concentration was close to IC_{50} (Figure 4f). Under visible light, currents induced by saturating GABA concentration (10 μ M) were decreased by 100- μ M Azo-NZ1 at -30 and $+30$ mV, respectively (Figure 4d,e), that is, the inhibitory action of Azo-NZ1 on p2 receptors is also voltage-dependent. UV illumination restored the amplitude of the current to the nearly control values. The amplitude of p2 currents induced by 10 μ M of GABA without addition of Azo-NZ1 was not affected by UV illumination (Figure 4c).

3.4 | Molecular determinants of Azo-NZ1 action

The well-defined voltage dependence of the Azo-NZ1 effect on GABA_A and GABA_C p2 receptors and its independence on the

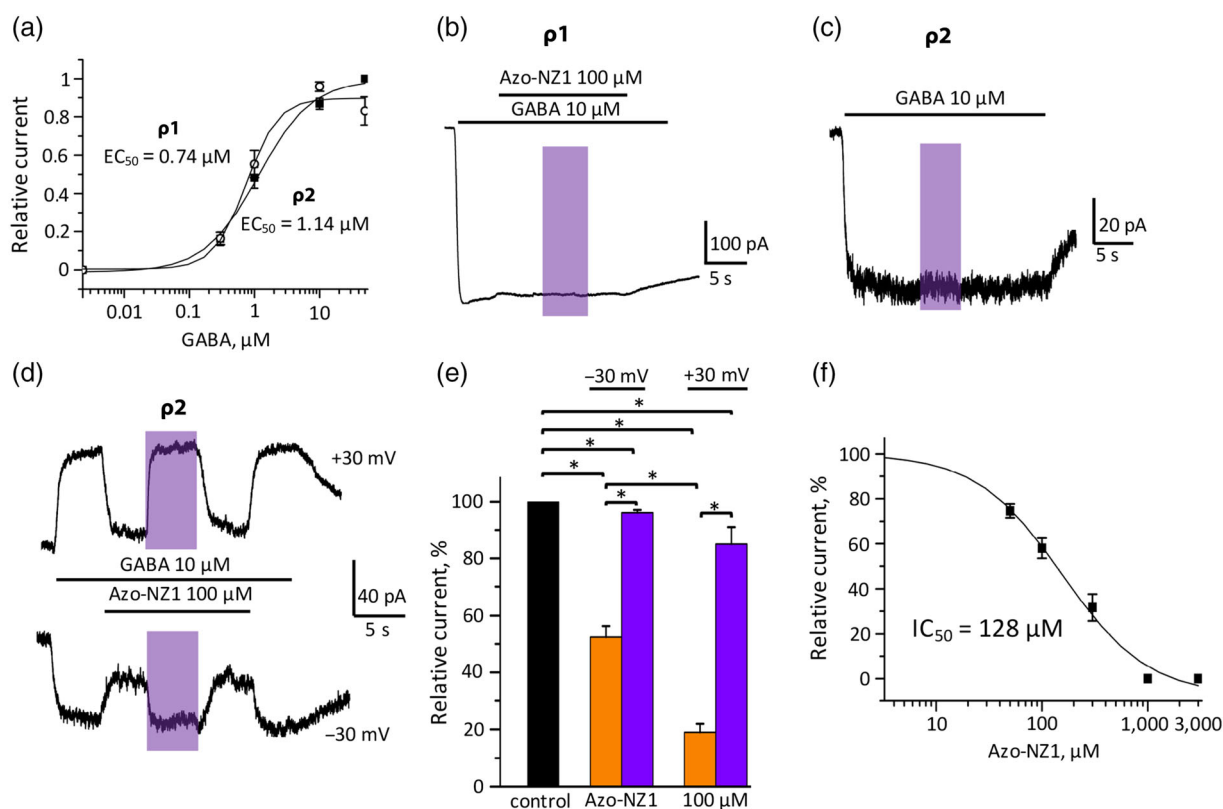


FIGURE 4 Azo-NZ1 decreases currents via GABA_C p2 receptors in a UV-dependent manner, while being not active at GABA_C p1 receptors. (a) Cumulative dose-response curves for GABA at GABA_C p1 and GABA_C p2 receptors. (b) Representative trace illustrating the absence of Azo-NZ1 effect on GABA_C p1 receptors at visible and UV light. (c) Representative trace of GABA p2-mediated current induced by application of GABA 10 μ M at visible light and upon UV illumination. Note the absence of UV effect on the amplitude of GABA-induced current. (d) Representative traces of GABA_C p2 currents induced by 10- μ M GABA and by a mixture of 10- μ M GABA with 100- μ M Azo-NZ1 at +30 mV (upper panel) and at -30 mV (lower panel). (e) The relative amplitudes of GABA_C p2-mediated currents suppressed by Azo-NZ1 (100 μ M): at +30 and -30 mV in control (GABA 10 μ M), after application of Azo-NZ1 in visible light (orange column) and upon UV illumination (violet column). Mean $\pm$ SEM, $n = 6-10$, $*P < .05$. (f) Cumulative dose-response curve for Azo-NZ1 at GABA p2 receptors ($n = 6$)

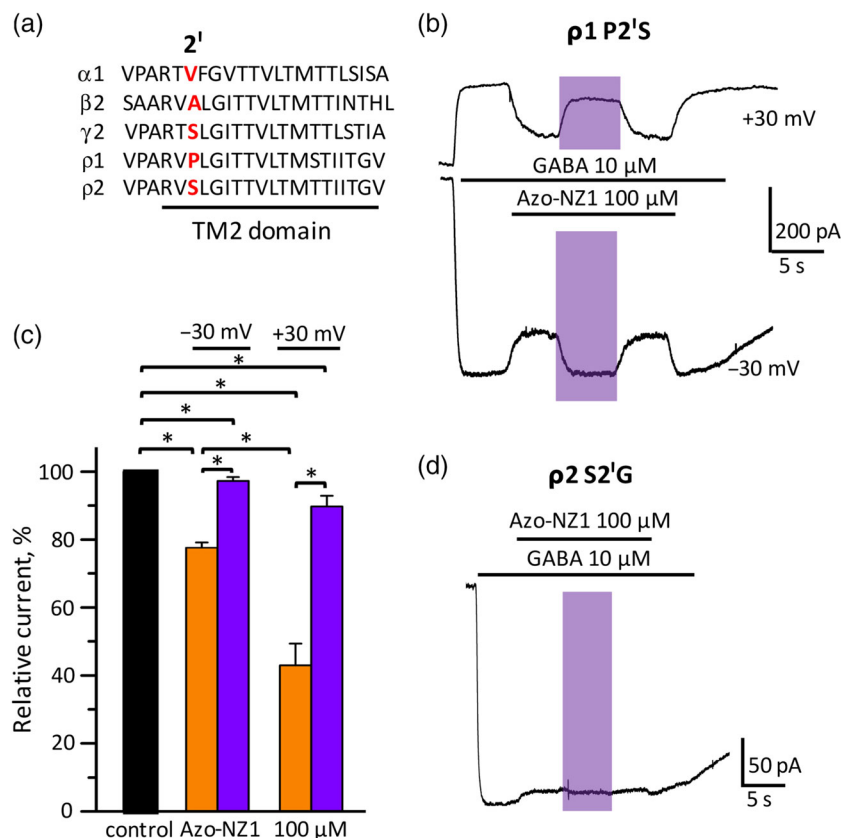


FIGURE 5 Effect of Azo-NZ1 on mutant p1 and p2 GABA_C receptors. (a) Amino acid sequence alignment of GABA receptor subunits TM2 Cl-selective pore. Amino acids in 2' position are highlighted in red. Note the high identity of the TM2 sequences between p1 and p2 subunits. (b) Representative traces of currents mediated by GABA p1 P2'S receptors suppressed by 100- μ M Azo-NZ1 application at -30 and +30 mV. (c) Relative amplitude of GABA-induced currents mediated by p1 P2'S receptors at +30 and -30 mV in control (10- μ M GABA), at application of 100- μ M Azo-NZ1 in visible light (orange column) and upon UV illumination (violet column). Mean $\pm$ SEM, $n = 7$, * $P < .05$. (d) Representative trace depicting the absence of Azo-NZ1 effect at p2 mutant S2'G receptors

agonist concentration strongly suggest that Azo-NZ1 in *trans*-configuration blocks the ion pore of these receptors. It is well documented that the position 2' of the pore-forming transmembrane domain 2 (TM2) is crucial for the action of several pore-blocking molecules in Cys-loop receptors, including GABA_C receptors (Xie, Song, Ripps, & Qian, 2008). An amino acid sequence alignment showed that TM2 domains of p1 and p2 subunits differ by the amino acids at the position 2': p1 contains proline while p2 contains a serine residue (Figure 5a).

To check the putative role of the 2' residue in Azo-NZ1 sensitivity, we substituted proline by serine at position 2' of the p1 subunit. P2'S mutation did not affect the apparent affinity of the p1 receptor to GABA ($EC_{50} = 0.98$, $n = 4$) but made it sensitive to the action of Azo-NZ1. Under visible light, 100- μ M Azo-NZ1 inhibited currents induced by saturating concentrations of 10- μ M GABA (Figure 5b,c). UV illumination caused recovery of currents to $97 \pm 1\%$ and $90 \pm 3\%$, respectively (Figure 5b).

Thus, we confirmed that Azo-NZ1 in its *trans*-configuration interacts with the pore of GABA receptors and identified the 2' position of the TM2 domain as key determinant of the light-dependent action of Azo-NZ1. This conclusion was additionally confirmed in experiments demonstrating that the S2'G mutation in the p2 subunit influenced its interaction with Azo-NZ1. Upon application of Azo-NZ1 currents amplitude decreased by $14 \pm 2\%$ (Figure 5d, $n = 5$, $P < .05$), which might be due to weak interaction between Azo-NZ1 and mutant receptor; importantly, UV effect was virtually absent. Results

of the modelling analysis presented below are in line with our observations.

3.5 | Interaction of Azo-NZ1 with other members of Cys-loop receptor family

GABA receptors and glycine receptors belong to the Cys-loop receptors family and amino acid sequences forming their Cl-selective pores are highly similar (Figure S1A). Thus, we asked if Azo-NZ1 also acted as a glycine receptor channel blocker.

Trans-Azo-NZ1 produced only minor inhibitory effect in $\alpha 1$ glycine receptors—the current amplitude was reduced to $86 \pm 3\%$ (Figure S1B, $n = 4$, $P < .05$), with slightly stronger reduction upon illumination with UV light (to $73 \pm 2\%$, $P < .05$). In contrast, in $\alpha 2$ glycine receptors, Azo-NZ1 induced a prominent decrease of the current amplitude (to $23 \pm 3\%$, $n = 10$, $P < .01$) that was altered by UV illumination ($95 \pm 7\%$, $P < .01$, $n = 10$; Figure S1B,C). The profile of $\alpha 2$ glycine receptor interaction with Azo-NZ1 strongly resembles the one of GABA_A and GABA_C receptors, suggesting the same pore-blocking mechanism of action.

In order to have a wider view of Azo-NZ1 selectivity profile, we also tested it in a cation-selective member of Cys-loop receptor family, the 5HT_{3A} receptor. In contrast to GABA receptors and glycine receptors, Azo-NZ1 in *trans* or *cis*-configurations did not interact in a specific manner with 5-HT_{3A} receptors (Figure S2, Supplementary

Text). Thus, Azo-NZ1 selectively interacts with anion-permeable channels of the Cys-loop receptor family, exhibiting a subunit specificity based on the nature of 2' residues in TM2 pore.

3.6 | Modelling of Azo-NZ1 molecular interactions with GABA receptors

To get further insights into the mechanism of action of Azo-NZ1 in GABA receptors, we performed molecular docking calculations. In view of the mutagenesis results indicating the relevance of the 2' residues, the docking was carried out in the transmembrane region of the ion channel pore. Four different GABA_C receptor structures were used: wild-type p2, S2'G p2 mutant, wild-type p1, and P2'S p1 mutant (see Section 2); all structures were modelled in the open channel state.

Docking of *trans*-Azo-NZ1 to the GABA_C p2 receptor (Figure 6a) showed that the preferred binding site of the sulfonate group is located in the narrow 2' position of the pore region (Figure S3A), at the centre of the ring formed by five serine residues (Figure 6b). Such a ligand orientation creates a strong hydrogen bond network involving the sulfonate oxygen atoms and the hydroxyl groups. In addition, a hydrogen bond between the hydroxyl group of one of the T13' residues and the carbonyl of the nitrazepam core of Azo-NZ1 holds the ligand in a linear orientation almost parallel to the pore axis. Further stabilization is provided by hydrophobic interactions involving the apolar region of the nitrazepam core, the side chain of L9' and the methyl group of T6' (Figure 6b). The length of the ligand (14 Å) is optimal to place its polar groups (sulfonate and amido) close to the polar side chains of S2' and T13', respectively, which are separated by 16 Å. In addition, the size of the sulfonate group is comparable with

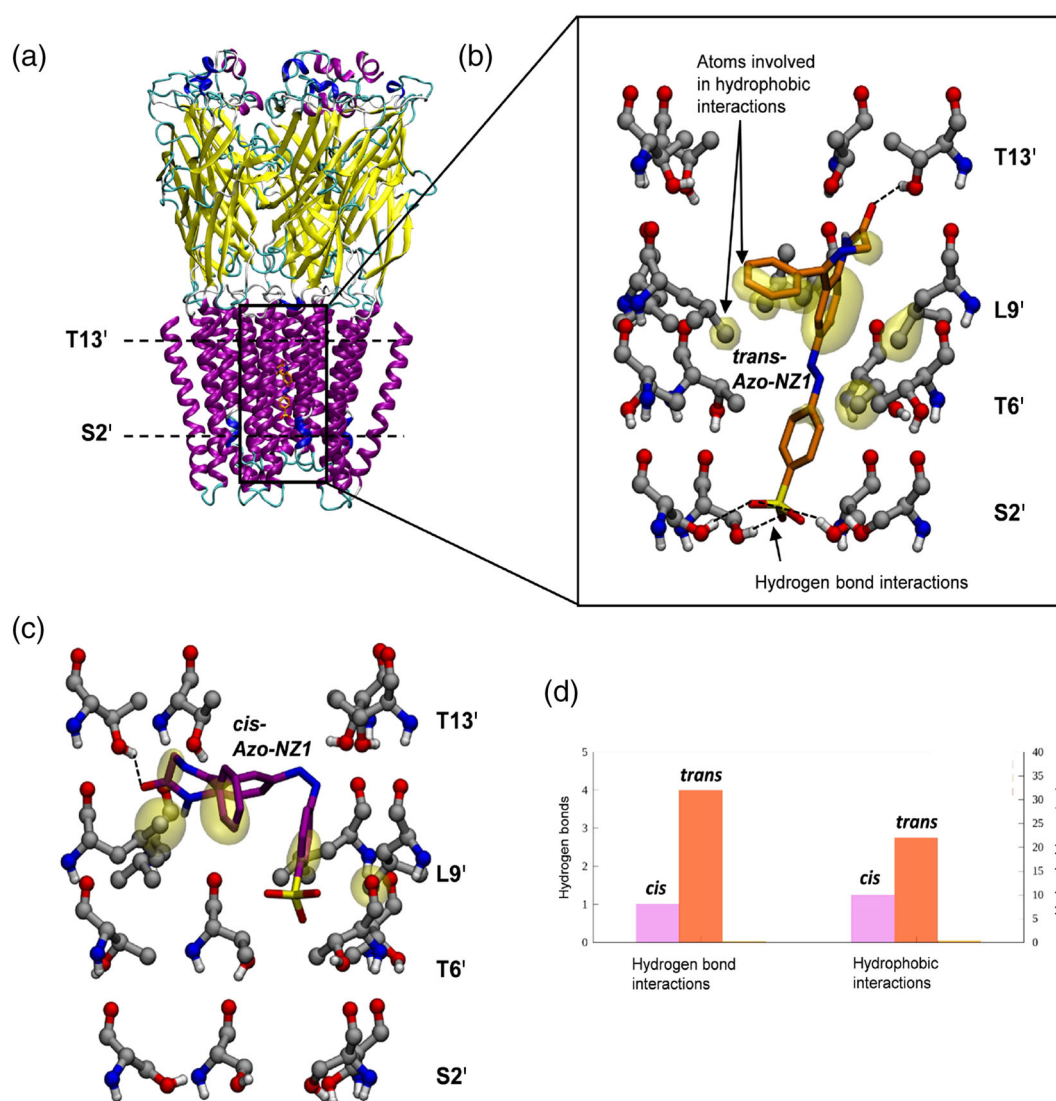


FIGURE 6 Results of the docking of Azo-NZ1 in the transmembrane part domain of p2 GABA_C receptor. (a) Tridimensional view of the p2 GABA_C receptor. The S2' and T13' positions are marked with dashed lines. (b) Most likely docking pose of the *trans*-isomer in the transmembrane domain. The interior of the pore is shown as viewed from the intracellular part. (c) Most likely pose for the *cis*-isomer in the transmembrane domain. The interior of the pore is shown as viewed from the extracellular part. (d) Relative number of intermolecular interactions formed by the *cis*- and *trans*-isomers of Azo-NZ1 with residues of the transmembrane domain of p2 GABA_C receptor

the pore diameter at the level of the 2' serine ring, thus creating an obstacle that hinders the passage of the chloride ions through the pore both sterically and electrostatically (Figure S4). Interestingly, the negatively charged sulfonate group occupies the same position as the chloride ion trapped in X-ray structures of GABA_A receptor (Lavery et al., 2017) and glycine receptor (Du, Lü, Wu, Cheng, & Gouaux, 2015). Altogether, the architecture of *trans*-Azo-NZ1 fits perfectly in the pore region of the GABA_C p2 receptor.

In contrast, the position of the sulfonate group of *cis*-Azo-NZ1 is more scattered all over the 2'-13' region of the pore (Figure S3B), and the number of poses with the sulfonate group in the 2' region (i.e., the ones able to block the pore) is decreased by half. Analysis of the preferred binding pose of the ligand (Figure 6c) shows that, due to the staple-like shape of *cis*-Azo-NZ1, the ligand is preferentially located further up in the pore and the number of interactions with the receptor is dramatically decreased compared to the *trans*-isomer (Figure 6d). The ligand forms only one hydrogen bond with the pore residues, involving the tertiary amine of the benzodiazepine core and one hydroxyl group of T13', and most importantly, the sulfonate group does not form any interaction with the pore-lining residues, since it is positioned near L9'. The smaller number of interactions of the *cis*-isomer compared to *trans* indicates that the ligand will have a

higher probability of dissociation upon UV irradiation. Moreover, the ligand is mostly placed in the regions where the diameter of the pore is wider (around 11 Å, see Figure S4), and thus, it cannot block chloride conduction by either steric or electrostatic effects. Therefore, we predict that, even if *cis*-Azo-NZ1 remains bound, it would not decrease the channel current of the receptor. These results explain the higher capability of *trans*-Azo-NZ1 to block the pore of the GABA_C p2 receptor compared to the *cis*-isomer.

The importance of 2' residues is also corroborated by the results obtained for the S2'G p2 mutant. In this case, the sulfonate group cannot form hydrogen bonds with the 2' residues. As a consequence, the population of *trans*-Azo-NZ1 in the 2' region decreases and the ligand is no longer able to block the pore (Figures S5B and S6A). Similar results were also obtained for the p1 receptor, which has proline at the 2' region (Figures S6B and S7A). Conversely, mutation of proline to serine (P2'S) in the p1 receptor increases the population of the sulfonate in the 2' region (Figure S7B) with respect to the WT receptor (Figure S7A). The associated binding mode of Azo-NZ1 to P2'S p1 (Figure S6C) is very similar to the one for wild-type p2 (Figure 6), consistent with the appearance of inhibition observed in our experiments (Figure 5).

We also performed docking calculations for the heteromeric $\alpha 1/\beta 2/\gamma 2$ GABA_A receptor, using a model of the open channel state

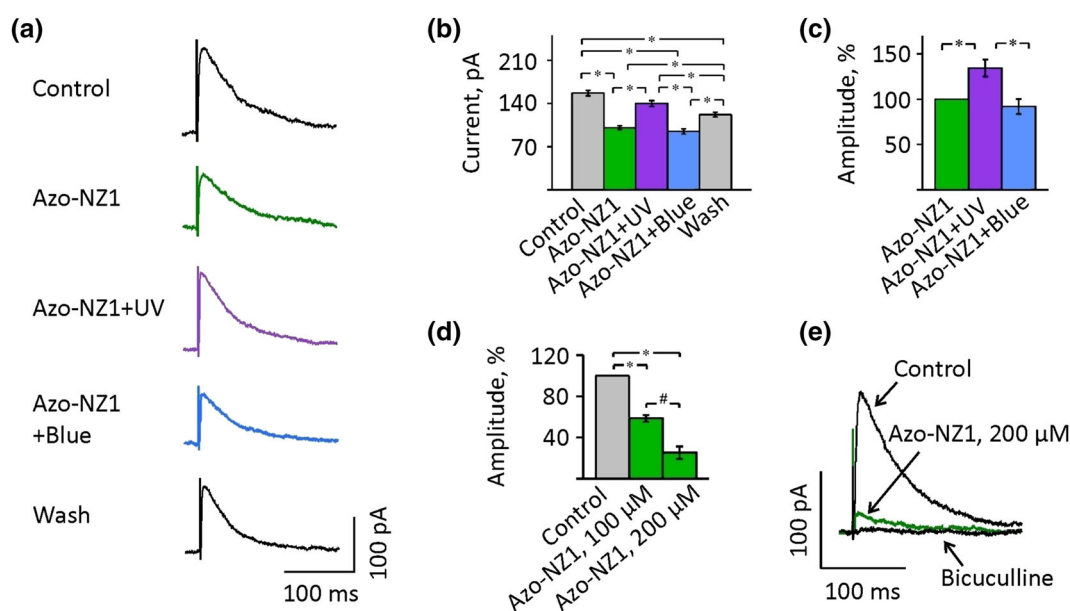


FIGURE 7 Effect of Azo-NZ1 on the GABAergic postsynaptic currents in brain slices. (a) Traces of average evoked GABAergic postsynaptic currents (eIPSCs) recorded from dentate gyrus granule cells in the adult mice hippocampal slices: in the control conditions (Control); after addition to aCSF of 100-μM Azo-NZ1 (Azo-NZ1); during perfusion of the slice with UV-illuminated aCSF +100-μM Azo-NZ1 (Azo-NZ1 + UV); during perfusion with blue light-illuminated aCSF +100-μM Azo-NZ1 (Azo-NZ1 + blue); 2–3 min of washing by aCSF (Wash). Each trace represents average of seven to 10 individual eIPSCs induced by stimulation with theta-tube electrode placed in the granule cell layer. $V_h = 0$ mV. (b) Summary of the mean amplitudes of GABAergic eIPSCs recorded in control conditions; in the presence of 100-μM Azo-NZ1, at illumination either at 365 nm (purple column) or 455 nm (green column). Data from the experiment presented in (a). Whiskers area reflects the standard error range. *Significant difference with $P < .05$ (two-sample t test, $n = 7$ –10). (c) Mean percentages of the eIPSCs amplitude potentiation in the presence of 100-μM Azo-NZ1 by UV illumination. *Significant difference with $P < .05$ (paired sample Wilcoxon signed rank test, $n = 5$). (d) Summary of the percentages of the eIPSC amplitude decrease in the presence of 100- and 200-μM Azo-NZ1 at blue light illumination. */#Significant difference with $P < .05$ (paired sample Wilcoxon signed rank test and Mann–Whitney U test, respectively; $n = 5$). (e) Superimposed traces of eIPSCs illustrating decrease of currents at addition of 200-μM Azo-NZ1 and 10-μM bicuculline. Note the complete suppression of events by bicuculline. Each trace represents average of six to 10 individual eIPSCs. $V_h = 0$ mV

(Bergmann, Kongsbak, Sørensen, Sander, & Balle, 2013). For this receptor, the density analysis results are noisier due to the heterogeneous subunit composition (Figure S8A). Therefore, we can only say with certainty that the *trans*-Azo-NZ1 binding mode with the sulphonate bound at position 2' is the most likely, similar to homomeric p2 GABA_C receptor. Even though there is only one serine in this region (belonging to the γ 2 subunit), the hydrogen bond interaction with the ligand is still present (Figure S8B). Moreover, although α 1-Val2' and β 2-Ala2' are not able to form hydrogen bonds, their volume is similar to that of Ser and thus still compatible with sulphonate binding at position 2'. In addition, the hydrogen bond between the carbonyl group of Azo-NZ1 and T13' is also maintained (Figure S8B). As a result, *trans*-Azo-NZ1 is clamped in the pore of the GABA_A receptor (Figure S8C), as it was in the GABA_C p2 receptor (Figure 6b) and thus is able to block chloride conduction.

Altogether, these observations demonstrate that Azo-NZ1 is a photoswitchable blocker of GABA_A and GABA_C receptors, whose *trans*-configuration interacts with residues located in the pore, in particular at positions 2' and 13'. In *trans*-Azo-NZ1, the distance between the sulphonate group and the carbonyl group of the benzodiazepine core is optimal for Azo-NZ1 to get clamped between these two residues, enabling pore blocking.

3.7 | Effect of Azo-NZ1 on synaptic currents in brain slices

Finally, to evaluate Azo-NZ1 as photochromic modulator of GABAergic synaptic events, we performed electrophysiological recordings from neurons in dentate gyrus granular layer, which possess a strong GABAergic synaptic inputs (Coulter & Carlson, 2007; Haselmann, Röpke, Werner, Kunze, & Geis, 2015).

Whole-cell recordings were performed at $V_h = 0$ mV and stimulation pulses induced by theta pipettes delivered at frequency 0.1 Hz, inducing a reliable GABAergic eIPSCs, which exhibited relatively low run-down (about 20%) during long-lasting recordings (>1 hr). In control conditions, amplitudes of eIPSCs varied in different neurons between 50 and 400 pA ($n = 10$).

For the neuron illustrated in the Figure 7a,b, amplitude of eIPSCs in control aCSF was 156.9 ± 4.5 pA, and it decreased to 100.6 ± 3.2 pA after addition of 100- μ M Azo-NZ1. Switching to the solution pre-illuminated with UV light (365 nm) caused an elevation of the amplitude, while the successive illumination with 455 nm resulted in its decrease. After washing with pure aCSF, the amplitude of eIPSCs recovered to near control value. Similar degrees of the light-induced modulation was observed on five other neurons (Figure 7c). Application of 200- μ M Azo-NZ1 caused much more pronounced inhibition (Figure 7d,e). On average, the suppression of eIPSCs amplitude by 100 and 200 μ M of Azo-NZ1 was to $58.6 \pm 3.3\%$ and $25.2 \pm 6.0\%$, respectively (Figure 7d). Bicuculline at 10 μ M caused complete inhibition of eIPSCs ensuring the GABAergic nature of the events (Figure 7e).

These observations demonstrate that Azo-NZ1 causes suppression of synaptic GABAergic currents in a light-dependent manner.

4 | DISCUSSION

Our study presents an azobenzene-nitrazepam-based photochromic compound (Azo-NZ1) and characterization of its action on Cys-loop receptors. Using electrophysiological, mutational, and molecular modelling analysis, we demonstrate that Azo-NZ1 is a light-controllable channel blocker of heteromeric GABA_A, homomeric GABA_C p2 receptors and α 2 glycine receptors, heterologally expressed in cultured cells, as well as modulator of synaptic GABAergic currents in dentate gyrus neurons of hippocampal brain slices. Our attention was mostly focused on the action of Azo-NZ1 at GABA receptors as they are the predominant subtype of inhibitory ligand-gated receptors in higher brain regions (Bowery et al., 1984; Sieghart & Sperk, 2002).

4.1 | Azo-NZ1 as Cl-channel blocker

Surprisingly, in our experiments, Azo-NZ1 caused an inhibitory effect on the GABA_A receptor, contrary to the potentiating effects via benzodiazepines binding sites. Moreover, similar inhibition was observed for GABA p2, which lacks the classical benzodiazepine site (Sieghart, 2015), and for α 2 glycine receptors. While we cannot entirely rule out the allosteric interaction of Azo-NZ1, the absence of modulation at nM concentrations and docking calculations (see Supporting Information) suggest that the functional effects of Azo-NZ1 via the classical benzodiazepine site are negligible.

Our results strongly suggest that the photochrome acts as a blocker of GABA receptors Cl-selective pore, interacting primarily with the 2' position of the TM2 domain of the channel. The following results support the role of Azo-NZ1 as GABA receptor pore blocker:

- Azo-NZ1-induced suppression becomes stronger upon elevation of GABA concentrations;
- the effect of Azo-NZ1 is voltage dependent, being much stronger at positive membrane potentials;
- mutation in the p1 GABA_C receptor of a single amino acid situated at the 2' level of the ion pore (P2'S) resulted in the appearance of blocking activity of Azo-NZ1, with features similar to GABA_C p2 receptors;
- the S2'G mutation in p2 subunits resulted in the loss of the Azo-NZ1 specific activity;
- molecular docking calculations show that the length of *trans*-Azo-NZ1 fits in the pore region, with the sulfonate group blocking ion passage at the 2' region.

In our experiments, we have achieved a robust photoswitching effect using Azo-NZ1. Upon UV irradiation, currents recovered to 85–97%. The remaining 15–3% might be explained by not complete switching of *trans*-Azo-NZ1 into *cis*-state, not complete dissociation of *cis*-Azo-NZ1 from the pore, or fast development of the receptor desensitization.

4.2 | Possible mechanism of Azo-NZ1 interaction with the channel

The 2' residue of the TM2 domain (see Figure 5a) is a key region that determines the action of several pore blockers of Cys-loop receptors, including cyanotriphenylborate (Rundström et al., 1994), picrotoxin (Lynch, Rajendra, Barry, & Schofield, 1995; Zhorov & Bregestovski, 2000), niflumic acid (Maleeva, Peiretti, Zhorov, & Bregestovski, 2017), and cyclothiazide (Xie, Song, Ripps, & Qian, 2008). The Azo-NZ1 construct contains two features that are essential to impart sensitivity to the photochrome: (a) the negative charge of the sulphonate group (mimicking that of the chloride ion) and (b) the distance between this sulphonate group and the carbonyl group of the benzo-diazepine core (see Figure S2). In *trans*-Azo-NZ1, this distance is optimal (~14.1 Å) to bridge the two pore-lining hydrogen bond residues S2' and T13'. Upon UV light irradiation, *cis*-Azo-NZ1 may either remain in the channel (assuming another binding conformation that unblocks the pore) or exit the channel (reaching the bath solution). Although with the data at hand we cannot distinguish between the two possibilities, it is feasible that *cis*-Azo-NZ1 remains partially bound in the pore, as can be suggested from Figures 3c and 7b and modelling analysis (Figure 6c).

4.3 | Effect of Azo-NZ1 on synaptic currents

Our analysis on dentate gyrus neurons from mouse brain slices demonstrated that Azo-NZ1 decreases amplitude of GABAergic synaptic events in a light-dependent manner. The inhibition was weaker in comparison with the one observed at heterologously expressed $\alpha 1/\beta 2/\gamma 2$ GABA_A receptors. This may result from the different composition of GABA receptors present in the dentate gyrus neurons, which can express up to 10 GABA_A receptor subunits, as single-cell mRNA analysis demonstrated (Brooks-Kayal, Shumate, Jin, Rikhter, & Coulter, 1998; Coulter & Carlson, 2007). Also, synaptic and extrasynaptic GABA_A receptors with distinctive properties and subunit composition can be differently modulated by the photochrome. The role of these discrepancies in the neuronal circuit modulation by Azo-NZ1 should be clarified in future studies.

Recently, several photochromic modulators of GABA_A receptors were developed (reviewed in Bregestovski, Maleeva, & Gorostiza, 2018): two potentiators (Stein et al., 2012; Yue et al., 2012) and several inhibitors (Huckvale, Mortensen, Pryde, Smart, & Baker, 2016; Lin et al., 2014, 2015; Lin, Tsai, Rajappa, & Kramer, 2018). The compounds developed by Lin et al. are required to be tethered to the receptor, which needs specific mutation of the GABA receptor, that is, genetic manipulation. The compound developed by Huckvale et al. is a soluble photochromic competitive GABA receptor antagonist based on combination of azobenzene and gabazine. Azo-NZ1, presented in our study, exhibits a completely different mode of action, being a light-controlled blocker of ionotropic GABA receptor channels. Moreover, Azo-NZ1 is the first known photochromic modulator of $\rho 2$ GABA_C receptors that are highly expressed in retina. Azo-NZ1 blocks

as well $\alpha 2$ glycine receptors in a similar UV-dependent manner, probably due to highly conserved amino acid sequences of pore-forming domains of GABA and glycine receptors. Indeed, the difference in Azo-NZ1 action on $\alpha 1$ and $\alpha 2$ glycine receptors, caused by glycine to alanine substitution in 2' position of their TM2 domains, confirms the pore-blocking effect of Azo-NZ1. In contrast, Azo-NZ1 was not able to block the 5-HT_{3A} receptor, a cation-selective member of Cys-loop receptor superfamily.

Our findings pave the way for development of a novel pharmacological toolset for photo-modulation, as well as functional and molecular physiology studies of these receptors. Azo-NZ1 is a freely diffusible ligand that binds to endogenous receptors, and such studies could be carried out without any genetic manipulations. Photocontrol of inhibitory neurotransmission should be readily accessible in acute preparations and in all kinds of vertebrate and invertebrate wild-type animals using Azo-NZ1.

ACKNOWLEDGEMENTS

We are grateful to Dr. M. Ernst for kind providing with $\rho 2$ GABA_C cDNA and Dr. S. Lummis for cDNA of 5-HT_{3A} subunit. This study was supported by ERA SynBio Grant MODULIGHTOR (PCIN-2015-163-C02-01), the Russian Science Foundation (Grant: 18-15-00313 for E. P. and P. B.), the program Algorish (Tatarstan, Russia, for E. P.), AGAUR/Generalitat de Catalunya (CERCA Programme 2017-SGR-1442 for C. R.), FEDER funds, Human Brain Project WAVESCALES, Fundaluce Foundation, Ramón Areces Foundation, and MINECO (Project CTQ2016-80066R). A. N.-H. thanks Generalitat de Catalunya for a PhD scholarship (FI-AGAUR). We also thankfully acknowledge the computer resources at MareNostrum III and MinoTauro and the technical support provided by the Barcelona Supercomputing Center (BCV-2016-2-0002 and BCV-2016-3-0005).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

G.M., E.P., and A.G.-J. performed the experiments. A.B.-B., D.W. and K.R. synthesized the compounds. A.N.-H. and M.A.-P. performed the molecular modelling. F.P. and P.S. performed the mutagenesis. G.M., K.R., A.N.-H., M.A.-P., C.R., B.K., P.G., P.B. analysed the data and wrote the manuscript. M.A.-P., C.R., B.K., P.G., P.B. supervised the project, reviewed, discussed, and conceptualized data.

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 as stated in the *BJP* guidelines for [Design & Analysis](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Maleeva G, Wutz D, Rustler K, et al. A photoswitchable GABA receptor channel blocker. *Br J Pharmacol*. 2019;176:2661–2677. <https://doi.org/10.1111/bph.14689>