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Functional Characterization of the 1,5-Benzodiazepine Clobazam and Its Major Active Metabolite *N*-Desmethylclobazam at Human $GABA_A$ Receptors Expressed in *Xenopus laevis* Oocytes

Harriet Hammer[1], Bjarke Ebert[2], Henrik Sindal Jensen[2], Anders A. Jensen[1]*

1 Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, **2** H. Lundbeck A/S, Valby, Denmark

* aaj@sund.ku.dk

Citation: Hammer H, Ebert B, Jensen HS, Jensen AA (2015) Functional Characterization of the 1,5-Benzodiazepine Clobazam and Its Major Active Metabolite *N*-Desmethylclobazam at Human $GABA_A$ Receptors Expressed in *Xenopus laevis* Oocytes. PLoS ONE 10(3): e0120239. doi:10.1371/journal.pone.0120239

Academic Editor: Uwe Rudolph, McLean Hospital/Harvard Medical School, UNITED STATES

Received: August 16, 2014

Accepted: February 4, 2015

Published: March 23, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: HH and AAJ received an unrestricted grant from Lundbeck (US) to conduct this work. AAJ thanks the Novo Nordisk Foundation for financial support. Lundbeck (Denmark) provided support in the form of salaries for authors HSJ and BE, but neither Lundbeck (US) or Lundbeck (Denmark) have had any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are

Abstract

The 1,5-benzodiazepine clobazam is indicated for the adjunctive treatment of seizures associated with Lennox-Gastaut syndrome in patients 2 years of age or older in the United States, and for treatment of anxiety and various forms of epilepsy elsewhere. Clobazam has been reported to exhibit different *in vivo* adverse effects and addiction liability profile than the classic 1,4-benzodiazepines. In this study, it was investigated whether the *in vitro* pharmacological properties of clobazam and its major active metabolite *N*-desmethylclobazam could explain some of these clinical differences. The functional properties of the two 1,5-benzodiazepines were characterized at the human γ-aminobutyric acid type A receptor ($GABA_AR$) subtypes $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ expressed in *Xenopus laevis* oocytes by use of two-electrode voltage-clamp electrophysiology and compared to those exhibited by the 1,4-benzodiazepine clonazepam. All three compounds potentiated GABA EC_{20}-evoked responses through the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_ARs$ in a reversible and concentration-dependent manner, with each displaying similar EC_{50} values at the four subtypes. Furthermore, the degrees of potentiation of the GABA EC_{20} currents through the four receptors mediated by saturating modulator concentrations did not differ substantially for any of the three benzodiazepines. The three compounds were substantially less potent (200-3900 fold) as positive allosteric modulators at the $\alpha_6\beta_2\delta$ $GABA_AR$ than at the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors. Interestingly, however, clobazam and especially *N*-desmethylclobazam were highly efficacious potentiators of $\alpha_6\beta_2\delta$ receptor signaling. Although this activity component is unlikely to contribute to the *in vivo* effects of clobazam/*N*-desmethylclobazam, the 1,5-benzodiazepine could constitute an interesting lead for novel modulators targeting this low-affinity binding site in $GABA_ARs$. In conclusion, the non-selective modulation exerted by clobazam, *N*-desmethylclobazam and clonazepam at the $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_ARs$ indicate that the observed clinical differences between

articulated in the 'author contributions' section. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: HS Jensen and BE are full-time employees of Lundbeck, whose company partly funded this study, and Clobazam (Onfi) is a Lundbeck product. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

clobazam and 1,4-benzodiazepines are likely to arise from factors other than their respective pharmacological properties at the $GABA_A$Rs as investigated here.

Introduction

As the main inhibitory neurotransmitter in the central nervous system (CNS), γ-aminobutyric acid (GABA) is directly involved in, or contributes to, an exhaustive number of physiological processes and pathophysiological states. GABA exerts its effects through two receptor classes, the $GABA_A$ and $GABA_B$ receptors [1, 2]. The $GABA_A$ receptors ($GABA_A$Rs) are membrane-bound, chloride-permeable ligand-gated ion channels belonging to the Cys-loop receptor superfamily, which also includes receptors for acetylcholine, serotonin, and glycine [2–5]. The $GABA_A$R complex is composed of five subunits, and the existence of a total of 19 human $GABA_A$ subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ and ρ_{1-3}) gives rise to an array of physiologically relevant receptor subtypes [6]. It is estimated that approximately 80% of all $GABA_A$Rs are αβγ receptors predominantly composed of $\alpha_{1/2/3/5}$, $\beta_{2/3}$, and γ_2 subunits in an anticlockwise α β α γ β arrangement (viewed from the extracellular space) [6–9]. However, numerous other physiologically important receptor subtypes exist, including the $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ receptors that through their predominant expression as extra- and perisynaptic receptors are key mediators of the GABAergic tonic inhibition [10, 11].

The signaling through the $GABA_A$R is susceptible to modulation by numerous ligands acting through different allosteric sites in the receptor complex, and many of these ligands are used to treat human pathologies [12–16]. Delineation of the molecular modes of action of these modulators at the receptors has provided considerable insight into the signal transduction mechanism of the $GABA_A$R as well as the molecular compositions of its allosteric sites. The allosteric modulation of the $\alpha_{1,2,3,5}\beta\gamma$ $GABA_A$Rs exerted by benzodiazepines is predominantly mediated through a high-affinity binding site located in the extracellular $\alpha^{(+)}/\gamma^{(-)}$ subunit interface of the receptor [14, 17]. However, benzodiazepines have also been proposed to target a low-affinity binding site located in the transmembrane domains of both αβ and αβγ $GABA_A$Rs [18]. Thus, the benzodiazepines have been proposed to possess a nM activity component arising exclusively from $\alpha_{1,2,3,5}\beta\gamma$ $GABA_A$Rs and a μM activity component that potentially could involve all $GABA_A$Rs [18]. Furthermore, several recent studies have proposed the existence of a low-affinity binding site for some benzodiazepines and other benzodiazepine-site ligands such as CGS 9895, LAU 177 and Ro 15–4513 in the extracellular $\alpha^{(+)}/\beta^{(-)}$ subunit interface of the $GABA_A$R, a site homologous to the extracellular high-affinity $\alpha^{(+)}/\gamma^{(-)}$ binding site for benzodiazepines in the αβγ $GABA_A$R [19–23].

The selectivity profile of a particular benzodiazepine at the different $\alpha_{1,2,3,5}\beta\gamma$ $GABA_A$R subtypes is believed to correlate to its clinical efficacy and adverse effects. Insights gained from studies of knock-in mice expressing benzodiazepine-insensitive subtypes have provided the rationale for the development of positive allosteric modulators (PAMs) of α_1-containing subtypes as hypnotics, of PAMs of α_2- or α_3-containing subtypes as anxiolytics and analgesics, and of negative allosteric modulators (NAMs) of α_5-containing receptors as cognitive enhancers [12, 13, 15, 16, 24]. Moreover, other studies suggest that the anticonvulsive and anxiolytic effects of benzodiazepines may be mediated via α_2-containing $GABA_A$Rs, whereas modulation of these subtypes has little or no sedative effects [25–29]. However, experiments in rodents indicate that the anticonvulsant effects require modulation of more than one specific α-subunit-containing $GABA_A$R and that modulation of different subtypes may act synergistically [29].

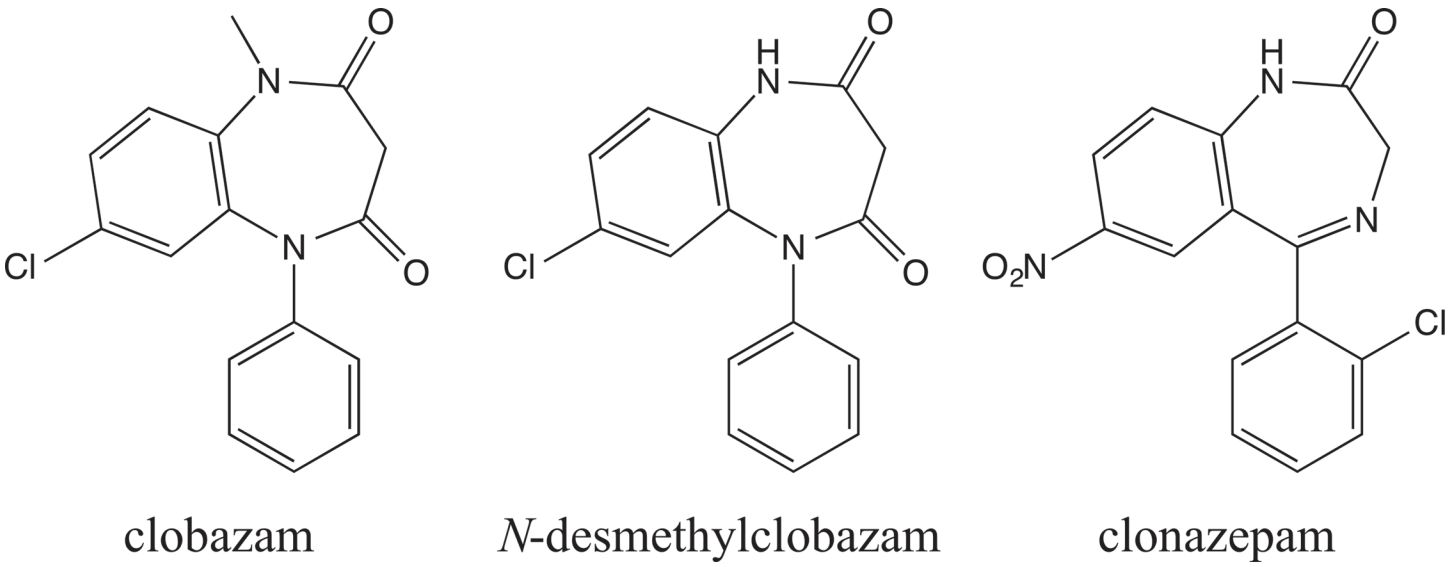

Fig 1. Chemical structures of clobazam, *N*-desmethylclobazam and clonazepam.

doi:10.1371/journal.pone.0120239.g001

To date, most benzodiazepines reported capable of modulating $GABA_AR$ signaling are 1,4-benzodiazepines. One of the few exceptions is the 1,5-benzodiazepine clobazam (Fig. 1). Clobazam (Onfi) has recently been approved in the United States and is indicated for the adjunctive treatment of seizures associated with Lennox-Gastaut syndrome in patients aged 2 years and older, and outside the United States the drug is routinely administered for anxiety disorders and epilepsy [30–32]. Interestingly, clobazam has been reported to exhibit different *in vivo* adverse effects and addiction liability profile than the 1,4-benzodiazepines, including clonazepam (Klonopin, Fig. 1), an antiepileptic drug approved for the treatment of Lennox-Gastaut syndrome (petit mal variant), and akinetic and myoclonic seizures. For example, studies suggest that clobazam induces fewer psychomotor disturbances than clonazepam when dosed at clinically effective concentrations in healthy volunteers [33, 34]. Moreover, preclinical studies suggest that clobazam in contrast to clonazepam and other 1,4-benzodiazepines may exert more specific anticonvulsant/antiepileptic over sedative effects [35, 36]. The major active metabolite of clobazam, *N*-desmethylclobazam (Fig. 1), has been shown to have a longer plasma half-life than the parent compound (79 h vs. 36 h), resulting in greater metabolite plasma concentrations following long-term clobazam dosing in humans [37]. Hence, the metabolite is likely to contribute significantly to the clinical efficacy of clobazam, and interestingly *N*-desmethylclobazam has been reported to produce fewer adverse effects than clobazam [38, 39].

Considering that clobazam has been administered for various indications in the clinic for decades, the current insight into the pharmacological characteristics of clobazam and *N*-desmethylclobazam at $GABA_ARs$ is surprisingly limited [40]. In a recent study, we delineated the binding characteristics of clobazam, *N*-desmethylclobam and the 1,4-benzodiazepine clonazepam at native $GABA_ARs$ in rat brain membranes and at human $\alpha_{1,2,3,5}\beta_2\gamma_{2S}GABA_ARs$ expressed in HEK293 cells in a [^{3}H]flumazenil competition binding assay [41]. In the present study, we have characterized the functional properties of clobazam and *N*-desmethylclobazam at human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ $GABA_ARs$ expressed in *Xenopus laevis* oocytes using two-electrode voltage clamp (TEVC) electrophysiology and compared these functionalities to that exhibited by clonazepam.

Materials and Methods

Materials

GABA, $ZnCl_2$ and chemicals for buffers were obtained from Sigma-Aldrich (Denmark), and DS2 was obtained from Tocris Cookson (Bristol, UK). Clobazam (synthesized at H. Lundbeck A/S, Denmark), *N*-desmethylclobazam (from Johnson Matthey Pharma Services, MA, USA), clonazepam (from Lipomed AG, Switzerland), diazepam, and zolpidem (both from Sigma-Aldrich, Denmark) were dissolved in DMSO and diluted in Ringer buffer on the given experimental day. The cDNAs encoding human α_1, α_2, α_3, α_5, α_6, β_2, γ_{2S} and δ $GABA_AR$ subunits were kind gifts from Dr. P J Whiting and Merck, Sharp & Dohme (Harlow, Essex, UK), and they were subcloned into mammalian expression vector pcDNA3.1 (Invitrogen, Denmark) as described previously [42, 43].

Preparation of cRNA and injection in *Xenopus laevis* oocytes

The cDNAs encoding the human $GABA_AR$ subunits were linearized with *Dra*III (α_1, α_2, α_3, α_5 and α_6), *Sma*I (β_2 and γ_{2S}) or *Stu*I (δ) and used as templates for *in vitro* cRNA synthesis using the T7 mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA). Except where otherwise indicated, *Xenopus* oocytes were injected with 36.8 nL cRNA solution encoding for $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_ARs$ in a 1:1:1 α:β_2:γ_{2S} ratio (2.7 ng/μL of each subunit), with 46 nL cRNA solution encoding for the $\alpha_6\beta_2\delta$ $GABA_AR$ in a 10:1:10 α_6:β_2:δ ratio (1:0.1:1 μg/μL), with 18 nL cRNA solution encoding for the $\alpha_1\beta_2$ $GABA_AR$ in a 1:1 α_1:β_2 ratio (0.6:0.6 μg/μL), or with 46 nL cRNA encoding for $\alpha_6\beta_2$ (α_6:β_2 ratio: 1:0.1 μg/μL). Following injection, the oocytes were incubated at 18°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 15 mM HEPES (pH 7.5), 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 100 U/mL penicillin and 100 μg/mL streptomycin]. Electrophysiological recordings were performed 3 to 6 days after injection.

Electrophysiological recordings

Electrophysiological recordings were performed using the TEVC technique on *Xenopus* oocytes expressing various $GABA_AR$ combinations using a protocol adapted from previous studies [44, 45]. Oocytes were placed in a recording chamber and gravity perfused with Ringer buffer [115 mM NaCl, 2.5 mM KCl, 10 mM HEPES (pH 7.5), 1.8 mM $CaCl_2$, 0.1 mM $MgCl_2$]. Cells were impaled with agar-plugged 0.5–1 MΩ electrodes containing 3 M KCl and voltage clamped at −70 mV by a Gene Clamp 500B amplifier (Axon Instruments, Union City, CA, USA) and recorded with pClamp 10 (Windows version, Molecular Devices, LLC, Sunnyvale, CA, USA). The oocytes were continuously perfused with Ringer buffer, and the test compounds were applied in the perfusate. Experiments were performed at room temperature and each data point represents the mean ± S.E.M. value of recordings performed on at least two oocytes from at least two different batches of oocytes. The recorded baseline-to-peak current amplitudes were analyzed using Clampfit 10.1 (Axon Instruments, Union City, CA, USA). Analogously to the procedures used in a recent study [43], the incorporation of the γ_{2S} subunit into the $GABA_ARs$ assembled at the cell surface of $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$-expressing oocytes was confirmed on a routinely basis with 100 μM $ZnCl_2$ [46]. The presence of δ in cell surface-expressed receptors in $\alpha_6\beta_2\delta$-injected oocytes was confirmed on a routinely basis using the δ-$GABA_AR$ selective PAM DS2 (1 μM) [47]. Furthermore, taking advantage of the differential sensitivity of αβ and αβδ receptors to Zn^{2+} as antagonist [45, 48–50], 1 μM $ZnCl_2$ was used to verify that a homogenous population of ternary $\alpha_6\beta_2\delta$ receptor complexes was expressed in these oocytes.

The GABA EC_{20} (the GABA concentration eliciting 20% of the maximum effect) for each receptor subtype was determined on two oocytes on each day of the experiment. A maximum concentration of GABA was applied until the peak of the response was observed, usually within 30 seconds. When two consecutive applications of the maximum GABA concentration were observed to elicit responses of similar effect (± 5%), 3–4 different concentrations of GABA were applied to the perfusate until the peak of the response was observed. Two to five minutes of wash time between each application were allowed to prevent receptor desensitization. Data for the GABA concentrations was normalized to the maximal response elicited by GABA on each oocyte, and the concentration-response curves were fitted in Prism GraphPad 5.0a (GraphPad Software, Inc. La Jolla, CA, USA) by nonlinear regression using the equation for sigmoidal dosage-response with variable slope (Equation 1): (1) Y = Bottom + [(Top—Bottom)/(1 + $10^{(logEC50-X)Hillslope}$)].

Bottom = response at the bottom plateau; EC_{50} = concentration giving rise to 50% of the maximum response; Top = response at the top plateau; X = logarithm of the concentration; Y = response.

The GABA EC_{20} response was calculated using Equation 2 (F = 20), and this concentration was subsequently used on a given experimental day. (2) log EC_{50} = (1/HillSlope) [log (F/(100-F)].

The functional characteristics of clobazam, *N*-desmethylclobazam, clonazepam, diazepam, and zolpidem on the $GABA_A$Rs were determined by co-application of different concentrations of the compounds with GABA EC_{20}. The test compounds were pre-applied 30 seconds prior to the co-application with GABA EC_{20}. At the end of an experiment, a maximum concentration of GABA was applied in the perfusate to determine the maximum response elicited by GABA through the receptor, which served as the internal standard and as a control of any potential drift in the system during the recordings. Two to five minutes of wash time between each application were permitted to overcome receptor desensitization. Data for the benzodiazepines was normalized to the responses elicited by GABA EC_{20} at the receptor (the EC_{20} response was defined as 100%). Concentration-response curves for the benzodiazepines were fitted using Equation 1.

Data analysis

Using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA), the pEC_{50} and I_{max} values were evaluated for statistical differences across the receptor subtypes per compound using one-way ANOVA with Tukey's Multiple-Comparison *Post-hoc* Test, where $P<0.05$ was considered significant.

Results

Functional characterization of GABA and determination of GABA EC_{20} values at human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ $GABA_A$Rs expressed in *Xenopus* oocytes. Prior to the functional characterization of the benzodiazepines at the five $GABA_A$R subtypes, the pharmacological properties of GABA at the receptors were determined. The concentration-response relationships displayed by GABA at the receptors are given in **Fig. 2**, and the pharmacological data are summarized in Table 1. The EC_{50} and Hill slope values determined for the agonist were in good agreement with those observed in previous studies of these five receptors expressed in *Xenopus* oocytes [46, 51].

For the functional characterization of the benzodiazepines at the receptors, the GABA EC_{20} values were determined on the days of the experiments. The actual GABA concentrations constituting the EC_{20} values for the respective receptor subtypes varied within 2- to 4-fold from

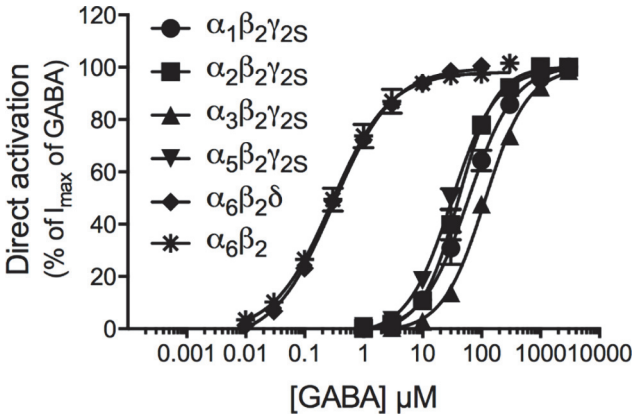

Fig 2. Functional properties of GABA at six human $GABA_A$Rs expressed in *Xenopus* oocytes. Concentration-response curves of GABA at the $\alpha_1\beta_2\gamma_{2S}$ (circle), $\alpha_2\beta_2\gamma_{2S}$ (square), $\alpha_3\beta_2\gamma_{2S}$ (triangle), $\alpha_5\beta_2\gamma_{2S}$ (inverted triangle), $\alpha_6\beta_2\delta$ (diamond) and $\alpha_6\beta_2$ (asterisk) $GABA_A$Rs (means ± S.E.M.; N = 4–7).

doi:10.1371/journal.pone.0120239.g002

day to day ($\alpha_1\beta_2\gamma_{2S}$: 20–30 μM; $\alpha_2\beta_2\gamma_{2S}$: 25–45 μM; $\alpha_3\beta_2\gamma_{2S}$: 25–60 μM; $\alpha_5\beta_2\gamma_{2S}$: 15–45 μM; $\alpha_6\beta_2\delta$0.05–0.20 μM). Thus, this procedure enabled us to use very accurate GABA EC_{20} concentrations for these studies. In fact, retrospective evaluation of the specific GABA concentrations used for characterization of the benzodiazepines in these subsequent studies revealed that these varied very little from the calculated EC_{20} (as percentage of GABA I_{max}, means ± S.E.M., N): $\alpha_1\beta_2\gamma_{2S}$ (20.2 ± 0.74, N = 15); $\alpha_2\beta_2\gamma_{2S}$ (19.8 ± 0.89, N = 17); $\alpha_3\beta_2\gamma_{2S}$ (21.0 ± 1.07, N = 11); $\alpha_5\beta_2\gamma_{2S}$ (18.6 ± 1.30, N = 13); and $\alpha_6\beta_2\delta$ (15.7 ± 1.47, N = 16).

Functional properties of clobazam, *N*-desmethylclobazam, and clonazepam at human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ $GABA_A$Rs expressed in *Xenopus* oocytes. The functional properties of clobazam, *N*-desmethylclobazam, and clonazepam when preincubated and co-applied with EC_{20} GABA at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_A$Rs expressed in *Xenopus* oocytes are given in Table 2. Representative traces for each of the three compounds at the $\alpha_5\beta_2\gamma_{2S}$subtype are given in Fig. 3A, and the concentration-response relationships obtained for the three compounds at the four receptors are outlined in Fig. 3B.

EC_{50} values are given in nM with pEC_{50} ± S.E.M. values in brackets, I_{max} values are given as % of the response evoked by GABA EC_{20} at the receptor, and the numbers of experiments (N) are also given.

Clobazam, *N*-desmethylclobazam, and clonazepam all potentiated GABA EC_{20}-mediated responses through the $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$Rs in a reversible and concentration-dependent manner (Fig. 3A and 3B). Clobazam and *N*-desmethylclobazam

Table 1. Functional properties of GABA at the human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$, $\alpha_6\beta_2\delta$ and $\alpha_6\beta_2$$GABA_A$Rs expressed in *Xenopus* oocytes. The EC_{50} values are given in μM with pEC_{50} ± S.E.M. values in brackets, and the Hill slopes (n_H ± S.E.M.) and the numbers of experiments (N) are also given.

Receptor	EC_{50} [pEC_{50} ± S.E.M.]	n_H ± S.E.M.	N
$\alpha_1\beta_2\gamma_{2S}$	57 [4.25 ± 0.10]	1.19 ± 0.05	6
$\alpha_2\beta_2\gamma_{2S}$	40 [4.40 ± 0.07]	1.44 ± 0.07	4
$\alpha_3\beta_2\gamma_{2S}$	120 [3.94 ± 0.03]	1.22 ± 0.05	7
$\alpha_5\beta_2\gamma_{2S}$	31 [4.50 ± 0.06]	1.19 ± 0.08	6
$\alpha_6\beta_2\delta$	0.30 [6.52 ± 0.04]	0.89 ± 0.02	6
$\alpha_6\beta_2$	0.29 [6.54 ± 0.07]	0.89 ± 0.09	7

doi:10.1371/journal.pone.0120239.t001

Table 2. Functional properties of clobazam, *N*-desmethylclobazam, and clonazepam at the human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$, $\alpha_6\beta_2\delta$ and $\alpha_6\beta_2$GABA$_A$Rs expressed in *Xenopus* oocytes.

Receptor	EC_{50} [pEC_{50} ± S.E.M.]	I_{max} ± S.E.M. (%)	N
Clobazam			
$\alpha_1\beta_2\gamma_{2S}$	132 [6.88 ± 0.05]	256 ± 12	5
$\alpha_2\beta_2\gamma_{2S}$	138 [6.86 ± 0.04]	261 ± 6.7	6
$\alpha_3\beta_2\gamma_{2S}$	240 [6.62 ± 0.10]	269 ± 0.8	4
$\alpha_5\beta_2\gamma_{2S}$	174 [6.76 ± 0.03]	216 ± 7.0	6
$\alpha_6\beta_2\delta$	55,000 [4.26 ± 0.04]	528 ± 46	6
N-desmethylclobazam			
$\alpha_1\beta_2\gamma_{2S}$	151 [6.82 ± 0.07]	203 ± 8.5	6
$\alpha_2\beta_2\gamma_{2S}$	138 [6.86 ± 0.18]	270 ± 20	6
$\alpha_3\beta_2\gamma_{2S}$	282 [6.55 ± 0.11]	270 ± 27	5
$\alpha_5\beta_2\gamma_{2S}$	98 (123 and 79) [b]	233 (248 and 217) [b]	2
$\alpha_6\beta_2\delta$	~300,000 [~3.5] [a]	2420 ± 210 [a]	5
$\alpha_6\beta_2$	~300,000 [~3.5] [a]	2350 ± 200 [a]	4
Clonazepam			
$\alpha_1\beta_2\gamma_{2S}$	15 [7.81 ± 0.12]	209 ± 14	4
$\alpha_2\beta_2\gamma_{2S}$	7.4 [8.13 ± 0.04]	253 ± 17	5
$\alpha_3\beta_2\gamma_{2S}$	16 (20 and 13) [b]	316 (334 and 297) [b]	2
$\alpha_5\beta_2\gamma_{2S}$	21 [7.68 ± 0.17]	255 ± 25	5
$\alpha_6\beta_2\delta$	29,000 [4.53 ± 0.10]	283 ± 38	5

[a] The concentration-response curves for *N*-desmethylclobazam at the $\alpha_6\beta_2\delta$ and $\alpha_6\beta_2$GABA$_A$Rs were not saturated at the maximal concentration used (1 mM). Thus, for this receptors the EC_{50} and pEC_{50} values for *N*-desmethylclobazam are estimated from the data, and the currents evoked by 1 mM *N*-desmethylclobazam (in % of the GABA EC_{20} response) is given instead of I_{max}.

[b] The properties for *N*-desmethylclobazam at $\alpha_5\beta_2\gamma_{2S}$ and for clonazepam at $\alpha_3\beta_2\gamma_{2S}$ are based on two independent experiments (n = 2), and thus the mean EC_{50} and I_{max} values for the modulators are given with specific EC_{50} and I_{max} values determined in the two experiments in parantheses.

doi:10.1371/journal.pone.0120239.t002

displayed EC_{50} values within the 100–300 nM range at all four receptors, whereas clonazepam was 9-, 19-, 15-, and 8-fold more potent than clobazam and 10-, 19-, 18-, and 5-fold more potent than *N*-desmethylclobazam as a PAM at $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$, respectively (Table 2). In terms of modulatory efficacy, saturating concentrations of clobazam and *N*-desmethylclobazam potentiated the GABA EC_{20}-evoked currents through the receptors 203–270%, corresponding to 40–54% of the maximum responses evoked by GABA through the respective receptors. Clonazepam potentiated the GABA EC_{20}-evoked responses through the receptors of 209–316%, corresponding to 42–63% of the maximum GABA responses (Fig. 3B, Table 2).

Statistical evaluation of the differences in pEC_{50} and I_{max} values exhibited by clobazam, *N*-desmethylclobazam, and clonazepam at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ GABA$_A$R subtypes was performed (Table 3). Significant differences ($P<0.05$) were identified for the pEC_{50} values for clobazam between $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_3\beta_2\gamma_{2S}$ subtypes as well as between the $\alpha_2\beta_2\gamma_{2S}$ and $\alpha_3\beta_2\gamma_{2S}$ receptors. The I_{max} value of clobazam at $\alpha_5\beta_2\gamma_{2S}$ was significantly smaller than those at $\alpha_1\beta_2\gamma_{2S}$ ($P<0.05$), $\alpha_2\beta_2\gamma_{2S}$ ($P<0.01$), and $\alpha_3\beta_2\gamma_{2S}$ ($P<0.01$). The pEC_{50} value of *N*-desmethylclobazam at $\alpha_3\beta_2\gamma_{2S}$ was significantly smaller than those at $\alpha_2\beta_2\gamma_{2S}$ ($P<0.01$) and $\alpha_5\beta_2\gamma_{2S}$ ($P<0.05$), and its I_{max} value at $\alpha_1\beta_2\gamma_{2S}$ was significantly smaller than those obtained at $\alpha_2\beta_2\gamma_{2S}$ and $\alpha_3\beta_2\gamma_{2S}$ receptors

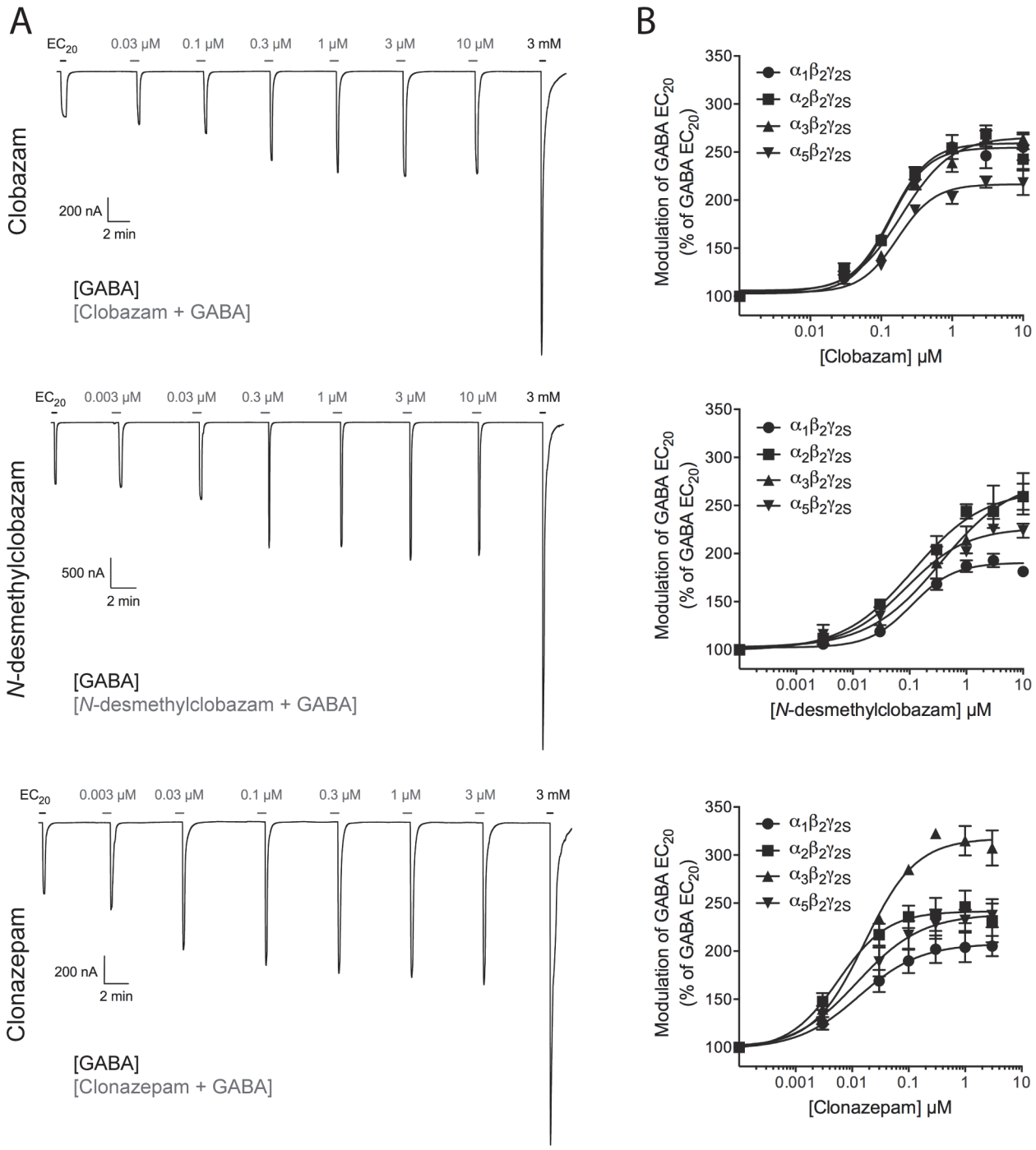

Fig 3. Functional properties of clobazam, *N*-desmethylclobazam and clonazepam at four human $GABA_A$Rs expressed in *Xenopus* oocytes. (A) Representative traces for various concentrations of clobazam (top), *N*-desmethylclobazam (middle) and clonazepam (bottom) co-applied with GABA EC_{20} to oocytes expressing the $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$R. The black bars represent applications of GABA EC_{20} and of 3 mM GABA that elicits maximal current through the receptor. The grey bars represent applications of various concentrations of clobazam, *N*-desmethylclobazam or clonazepam (a 30 s pre-incubation with the compound followed by co-application of the compound and GABA EC_{20}). (B) Concentration-response relationships for clobazam (top), *N*-desmethylclobazam (middle) and clonazepam (bottom) at $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$Rs in the presence of GABA EC_{20} (means ± S.E.M.; N = 2–6).

doi:10.1371/journal.pone.0120239.g003

Table 3. Statistical analysis of the functional properties of clobazam, *N*-desmethylclobazam, and clonazepam at the human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$Rs.

								pEC_{50} Values						
Clobazam	**$\alpha_1\beta_2\gamma_{2S}$**	**$\alpha_2\beta_2\gamma_{2S}$**	**$\alpha_3\beta_2\gamma_{2S}$**	**$\alpha_5\beta_2\gamma_{2S}$**	***N*-desmethylclobazam**	**$\alpha_1\beta_2\gamma_{2S}$**	**$\alpha_2\beta_2\gamma_{2S}$**	**$\alpha_3\beta_2\gamma_{2S}$**	**$\alpha_5\beta_2\gamma_{2S}$**	**Clonazepam**	**$\alpha_1\beta_2\gamma_{2S}$**	**$\alpha_2\beta_2\gamma_{2S}$**	**$\alpha_3\beta_2\gamma_{2S}$**	**$\alpha_5\beta_2\gamma_{2S}$**
$\alpha_1\beta_2\gamma_{2S}$	—	NS	$P<0.05$	NS	$\alpha_1\beta_2\gamma_{2S}$	—	NS	NS	NS	$\alpha_1\beta_2\gamma_{2S}$	—	NS	NS	NS
$\alpha_2\beta_2\gamma_{2S}$	—	—	$P<0.05$	NS	$\alpha_2\beta_2\gamma_{2S}$	—	—	$P<0.01$	NS	$\alpha_2\beta_2\gamma_{2S}$	—	—	NS	NS
$\alpha_3\beta_2\gamma_{2S}$	—	—	—	NS	$\alpha_3\beta_2\gamma_{2S}$	—	—	—	$P<0.05$	$\alpha_3\beta_2\gamma_{2S}$	—	—	—	NS
$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—	$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—	$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—
I_{max} Values														
Clobazam	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_2\beta_2\gamma_{2S}$	$\alpha_3\beta_2\gamma_{2S}$	$\alpha_5\beta_2\gamma_{2S}$	*N*-desmethylclobazam	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_2\beta_2\gamma_{2S}$	$\alpha_3\beta_2\gamma_{2S}$	$\alpha_5\beta_2\gamma_{2S}$	Clonazepam	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_2\beta_2\gamma_{2S}$	$\alpha_3\beta_2\gamma_{2S}$	$\alpha_5\beta_2\gamma_{2S}$
$\alpha_1\beta_2\gamma_{2S}$	—	NS	NS	$P<0.05$	$\alpha_1\beta_2\gamma_{2S}$	—	$P<0.05$	$P<0.05$	NS	$\alpha_1\beta_2\gamma_{2S}$	—	NS	$P<0.05$	NS
$\alpha_2\beta_2\gamma_{2S}$	—	—	NS	$P<0.01$	$\alpha_2\beta_2\gamma_{2S}$	—	—	NS	NS	$\alpha_2\beta_2\gamma_{2S}$	—	—	NS	NS
$\alpha_3\beta_2\gamma_{2S}$	—	—	—	$P<0.01$	$\alpha_3\beta_2\gamma_{2S}$	—	—	—	NS	$\alpha_3\beta_2\gamma_{2S}$	—	—	—	NS
$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—	$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—	$\alpha_5\beta_2\gamma_{2S}$	—	—	—	—

P-values from one-way ANOVA testing if mean values (pEC_{50} and I_{max}) experimentally determined for the compounds with Tukey's Multiple Comparison *Post-hoc* Test. NS; not significant.

doi:10.1371/journal.pone.0120239.t003

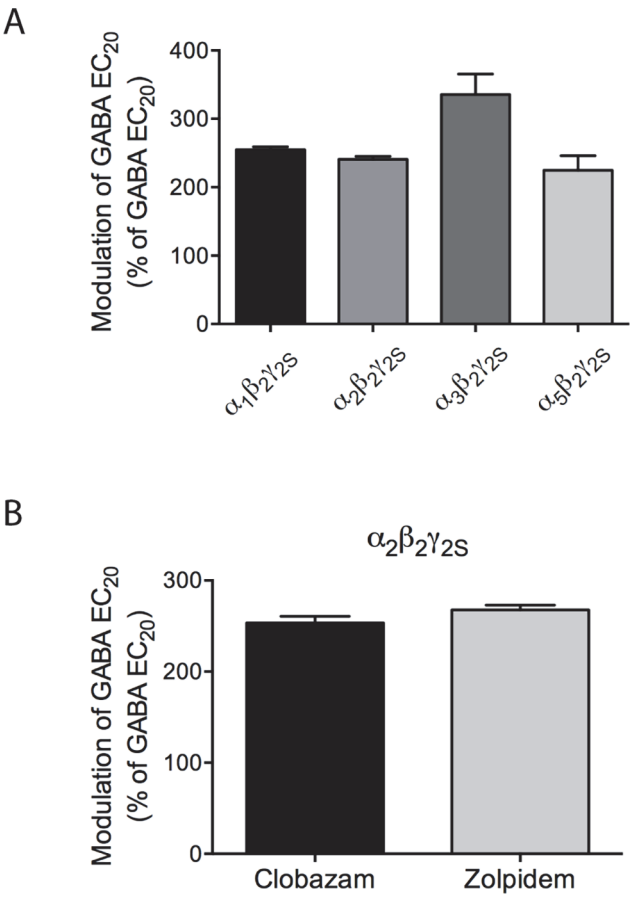

Fig 4. Comparison of the functional efficacies of clobazam, *N*-desmethylclobazam, and clonazepam at $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_A$Rs with those of diazepam and zolpidem. (A) Potentiation of the response elicited by GABA EC_{20} by 3 μM diazepam in Xenopus oocytes injected with cRNAs encoding for $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$Rs in a subunit ratio of 1:1:1 (means ± S.E.M.; N = 2–4) (B) Potentiation of the response elicited by EC_{20} GABA by 3 μM clobazam and 3 μM zolpidem in Xenopus oocytes injected with cRNAs encoding the $\alpha_2\beta_2\gamma_{2S}$ $GABA_A$R injected in a subunit ratio of 1:1:5 (means ± S.E.M.; N = 2).

doi:10.1371/journal.pone.0120239.g004

($P<0.05$). As for the functional properties of clonazepam at the four receptors, only a significant difference was identified for its I_{max} values at $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_1\beta_2\gamma_{2S}$ ($P<0.05$).

Investigations into the benzodiazepine-mediated modulation of the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_A$Rs

As will be outlined in the *Discussion* section, the modulatory efficacies exhibited by benzodiazepines at αβγ $GABA_A$Rs in recombinant expression systems in previous studies have varied considerably. To address this aspect, we compared the maximum responses mediated by saturating concentrations (3 μM) of clobazam, *N*-desmethylclobazam, and clonazepam at the $GABA_A$Rs with the maximal responses mediated by a saturating concentration of diazepam, a 1,4-benzodiazepine often referred to as a "full benzodiazepine agonist". The maximum responses evoked by clobazam and *N*-desmethylclobazam when co-applied with GABA EC_{20} at $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_2\beta_2\gamma_{2S}$ $GABA_A$Rs, respectively, were slightly but significantly smaller than those mediated by diazepam at the respective receptors ($p<0.1$; ordinary one-way ANOVA) (Table 2, Figs. 3B and 4A). In contrast, the maximum responses mediated by clobazam at $\alpha_{2,3,5}\beta_2\gamma_{2S}$, by *N*-desmethylclobazam at $\alpha_{1,3,5}\beta_2\gamma_{2S}$ and by clonazepam at all the four receptors

did not differ significantly from those exhibited by diazepam (Table 2, Figs. 3B and 4A). We also compared the maximum degree of potentiation mediated by clobazam at the $\alpha_2\beta_2\gamma_{2S}$ $GABA_AR$ with the maximal responses induced by a saturating concentration of zolpidem. Albeit not a benzodiazepine, zolpidem acts as a PAM through the high-affinity benzodiazepine site in $\alpha_{1,2,3,5}\beta\gamma$ $GABA_ARs$, and the compound has previously been reported to be a "full benzodiazepine-site agonist" at the $\alpha_2\beta_2\gamma_2$ subtype [52]. In this experiment cRNA for $\alpha_2\beta_2\gamma_{2S}$ was injected in a ratio of 1:1:5 (α_2:β_2:γ_{2S}) to facilitate the expression of a homogenous population of γ_{2S}-containing complexes. The modulatory efficacies displayed by clobazam and zolpidem at the $\alpha_2\beta_2\gamma_{2S}$ receptors in these experiments did not differ significantly (Fig. 4B). Moreover, the respective degrees of potentiation mediated by clobazam and zolpidem at the receptors expressed in these oocytes did not differ significantly from those at oocytes injected with an α_2:β_2:γ_{2S} cRNA ratio of 1:1:1 (Fig. 4B and data not shown).

In another series of experiments, we took advantage of the well-documented ability of zinc to discriminate between αβand αβγ $GABA_ARs$ [46, 53, 54] to investigate whether oocytes injected with $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ cRNAs in a 1:1:1 subunit ratio express homogeneous populations of ternary receptor complexes. As can be seen from Fig. 5, the GABA EC_{80}-evoked response through the $\alpha_1\beta_2$ $GABA_AR$ was almost completely eliminated by 100 μM Zn^{2+}, whereas the presence of this concentration of the metal ion had negligible effect on the currents elicited in $\alpha_1\beta_2\gamma_{2S}$-expressing oocytes. This strongly suggests that the functional properties of clobazam, *N*-desmethylclobazam and clonazepam at the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_ARs$ have been determined at homogenous populations of γ_{2S}-containing receptors.

Functional properties of clobazam, *N*-desmethylclobazam, and clonazepam at the human $\alpha_6\beta_2\delta$ $GABA_AR$ expressed in *Xenopus* oocytes

To investigate whether the functional properties of clobazam and its metabolite at αβδ $GABA_ARs$ potentially differ from those of clonazepam, the three compounds were tested at a representative of these receptors, the human $\alpha_6\beta_2\delta$ subtype (Table 2). Representative traces recorded for clobazam, *N*-desmethylclobazam and clonazepam when pre-incubated and co-applied with GABA EC_{20} at the receptor are presented in Fig. 6A, and concentration-response relationships determined for the compounds at the receptor are given in Fig. 6B.

All three modulators potentiated GABA EC_{20}-mediated signaling in $\alpha_6\beta_2\delta$-oocytes in a concentration-dependent and reversible manner, exhibiting EC_{50} values in the mid-to-high micromolar range at the receptor (Table 2). Thus, the modulatory potencies of clobazam, *N*-desmethylclobazam and clonazepam at this receptor were 200–400, 1100–3100 and 1400–3900 fold lower than those exhibited by the respective benzodiazepines at the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_ARs$, respectively (Table 2). Strikingly, however, the modulatory efficacies of the two 1,5-benzodiazepines at this receptor were very different from those at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors. Whereas the degree of potentiation of the GABA EC_{20}-evoked response through $\alpha_6\beta_2\delta$ mediated by clonazepam was comparable to those at the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_ARs$, clobazam and in particular *N*-desmethylclobazam were much more efficacious PAMs at the receptor (Table 2, Fig. 6). Albeit the concentration-response relationship for *N*-desmethylclobazam at the $\alpha_6\beta_2\delta$ receptor was not saturated within the concentration range tested, 1 mM of the drug was found to potentiate the GABA EC_{20}-evoked response through the receptor by ∼24-fold (Fig. 6A and 6B). Interestingly, a distinct inhibition phase in the concentration-response relationship was observed in some of these recordings for clobazam and clonazepam, with 1 mM of the modulator resulting in a lower degree of potentiation than 300 μM (Fig. 6A). Moreover, rebound currents were observed at these high modulator concentrations (Fig. 6A).

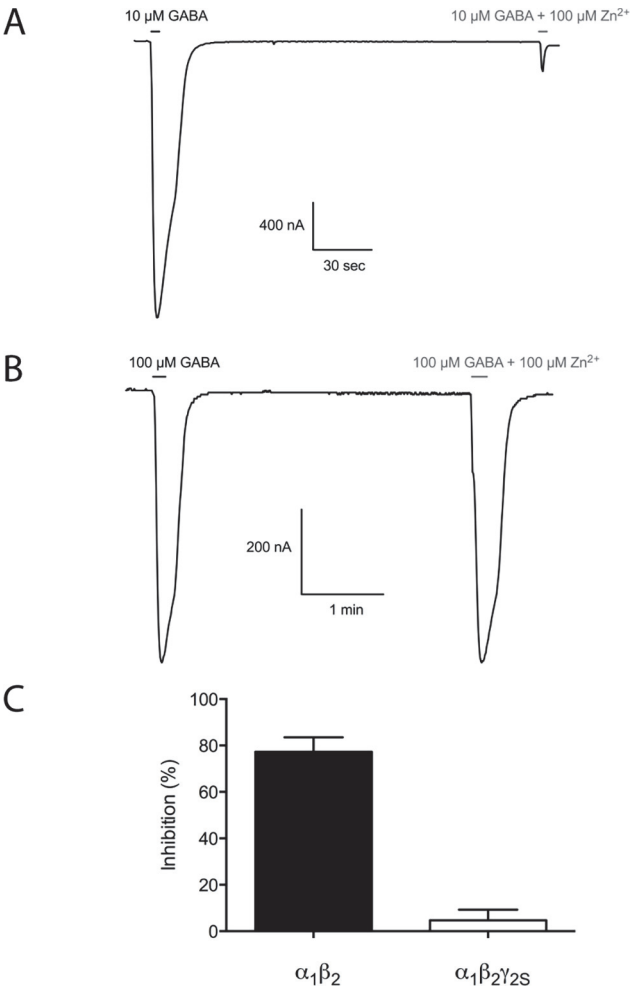

Fig 5. Zinc-mediated inhibition of human $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ $GABA_AR$ signalling in *Xenopus* oocytes. (A) Representative trace of the inhibition mediated 100 μM Zn^{2+} of the currents elicited by 10 μM GABA (EC_{80}) through the $\alpha_1\beta_2$ $GABA_AR$. (B) Representative trace of the inhibition mediated 100 μM Zn^{2+} of the currents induced by 100 μM GABA (EC_{80}) through the $\alpha_1\beta_2\gamma_{2S}$ $GABA_AR$. (C) The degree of inhibition mediated by 100 μM Zn^{2+} of GABA EC_{80}-evoked currents in oocytes expressing $\alpha_1\beta_2$ mean ± S.E.M.; 77 ± 6.3%; N = 7and $\alpha_1\beta_2\gamma_{2S}$ (mean ± S.E.M.; 4.7 ± 4.6%; N = 6) $GABA_ARs$.

doi:10.1371/journal.pone.0120239.g005

To elucidate the molecular basis for the high-efficacious modulation exerted by *N*-desmethylclobazam at the $\alpha_6\beta_2\delta$ $GABA_AR$, we characterized the functional characteristics of the compound at the binary $\alpha_6\beta_2$ receptor. *N*-desmethylclobazam also potentiated the GABA EC_{20}-mediated signaling through this receptor in a concentration-dependent manner, exhibiting modulatory potency and efficacy not significantly different from those displayed at $\alpha_6\beta_2\delta$ (Table 2, Fig. 6B). However, it should be stressed that the EC_{50} and I_{max} values given for *N*-desmethylclobazam at $\alpha_6\beta_2\delta$ and $\alpha_6\beta_2$ are estimates, since neither of the concentration-response curves for the modulator at these two receptors reached saturation.

Discussion

In view of the clinical use of clobazam for the treatment of various diseases over the last decades, surprising little is known about the *in vitro* pharmacology of the drug. In the present study, we have performed an elaborate functional characterization of clobazam, its major active

Figure 5

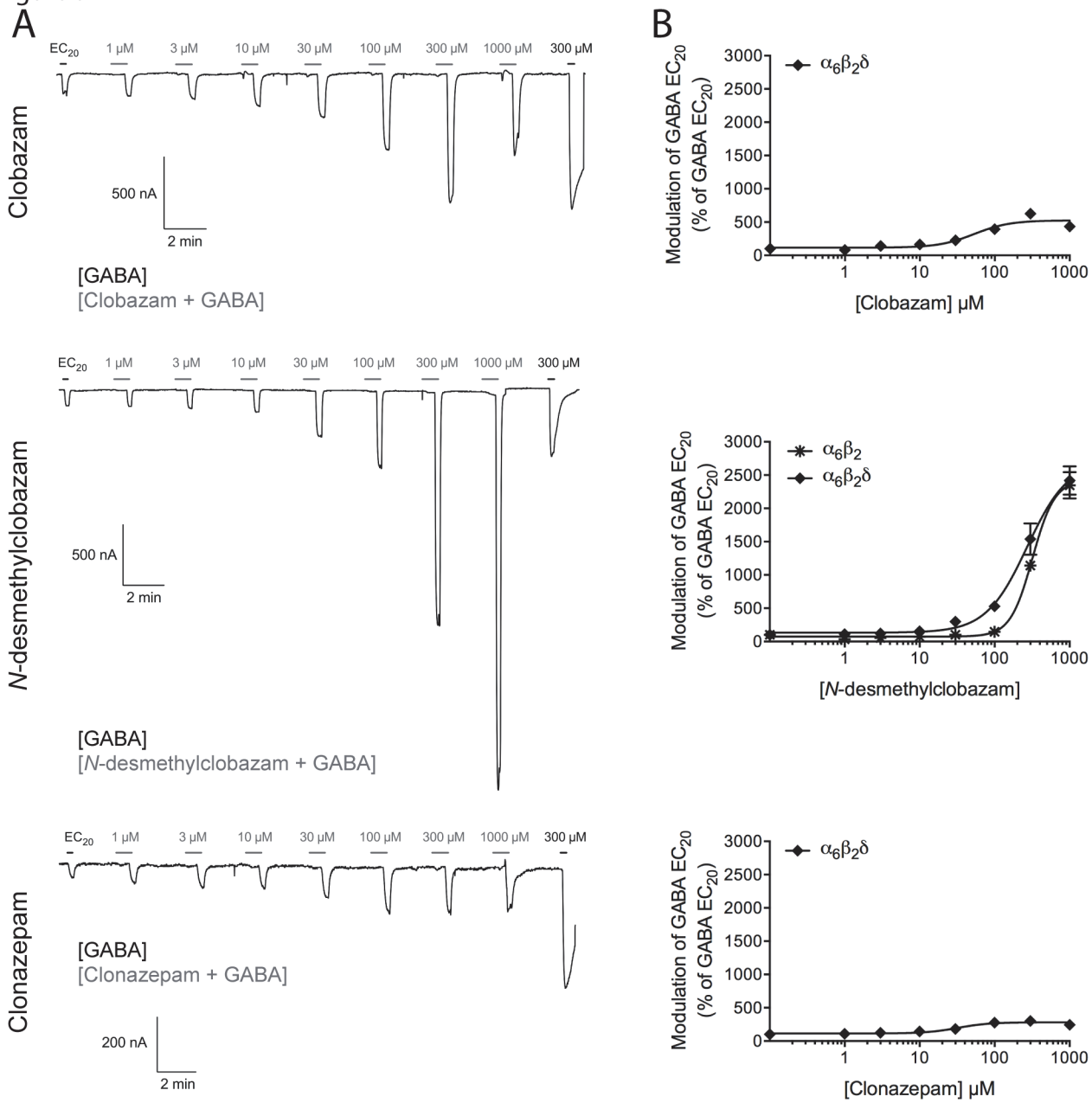

Fig 6. . Functional properties of clobazam, *N*-desmethylclobazam and clonazepam at the human $\alpha_6\beta_2\delta$ and $\alpha_6\beta_2$ $GABA_A$Rs expressed in *Xenopus* oocytes. (A) Representative traces for various concentrations of clobazam (top), *N*-desmethylclobazam (middle) and clonazepam (bottom) co-applied with GABA EC_{20} to oocytes expressing the $\alpha_6\beta_2\delta$ $GABA_A$R. The black bars represent applications of GABA EC_{20} and of 300 M GABA that elicits maximal current through the receptor. The grey bars represent applications of various concentrations of clobazam, *N*-desmethylclobazam or clonazepam (a 30 s pre-incubation with the compound followed by co-application of the compound and GABA EC_{20}). (B) Concentration-response relationships for clobazam (top), *N*-desmethylclobazam (middle) and clonazepam (bottom) at the $\alpha_6\beta_2\delta$ $GABA_A$R and for *N*-desmethylclobazam at the $\alpha_6\beta_2$ $GABA_A$R (middle) in the presence of GABA EC_{20} (means ± S.E.M.; N = 4–6).

doi:10.1371/journal.pone.0120239.g006

metabolite *N*-desmethylclobazam and the 1,4-benzodiazepine clonazepam at the human $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ $GABA_A$Rs expressed in *Xenopus* oocytes. A detailed functional characterization of clonazepam at recombinant $GABA_A$Rs has to our

knowledge not been published previously, and thus this study provides substantial insights into the molecular pharmacology of all three modulators.

Each of the three benzodiazepines exhibited similar EC_{50} values as a PAM at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ $GABA_A$Rs, and the maximum responses evoked by saturating concentrations of each of the modulators upon co-application with GABA EC_{20} at these four subtypes were also very similar (Table 2). Although statistical analysis identified differences between some of the potencies and efficacies displayed by the respective compounds at the four receptors (Table 3), these differences are not considered pertinent from a biological perspective. It is important to stress that the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors included in this study only constitute a selection of all αβγ receptors targeted by benzodiazepines in clinically relevant concentrations. However, the fact that benzodiazepines have been reported to be less potent and less efficacious modulators of $\alpha\beta\gamma_1$ and $\alpha\beta\gamma_3$ receptors than of the corresponding $\alpha\beta\gamma_2$ receptors [55–57] combined with the very restricted expression of γ_1 and γ_3 in the CNS [58, 59] strongly suggests that the contributions of these receptor assemblies to the overall clinical effects of benzodiazepines are negligible. More importantly, the identity of the β subunit in the $\alpha\beta\gamma_2$ $GABA_A$R complex is not believed to influence benzodiazepine pharmacology substantially [56, 57], just as there to our knowledge are no reports of benzodiazepines exhibiting significantly different pharmacological properties at γ_{2S}- and γ_{2L}-containing $GABA_A$Rs. Thus, although we can not exclude the possibility that the functional properties of clobazam, *N*-desmethylclobazam and/or clonazepam at $\alpha_{1,2,3,5}\beta\gamma_2$ complexes comprising $\beta_1\beta_3$ and/or γ_{2L} subunits could differ from those observed at the receptors in this study, we propose that the three benzodiazepines are likely to act as non-selective PAMs at all $\alpha_{1,2,3,5}\beta\gamma_2$ receptors.

In our recent study of the binding properties of the three benzodiazepines, clobazam and *N*-desmethylclobazam displayed slightly but significantly higher binding affinities at the $\alpha_2\beta_2\gamma_{2S}$ $GABA_A$R compared to the $\alpha_1\beta_2\gamma_{2S}$ subtype (2.5- and 4.3-fold, respectively), whereas clonazepam exhibited significantly higher binding affinities to $\alpha_{1,2,5}\beta_2\gamma_{2S}$ subtypes than to the $\alpha_3\beta_2\gamma_{2S}$ receptor (2.8- to 3.4-fold) [41]. The fact that these binding subtype-preferences are not mirrored in the functional profiles of the modulators is not particular surprising, given the different methodologies used to assess binding affinities and functional potencies of modulators. In support of this, another interesting observation that can be extracted from the two studies is that the 200–1000 fold higher binding affinities displayed by clonazepam compared to clobazam and *N*-desmethylclobazam at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors in the [^{3}H]flumazenil binding assay translate into considerably less pronounced differences (5–19 fold) between the EC_{50} values of the 1,4-benzodiazepine and the two 1,5-benzodiazepines at the receptors in the oocyte recordings (Table 2) [41]. These observations may reflect differences in the degrees of receptor desensitization in the two assays.

A comprehensive literature search has only identified two previous studies of the functional properties of clobazam and *N*-desmethylclobazam at recombinant $GABA_A$Rs [55, 60]. Clobazam has been reported to exert negligible potentiation of GABA EC_5-EC_{10}-evoked responses through $\alpha_1\beta_2\gamma_1$ receptors in *Xenopus* oocytes, suggesting that the presence of this γ subunit in the $GABA_A$R complex alters the functionality of the 1,5-benzodiazepine substantially [55]. Of particular interest for this study, Fisher has performed a direct comparison of the modulation mediated by clonazepam, clobazam, and *N*-desmethylclobazam on the current elicited by 3 μM GABA (EC_{10}–EC_{20}) through the rat $\alpha_3\beta_3\gamma_{2L}$ $GABA_A$R expressed in HEK-293T cells by patch clamp electrophysiology [60]. The EC_{50} values determined for clonazepam, clobazam, and *N*-desmethylclobazam at the rat $\alpha_3\beta_3\gamma_{2L}$ receptor in the Fisher study were 90 nM, 490 nM and 550 nM, respectively, which are in good agreement with the potencies exhibited by three benzodiazepines at the human $\alpha_3\beta_2\gamma_{2S}$ receptor in this study (Table 2) [60]. However, Fisher found clobazam and diazepam to be substantially more efficacious potentiators of rat $\alpha_3\beta_3\gamma_{2L}$

currents (I_{max} values of 487% and 508% of the responses evoked by GABA EC_{10}–EC_{20}, respectively) than *N*-desmethylclobazam and clonazepam (I_{max} values of 270% and 263%, respectively) [60]. Whereas the modulatory efficacies of *N*-desmethylclobazam and clonazepam at $\alpha_3\beta_3\gamma_{2L}$ are in good agreement with those at the $\alpha_3\beta_2\gamma_{2S}$ receptor in the present study, the higher efficacies exhibited by clobazam and diazepam in the Fisher study clearly contrast the comparable I_{max} values determined for the four PAMs at the $\alpha_3\beta_2\gamma_{2S}$ receptor in this study (Table 2, Figs. 3 and 4). Several factors might explain this apparent discrepancy, including the different receptors studied (rat $\alpha_3\beta_3\gamma_{2L}$ vs. human $\alpha_3\beta_2\gamma_{2S}$), the different expression systems (HEK-293T cells vs. *Xenopus* oocytes), and the different recording techniques (patch clamp vs. TEVC electrophysiology). We propose that the precise determination of the GABA EC_{20} used for the characterization of the benzodiazepines in the present study can have facilitated a more precise determination of the degree of maximum potentiation of the GABA-evoked responses than the GABA EC_{10}–EC_{20} concentrations employed in the Fisher study. On the other hand, the faster application rate of the compounds in patch clamp recordings using HEK-293T cells may have resulted in less concomitant desensitization of the receptors during application than in the *Xenopus* oocyte recordings system, which is characterized by slower exchange rates. Thus, we cannot exclude the possibility that concurrent desensitization of the receptors upon co-application of GABA EC_{20} with high benzodiazepine concentrations could constitute a ceiling effect with respect to the degree of maximum response elicited by the benzodiazepine-bound receptor, and that this effect potentially can have masked putative differential efficacies of the benzodiazepines at the receptors. However, as will be outlined below, the divergent efficacies reported for benzodiazepines at $GABA_A$Rs expressed in oocytes in the literature strongly suggest that establishing a "true efficacy" for any given benzodiazepine is not trivial.

In the absence of other previous studies of the functional characteristics of clobazam, *N*-desmethylclobazam and/or clonazepam at $GABA_A$Rs expressed in *Xenopus* oocytes, we investigated whether the determined modulatory efficacies for these compounds could be considered reliable by comparing them to the efficacies mediated by two reference benzodiazepine-site modulators. A saturating concentration of the prototypic benzodiazepine diazepam was observed to induce comparable or slightly higher responses than those mediated by saturating concentrations of clobazam, *N*-desmethylclobazam and clonazepam at the four $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ subtypes (Figs. 3B and 4A). Furthermore, the maximum responses induced by zolpidem at $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_2\beta_2\gamma_{2S}$ receptors were comparable to those mediated by clobazam (Fig. 4B and data not shown). Unfortunately, the efficacies determined for diazepam and zolpidem at $GABA_A$Rs expressed in oocytes in previous studies have varied considerably. Although often referred to as a "full benzodiazepine agonist," diazepam has exhibited very different efficacies as a PAM of GABA EC_{10}- to EC_{20}-evoked currents through $\alpha_1\beta_2\gamma_2$ receptors in previous studies, including degrees of potentiation in the same 2.3- to 3.4-fold range as exhibited by the modulator at the $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors in this study [61–63]. Likewise, whereas zolpidem has been reported to potentiate the GABA EC_{15}- to EC_{20}-evoked responses through $\alpha_1\beta_2\gamma_2$ and $\alpha_2\beta_2\gamma_2$ receptors 4- to 6-fold in some studies [52, 64, 65], its maximum modulation of GABA-evoked currents through the receptors in other studies have been very similar to the 2.7-fold potentiation observed at $\alpha_2\beta_2\gamma_{2S}$ in this study [61, 62, 66]. In conclusion, the results in this study indicate that clobazam and *N*-desmethylclobazam are equally efficacious or almost as efficacious as the 1,4-benzodiazepines clonazepam and diazepam at the human $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors. However, considering the variation in the efficacies reported for standard benzodiazepines in the literature, the absolute degrees of potentiation exerted by the two 1,5-benzodiazepines at the receptors in other recording set-ups could potentially differ from those observed in this study.

The $\alpha_6\beta_2\delta$ receptor was included in this study as a representative of the δ-containing $GABA_A$Rs, and clobazam, *N*-desmethylclobazam and clonazepam all displayed mid-to-high micromolar potencies as PAMs of this receptor (Fig. 6, Table 2). Apart from the obvious absence of an $\alpha^{(+)}/\gamma^{(-)}$ subunit interface in the $\alpha_6\beta_2\delta$ complex, the distinct functional characteristics exhibited by the three benzodiazepines at this receptor support the notion of them acting through another allosteric site than the classical high-affinity benzodiazepine binding site, and the comparable potencies displayed by *N*-desmethylclobazam as a PAM at the $\alpha_6\beta_2$ and $\alpha_6\beta_2\delta$ receptors strongly suggest that this site is comprised within the αβ regions of the αβδ complex (Fig. 6B, Table 2). Some of the characteristics displayed by the benzodiazepines at the $\alpha_6\beta_2\delta$ $GABA_A$R could be argued to be indicative of a binding site in the transmembrane domain of the receptor. The tendencies towards bell-shaped concentration-response curves observed for clobazam and clonazepam as well as the rebound currents observed at high concentrations of the modulators are certainly reminiscent of the characteristics previously reported for PAMs/ago-PAMs acting through transmembrane domains of $GABA_A$Rs [55, 67–72]. Moreover, whereas PAMs targeting extracellular non-cannonical subunit interfaces in $GABA_A$Rs and other Cys-loop receptors historically predominantly have been found to increase agonist potency without affecting the maximal agonist response through the receptors significantly [20, 73, 74], several PAMs acting through the transmembrane domains of the receptors have been shown capable of increasing agonist-evoked maximal peak currents, analogously to the high-efficacious potentiation of $\alpha_6\beta_2\delta$ signaling mediated by *N*-desmethylclobazam [67, 74–76]. However, this phenotypic difference between PAMs acting through extracellular and transmembrane regions of Cys-loop receptors does not appear to be black-and-white, as illustrated by the pronounced enhancement of GABA efficacy at the $\alpha_1\beta_3\delta$ $GABA_A$R mediated by LAU 177 through a site in the extracellular $\alpha_1^{(+)}/\beta_3^{(-)}$ interface [22] and by the augmentation of agonist efficacy exerted by the anthelmintic drug morantel through the extracellular $\beta_2^{(+)}/\alpha_3^{(-)}$ interface of the $\alpha_3\beta_2$ nicotinic acetylcholine receptor [77]. Thus, not having explored the modes of action of clobazam, *N*-desmethylclobazam and clonazepam at the $\alpha_6\beta_2\delta$ $GABA_A$R in detail, we will refrain from speculations about whether the modulators target a low-affinity binding site located in the transmembrane domain, in the extracellular $\alpha^{(+)}/\beta^{(-)}$ subunit interface, or in another region of the receptor complex.

The nature and efficacies of the modulation exerted by clobazam and *N*-desmethylclobazam at other αβ and αβδ receptors could potentially differ from those observed for the compounds at $\alpha_6\beta_2$ and $\alpha_6\beta_2\delta$. However, the two 1,5-benzodiazepines are likely to be weak modulators at all αβand αβδ $GABA_A$Rs, thus mediating their effects at these receptors at substantially higher concentrations than those required to modulate αβγ receptors. Although the μM activity component of benzodiazepines previously has been proposed to contribute to the CNS depression observed at high *in vivo* concentrations [18], we find it highly improbable that this component contributes significantly to the clinical efficacy of clobazam/*N*-desmethylclobazam. On the other hand, the highly efficacious potentiation of $\alpha_6\beta_2\delta$ $GABA_A$R signaling exerted by *N*-desmethylclobazam is quite interesting from a molecular perspective, and we propose that the 1,5-benzodiazepine could constitute an interesting lead scaffold for the development of novel allosteric modulators targeting this elusive low-affinity benzodiazepine binding site in the $GABA_A$Rs.

Conclusion

The present study represents the first elaborate *in vitro* functional characterization of clobazam, *N*-desmethylclobazam and clonazepam at recombinant human $GABA_A$Rs. While both 1,5-benzodiazepines are potent PAMs of human $\alpha_{1,2,3,5}\beta_2\gamma_{2S}$ receptors, they appear to be

non-selective both in terms of potency and efficacy, a characteristic they share with many classical 1,4-benzodiazepines including clonazepam (Table 2) [15, 16]. Obviously we can not completely exclude the possibility that the functionalities of clobazam and/or *N*-desmethylclobazam at one or a few of the $GABA_AR$ subtypes not included in this study could differ substantially from those of clonazepam and other 1,4-benzodiazepines, and that this difference could contribute to the distinct properties observed for clobazam compared with the classical 1,4-benzodiazepines in the clinic. However, judging from the results in this study we propose that these clinical differences are more likely to be rooted in other factors than the *in vitro* pharmacological properties of the modulators, such as their respective pharmacokinetic characteristics.

Acknowledgments

Dr. Paul J. Whiting and Merck, Sharp & Dohme are thanked for their generous gifts of the $GABA_AR$ subunit cDNAs. Anders A. Jensen thanks the Novo Nordisk Foundation for financial support. Editorial support during manuscript preparation was provided by Apurva Davé, PhD, of Prescott Medical Communications Group (Chicago, IL), and Michael A. Nissen, ELS, Lundbeck LLC (Deerfield, IL).

Author Contributions

Conceived and designed the experiments: HH BE HSJ AAJ. Performed the experiments: HH. Analyzed the data: HH HSJ. Wrote the paper: HH AAJ.

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