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RESEARCH PAPER

The orthosteric $GABA_A$ receptor ligand Thio-4-PIOL displays distinctly different functional properties at synaptic and extrasynaptic receptors

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BACKGROUND AND PURPOSE
Explorations into the heterogeneous population of native GABA type A receptors ($GABA_A$Rs) and the physiological functions governed by the multiple $GABA_A$R subtypes have for decades been hampered by the lack of subtype-selective ligands.

EXPERIMENTAL APPROACH
The functional properties of the orthosteric $GABA_A$ receptor ligand 5-(4-piperidyl)-3-isothiazolol (Thio-4-PIOL) have been investigated *in vitro*, *ex vivo* and *in vivo*.

KEY RESULTS
Thio-4-PIOL displayed substantial partial agonist activity at the human extrasynaptic $GABA_A$R subtypes expressed in *Xenopus* oocytes, eliciting maximal responses of up to ~30% of that of GABA at $\alpha_5\beta_3\gamma_{2S}$, $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ and somewhat lower efficacies at the corresponding $\alpha_5\beta_2\gamma_{2S}$, $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$ subtypes (maximal responses of 4–12%). In contrast, it was an extremely low efficacious agonist at the $\alpha_1\beta_3\gamma_{2S}$, $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_2\beta_3\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_3\beta_3\gamma_{2S}$ $GABA_A$Rs (maximal responses of 0–4%). In concordance with its agonism at extrasynaptic $GABA_A$Rs and its *de facto* antagonism at the synaptic receptors, Thio-4-PIOL elicited robust tonic currents in electrophysiological recordings on slices from rat CA1 hippocampus and ventrobasal thalamus and antagonized phasic currents in hippocampal neurons. Finally, the observed effects of Thio-4-PIOL in rat tests of anxiety, locomotion, nociception and spatial memory were overall in good agreement with its *in vitro* and *ex vivo* properties.

CONCLUSION AND IMPLICATIONS
The diverse signalling characteristics of Thio-4-PIOL at $GABA_A$Rs represent one of the few examples of a functionally subtype-selective orthosteric $GABA_A$R ligand reported to date. We propose that Thio-4-PIOL could be a useful pharmacological tool in future studies exploring the physiological roles of native synaptic and extrasynaptic $GABA_A$Rs.

Abbreviations
$GABA_A$R, $GABA_A$ receptor; GHB, γ-hydroxybutyric acid; mIPSC, miniature IPSC; PAM, positive allosteric modulator; RS, series resistance; TEVC, two-electrode voltage clamp; Thio-4-PIOL, 5-(4-piperidyl)-3-isothiazolol; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

Introduction

GABA is the predominant inhibitory neurotransmitter in the CNS. Decades of clinical use of benzodiazepines, barbiturates, neuroactive steroids and general anesthetics have established the $GABA_A$ receptors ($GABA_ARs$) as drug targets in anxiety, sleeping disorders, pain and epilepsy, and the receptors are still pursued as putative targets in numerous neurological and psychiatric disorders (Orser *et al.*, 2002; Nemeroff, 2003; Taylor *et al.*, 2003; Ebert *et al.*, 2006; Enna and McCarson, 2006; Korpi and Sinkkonen, 2006; Da Settimo *et al.*, 2007).

The $GABA_ARs$ are membrane-bound pentameric ligand-gated ion channels belonging to the Cys-loop receptor superfamily, and they facilitate the flux of anions across the cell membrane leading to hyperpolarization and inhibition of the cell (Miller and Smart, 2010). The multifaceted contributions of $GABA_ARs$ to inhibitory neurotransmission arise from the existence of 19 subunits (α_1-α_6, β_1-β_3, γ_1-γ_3, δ, ε, π, θ and ρ_1-ρ_3), as the numerous receptor subtypes formed from these display distinct regional and cell type-specific expression patterns (McKernan and Whiting, 1996; Pirker *et al.*, 2000; Whiting, 2003; Olsen and Sieghart, 2009). The synaptic $GABA_ARs$ mediating the phasic GABA signalling are predominantly composed of α_1, α_2 and/or α_3 in combination with β_2/β_3 and γ_2 subunits, $\alpha_1\beta_2\gamma_2$ being the most predominant subtype (McKernan and Whiting, 1996; Whiting, 2003; Olsen and Sieghart, 2009). The extrasynaptic receptors mediating tonic inhibition are predominantly $\alpha_4\beta_{2/3}\delta$ or $\alpha_6\beta_{2/3}\delta$ complexes, although other extrasynaptic subtypes, such as $\alpha_5\beta_{2/3}\gamma_2$ $GABA_ARs$ in hippocampal pyramidal cells, exist (McKernan and Whiting, 1996; Glykys and Mody, 2007; Glykys *et al.*, 2008; Belelli *et al.*, 2009; Olsen and Sieghart, 2009; Marowsky *et al.*, 2012). The therapeutic prospects in extrasynaptic $GABA_ARs$ are underlined by the *in vivo* effects of the $\alpha_4\beta\delta/\alpha_6\beta\delta$ agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP or gaboxadol) (Krogsgaard-Larsen *et al.*, 2004; Ebert *et al.*, 2006) and positive allosteric modulators (PAMs) of $\alpha_4\beta\delta/\alpha_6\beta\delta$ receptors (Wafford *et al.*, 2009; Hoestgaard-Jensen *et al.*, 2010), and by the increasing interest in hippocampal $\alpha_5\beta_{2/3}\gamma_2$ receptors as putative targets in cognitive disorders (Maubach, 2003; Glykys and Mody, 2007; Möhler, 2009; Atack, 2011b).

In the present study, the orthosteric $GABA_AR$ ligand 5-(4-piperidyl)-3-isothiazolol (Thio-4-PIOL) has been found to exhibit distinctly different functional properties at some extrasynaptic $GABA_ARs$ compared with the synaptic receptors, and the effects of Thio-4-PIOL on phasic and tonic currents in hippocampal CA1 pyramidal and thalamic principal neurons and in animal tests of anxiety, locomotion, nociception and spatial memory have been delineated.

Methods

Materials

Culture media, serum, antibiotics and buffers for cell culture were obtained from Invitrogen (Paisley, UK). Thio-4-PIOL and THIP were synthesized in-house, and SR95531 (gabazine) and DS2 were purchased from Tocris Bioscience (Bristol, UK). Human α_1, α_5, β_2 and γ_{2S} $GABA_AR$ subunit cDNAs in pcDNA3.1 were used for the experiments using HEK293 cells, whereas cDNAs encoding α_1, α_3, β_2, β_3, γ_{2S} and ™ in pGemHE, α_4 in pcDNAI, and α_2, α_5 and α_6 in pcDNA3.1 were used for the oocyte experiments. Drug and molecular target nomenclature conforms to the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Xenopus laevis *oocytes and two-electrode voltage clamp (TEVC)*

All cDNAs were transcribed and capped *in vitro* (mMessage mMachine T7 kit, Ambion, Foster City, CA, USA), and cRNAs were purified using RNeasy Mini columns (Qiagen, Hilden, Germany). Oocyte isolation, injection and TEVC were performed as described previously (Storustovu and Ebert, 2006). 32 nL cRNA encoding $\alpha_{1,2,3,5}\beta_{2,3}\gamma_{2S}$ (in a subunit ratio of 0.2; 0.2; 0.2 μg/μL) or 46 nL cRNA encoding $\alpha_{4,6}\beta_3\delta$ (in a subunit ratio of 1; 0.1; 1 μg/μL) were injected into the oocytes, which were then incubated for at least 72 h in modified Barth's saline. Oocytes were clamped at –40 to –70 mV by a GeneClamp 500B amplifier (Axon Instruments, Union City, CA, USA) and both voltage and current electrodes were filled with 3 M KCl. Using TEVC, agonists were applied until the peak of the response was observed, usually after 30 s or less. A 4 min washout period between agonist applications was allowed to minimize desensitization of $\alpha_{1,2,3}\beta_{2,3}\gamma_{2S}$ $GABA_ARs$, whereas a 7 min washout period was allowed at $\alpha_5\beta_{2,3}\gamma_{2S}$, $\alpha_4\beta_{2,3}\delta$ and $\alpha_6\beta_{2,3}\delta$ $GABA_ARs$. The presence of δ in cell surface-expressed $\alpha_4\beta_{2,3}\delta$ and $\alpha_6\beta_{2,3}\delta$ complexes was verified by using Zn^{2+} and DS2 (Storustovu and Ebert, 2006; Wafford *et al.*, 2009), and the incorporation of γ_{2S} into cell surface-expressed $\alpha_{1,2,3,5}\beta_{2,3}\gamma_{2S}$ $GABA_ARs$ was confirmed using diazepam. Experiments were performed on at least four oocytes from at least two different batches of oocytes for each subtype. Data were normalized to the maximum current elicited by GABA at the individual oocyte. Concentration-response curves were fitted by use of the non-linear regression, GraFit 5.0.13 (Erithacus Software, Horley, Surrey, UK). The parameters obtained were compared using Student's *t*-test (two-tailed, two-sample equal variance) and considered significant if $P < 0.05$.

Patch-clamp recordings

Transient transfections and whole-cell patch-clamp recordings were performed essentially as previously described (Madsen *et al.*, 2007). HEK293 cells were co-transfected with α_1-pcDNA3.1 or α_5-pcDNA3.1, β_2-pcDNA3.1 and γ_{2S}-pcDNA3.1 (1:1:5 ratio) and GFP-Targefect-293 (Targeting Systems, CA, USA) and recordings were performed 40–100 h after transfection. The presence of γ_{2S} in cell surface-expressed receptors was verified by the ability of 1 μM diazepam to potentiate the GABA response.

Slice electrophysiology

Protocols were approved by the Danish Authorities for Animal Experimentation. Slice electrophysiology was performed as previously described using brains from adult (42–60 days) male Lister hooded rats (Harlan, UK) (Hoestgaard-Jensen *et al.*, 2010). Briefly, a 5 min baseline recording was followed by Thio-4-PIOL application and recording for an additional 5 min, after which SR95531 was added to the bath (final concentration ~100 μM). Whole-cell capacitance and series resistance (RS) were noted every 3–4 min throughout the

recording, and RS were compensated by 70%. Recordings were discarded if the RS or cell capacitance deviated more than 20% from initial values. For assessment of tonic currents, a systematic sampling regimen was used plotting the mean holding current of a 1 ms period every 100 ms against time. The tonic current in hippocampus CA1 and thalamic neurons was measured as the difference in holding current for two 5 s windows at the peak of tonic current and after addition of SR95531 until full effect (10–30 s). In thalamus, the tonic current in the control situation was subtracted. A two-tailed, two-sample equal variance *t*-test was used for comparing tonic currents. Slices were post-recording processed for visualization of the recorded neuron using a Alexa-Flour® 488 streptavidin conjugate (Hoestgaard-Jensen *et al.*, 2010). Detection and analysis of IPSCs was carried out in Minianalysis (6.03, Synaptosoft, Decatur, GA, USA) (Hoestgaard-Jensen *et al.*, 2010). The miniature IPSC (mIPSC) frequency was assessed in a 2 min window at the end of baseline and drug perfusion period. The event amplitude, 10–90% rise-time and mono-exponential fit for decay-time constants were assessed for the averaged non-contaminated event. Statistical significance for effect was $P < 0.05$.

Animal studies

Animals. Eighty male Sprague Dawley rats (Harlan, UK) were used in these studies (40 were used in the Morris water maze experiment while a separate cohort of 40 rats was used for all other behavioural experiments). Upon arrival, rats weighed 250–300g. For the Morris water maze experiment animals were group housed 3–5 per cage post-surgery while for all other behavioural experiments animals were single-housed post-surgery. The holding room was temperature (22 ± 1°C) and humidity controlled (55 ± 5%) and under a 12-hour light/dark cycle (lights on 07:00) with food and water available *ad libitum*. All surgeries and experiments were approved by the Department of Health in Ireland in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation & Ethics Committee of University College Cork. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Stereotactic surgery. All surgical procedures were carried out under semi-sterile conditions. Anaesthesia was induced with a ketamine (90 $mg \cdot kg^{-1}$) and xyalizine (10 $mg \cdot kg^{-1}$) mixture for the experiments involving the Morris water maze while anaesthesia was induced and maintained with isoflurane for all other behavioural experiments. For cannula implantation, rats were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the incisor bars set at –3.3 mm. Two stainless steel screws were inserted into the skull. For i.c.v. cannula implantation, a steel guide cannula (Plastics One, Roanoke, VA, USA) was implanted 1 mm above the right lateral ventricle [0.8 mm anterior-posterior (AP), 1.3 mm medial lateral (ML); Paxinos and Watson, 1998] and for bilateral hippocampal implantation, a similar steel guide cannula was implanted 3 mm above both hippocampi (–4.0 mm AP, ±3.6 mm ML). Dental cement was applied for fixation and stabilization of the implants. Following surgery animals received 3 $mg \cdot kg^{-1}$ carprofen (s.c.) and were monitored for at least 10 days of recovery before experimental testing started.

Drugs. Thio-4-PIOL was dissolved in sterile saline (0.9%) to final concentrations of 1, 2 and 4 $nmol \cdot \mu L^{-1}$. Due to the unknown ability of Thio-4-PIOL to cross the blood–brain barrier, all solutions were delivered direct to the brain using cannula implantation. For i.c.v. administration, solutions were delivered in a 5 µL volume resulting in 5, 10 and 20 nmol of Thio-4-PIOL. For studies assessing the effects of Thio-4-PIOL on spatial memory, solutions were delivered bilaterally to the hippocampus. A volume of 1 µL was delivered to either hippocampus leading to concentrations of 1, 2 and 4 nmol (a smaller volume of drug was used due to the smaller diffusion area present in the hippocampus). All substances (Thio-4-PIOL/saline) were injected 10 min prior to testing. Drugs were administered using an infusion system (Plastics One). Correct surgical placement was confirmed at the end of the study by injection of blue dye.

Behavioural testing. Behavioural testing started after 10 days of recovery from surgery. All tests were carried out in all animals, with at least 10 days of experimental break between individual tests. Animals displaying post-surgical problems or severe side effects after drug administration, for example, seizures or respiratory depression, were excluded from the experiment.

Open field. The open field test was carried out as described previously (McKernan *et al.*, 2010; O'Malley *et al.*, 2010). Briefly, animals were placed into the equally lit (1000 lux) testing arena (90 cm diameter) for 40 min and locomotor activity (distance travelled) was analysed using EthoVision software (Noldus, Wageningen, The Netherlands).

Elevated plus maze (EPM). The EPM experiments were performed as described previously (Jacobson and Cryan, 2008). The EPM apparatus consisted of two open arms (51 × 10 cm) and two enclosed arms (51 × 10 × 40.5 cm) that radiated from a central platform (10 cm × 10 cm) raised 74.5 cm from the ground. Rats were placed into the neutral zone facing towards the closed arm and were allowed to freely explore the maze for five minutes. An entry was scored when the animal was inside an arm with all four paws.

Hot plate test. For assessment of pain threshold and sedative state, animals were exposed to a 55°C hot steel plate and latencies to retract of lick paws were measured (Allen and Yaksh, 2004; Gosselin *et al.*, 2010). Animals received one baseline session, were injected 35 min later and retested 10 min post-injection. The difference from baseline in individual latencies was recorded.

Morris water maze. The protocol was based on previously published studies (Zellner *et al.*, 1991; Vorhees *et al.*, 2000; Collinson *et al.*, 2006; Vorhees and Williams, 2006) with minor modifications. The apparatus consisted of a circular tank of 180 cm diameter filled with water to a depth of 31 cm. An opaque platform with a diameter of 10 cm was placed in the middle of one of the quadrants so that it was slightly submerged below the water level and not visible from the surface. Distal cues were arranged around the maze to provide landmarks by which the animals could to use to navigate to the platform. Animals received 4 days of training

that consisted of four trials per day. At the beginning of each trial, the animal was placed in one of the four distal start positions facing the wall of the tank and allowed to explore the maze for 180 s. A different starting position was used for each of the four trials on a given day arranged in a semi-random pattern. If the platform was not located within this time, the animal was gently assisted to the platform by the experimenter. On the fifth day of the procedure, the platform was removed and the animals were placed in a novel starting position and allowed to freely explore the pool for 60 s. The amount of time spent in the quadrant originally hosting the platform was recorded via EthoVision.

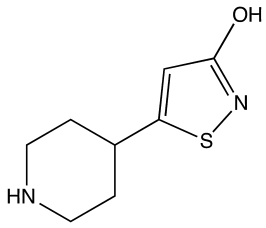
Statistical analysis. Statistical analysis was performed using a one-way ANOVA followed by *post hoc* comparison (least significant difference). For analysis of locomotor activity over 40 min in the open field and for analysis of performance during training sessions in the Morris water maze, a one-way repeated measures ANOVA was carried out in addition. All tests were carried out at a significance level of $P < 0.05$. All analysis was carried out using SPSS 15.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

Functional characterization of Thio-4-PIOL at recombinant $GABA_A$Rs in Xenopus *oocytes*

In a search for $GABA_A$R ligands with interesting functional properties, a number of previously published bioisosteric analogues of GABA were characterized functionally at six human $GABA_A$R subtypes expressed in tsA201 cells in the fluorescence-based FLIPR® Membrane Potential Blue assay (Jensen *et al.*, 2010). In this screening, the compound Thio-4-PIOL (Figure 1) was found to possess an interesting subtype-selectivity profile (data not shown).

To study the pharmacological properties of Thio-4-PIOL in a more sophisticated functional assay, the compound was characterized functionally at 12 human $GABA_A$R subtypes expressed in *Xenopus* oocytes by use of the TEVC technique. Here, Thio-4-PIOL was found to be a partial agonist at the extrasynaptic $\alpha_5\beta_3\gamma_{2S}$, $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ $GABA_A$R subtypes displaying EC_{50} values in the high nanomolar-low micromolar ranges and maximal responses of about 30% of that of GABA at the respective receptors (Figure 2 and Table 1). Thio-4-PIOL was also found to be a partial agonist at the β_2-containing extrasynaptic $GABA_A$R subtypes $\alpha_5\beta_2\gamma_{2S}$, $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$. However, the maximal responses exhibited by the compound at these receptors were somewhat lower than those at the corresponding β_3-containing subtypes, ranging from 4 to 12% of that of GABA at the respective receptors (Figure 2 and Table 1). The observed difference in efficacies obtained for Thio-4-PIOL at $\alpha_4\beta_2\delta$ and $\alpha_4\beta_3\delta$ did not seem to arise from a general trend of agonists exhibiting higher efficacies β_3-containing receptors than at β_2-containing receptors in the *Xenopus* oocyte expression system, since the reference $\alpha_4\beta\delta$ super agonist THIP displayed similar maximal responses (in % of the respective R_{max} values of GABA) at the two receptors (Supporting Information Fig. S1). In fact, the functional properties exhibited by THIP at the two receptors were in excellent agreement with those reported for the compound at the $\alpha_4\beta_3\delta$ $GABA_A$R in a previous study (Storustovu and Ebert, 2006).



Thio-4-PIOL

Figure 1
Chemical structure of Thio-4-PIOL.

It should be mentioned that while the maximal responses exhibited by Thio-4-PIOL in $\alpha_4\beta_3\delta$-, $\alpha_4\beta_2\delta$-, $\alpha_6\beta_2\delta$- and $\alpha_5\beta_2\gamma_{2S}$-expressing oocytes were consistent in size in all recordings, the same cannot be claimed to be the case for $\alpha_5\beta_3\gamma_{2S}$ and $\alpha_6\beta_3\delta$ (Table 1). The efficacies exhibited by Thio-4-PIOL at receptors assembled in oocytes injected with cRNAs encoding for these two subtypes differed substantially between oocyte batches, whereas the efficacies displayed by the compound at receptors in different oocytes from the same batch were very similar. Thus, two distinct receptor populations with high and low Thio-4-PIOL efficacy seemed to be formed in oocytes expressing $\alpha_5\beta_3\gamma_{2S}$ ($34 \pm 9\%$ and $3.9 \pm 0.8\%$) and $\alpha_6\beta_3\delta$ ($32 \pm 3\%$ and $9.2 \pm 1.6\%$). The presence of the γ_{2S} subunit in both 'high efficacy' and 'low efficacy' $\alpha_5\beta_3\gamma_{2S}$ receptors was verified using diazepam, and analogously, the potentiation of GABA-evoked currents in oocytes expressing either of the two $\alpha_6\beta_3\delta$ populations by DS2 confirmed the incorporation of the δ subunit at least some of these receptors (data not shown). Elaborate investigations using different cRNA preparations, different subunit injection ratios, different Thio-4-PIOL batches and oocytes from other sources did not elucidate the reasons for these different efficacies of Thio-4-PIOL at $\alpha_5\beta_3\gamma_{2S}$ and $\alpha_6\beta_3\delta$ in different oocyte batches further. Thus, except for the generally fickle nature of extrasynaptic $GABA_A$Rs in the *Xenopus* oocyte expression system, we cannot provide an explanation for this observation.

In contrast to its pronounced agonism at extrasynaptic $GABA_A$Rs, in particular the β_3-containing subtypes, Thio-4-PIOL displayed negligible agonist activity at the $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_2\beta_3\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_3\beta_3\gamma_{2S}$ subtypes at concentrations up to 1 mM, eliciting maximal responses of 0–4% of those of GABA at the respective receptors (Figure 3 and Table 1). Thus, Thio-4-PIOL must be considered a *de facto* antagonist at these receptors.

In a recent study, the GABA metabolite γ-hydroxybutyric acid (GHB) has been shown to be an agonist at the extrasynaptic $\alpha_4\beta\delta$ $GABA_A$R, and GHB has been proposed to act through a binding site distinct from but overlapping with the orthosteric site in the receptor (Absalom *et al.*, 2012). A substantial amount of evidence suggests that Thio-4-PIOL targets the orthosteric site in the $GABA_A$R complex and not this GHB site. Being a small unsubstituted GABA analogue, Thio-4-PIOL comprises all pharmacophore elements required for

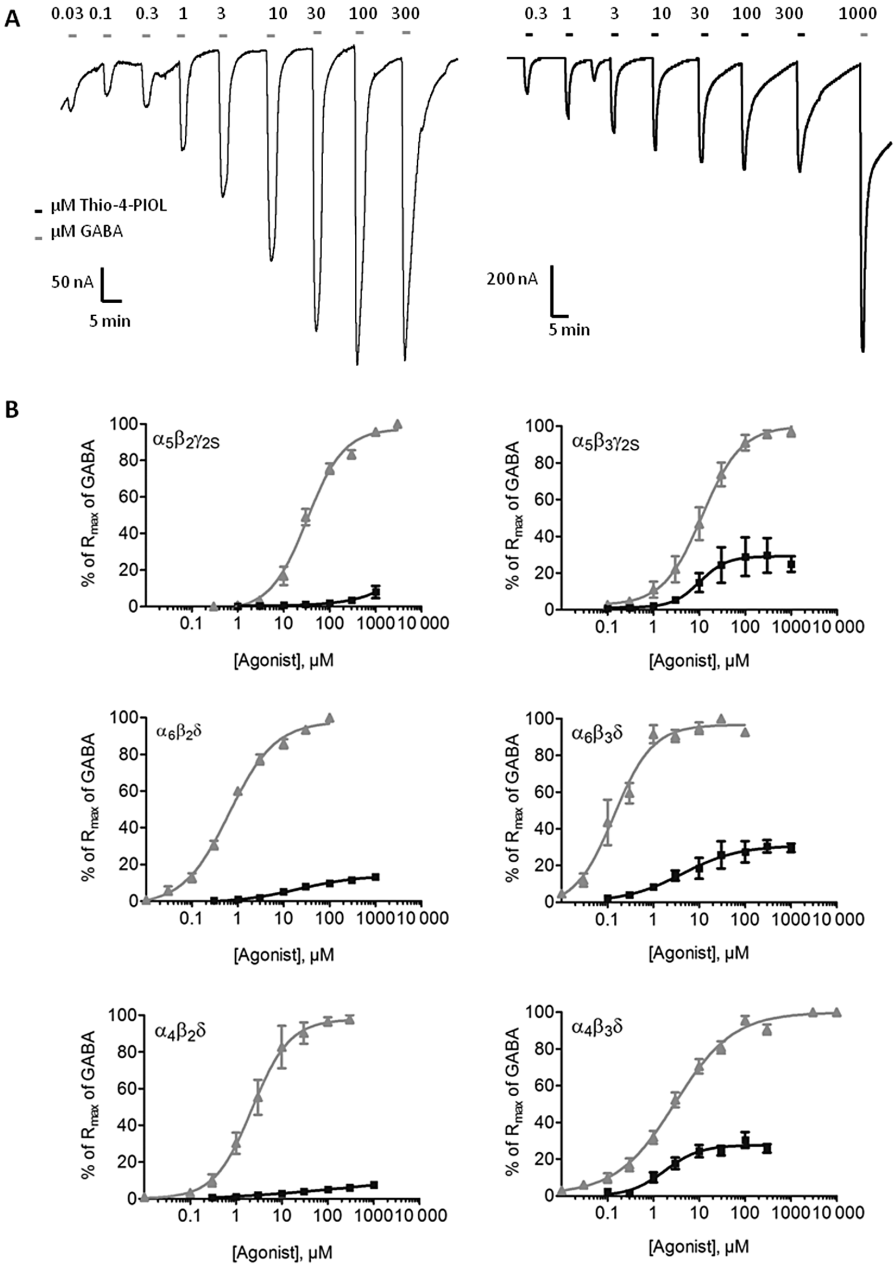


Figure 2

Functional properties of Thio-4-PIOL at extrasynaptic GABA$_A$Rs expressed in *Xenopus* oocytes. (A) Representative traces of the responses elicited GABA (left) and Thio-4-PIOL (right) in oocytes expressing the $\alpha_4\beta_3\delta$ GABA$_A$R. (B) Concentration-response curves for GABA (▲) and Thio-4-PIOL (■) at human $\alpha_5\beta_2\gamma_{2S}$, $\alpha_5\beta_3\gamma_{2S}$, $\alpha_6\beta_2\delta$, $\alpha_6\beta_3\delta$, $\alpha_4\beta_2\delta$ and $\alpha_4\beta_3\delta$ GABA$_A$Rs expressed in *Xenopus* oocytes. For $\alpha_5\beta_3\gamma_{2S}$ and $\alpha_6\beta_3\delta$, the curves are based on recordings on 'Thio-4-PIOL high efficacy' oocytes (see Table 1 for data for 'Thio-4-PIOL low efficacy' $\alpha_5\beta_3\gamma_{2S}$- and $\alpha_6\beta_3\delta$-expressing oocytes). Each data point represents the mean ± SEM values for 4–10 oocytes.

binding to the orthosteric site in the receptor, whereas the hydroxy group present in GHB and all other high-affinity GHB site ligands published to date is substituted by an amino group in Thio-4-PIOL. Furthermore, whereas Thio-4-PIOL displaces binding of the orthosteric radioligand [^{3}H]muscimol to native and recombinant GABA$_A$Rs in a competitive manner (Frølund *et al.*, 1995; Ebert *et al.*, 1997), [^{3}H]NCS-382 binding to rat brain tissue is not displaced by high concentrations of the orthosteric GABA$_A$R agonists muscimol and THIP, which both share high structural similarity to Thio-4-PIOL (Mehta *et al.*, 2001; Absalom *et al.*, 2012). In this study, we investigated whether the agonist activity of Thio-4-PIOL at the $\alpha_4\beta\delta$ GABA$_A$R is mediated by via the GHB site by use of NCS-382, a GHB site-specific ligand (Kaupmann *et al.*, 2003). The response elicited by 30 μM Thio-4-PIOL (EC_{70}-EC_{80}) in $\alpha_4\beta_2\delta$-expressing oocytes was not decreased (or increased) significantly by pre-incubation and co-application of 100 μM NCS-382 with Thio-4-PIOL (data not shown). This strongly

Table 1

Functional properties of GABA and Thio-4-PIOL at 12 human $GABA_AR$ subtypes expressed in *Xenopus* oocytes. The EC_{50} (in μM), pEC_{50} ± SEM, n_H ± SEM, R_{max} ± SEM (in % of the maximum response of GABA) and number of experiments performed (*n*) for Thio-4-PIOL and GABA are given

	EC_{50} (μM)	pEC_{50}	n_H	R_{max} (%)	*n*
$\alpha_1\beta_2\gamma_{2S}$					
GABA	37	4.43 ± 0.03	1.21 ± 0.07	100	6
Thio-4-PIOL	ND	ND	ND	ND	4
$\alpha_1\beta_3\gamma_{2S}$					
GABA	11	4.97 ± 0.13	0.97 ± 0.07	100	10
Thio-4-PIOL	23	4.63 ± 0.04	1.43 ± 0.11	4.4 ± 1.7	6
$\alpha_2\beta_2\gamma_{2S}$					
GABA	51	4.29 ± 0.05	1.35 ± 0.05	100	4
Thio-4-PIOL	85	4.07 ± 0.67	1.49 ± 0.19	1.0 ± 0.5	4
$\alpha_2\beta_3\gamma_{2S}$					
GABA	110	3.94 ± 0.20	0.77 ± 0.09	100	5
Thio-4-PIOL	33	4.49 ± 0.24	1.42 ± 0.27	4.2 ± 0.6	4
$\alpha_3\beta_2\gamma_{2S}$					
GABA	72	4.14 ± 0.05	1.19 ± 0.07	100	4
Thio-4-PIOL	320	3.50 ± 0.43	1.13 ± 0.51	1.2 ± 0.6	5
$\alpha_3\beta_3\gamma_{2S}$					
GABA	330	3.48 ± 0.38	0.86 ± 0.19	100	6
Thio-4-PIOL	17	4.78 ± 0.27	1.66 ± 0.73	1.2 ± 0.7	4
$\alpha_5\beta_2\gamma_{2S}$					
GABA	35	4.46 ± 0.08	1.05 ± 0.09	100	6
Thio-4-PIOL	91	4.04 ± 0.40	3.32 ± 2.21	3.9 ± 1.4	4
$\alpha_5\beta_3\gamma_{2S}$					
GABA	11	4.94 ± 0.14	1.13 ± 0.08	100	5
Thio-4-PIOL	24	4.61 ± 0.22	1.33 ± 0.12	34 ± 9[a]	7
				3.9 ± 0.8 [a]	6
$\alpha_4\beta_2\delta$					
GABA	2.6	5.59 ± 0.19	1.15 ± 0.07	100	4
Thio-4-PIOL	16	4.79 ± 0.31	0.89 ± 0.13	6.7 ± 0.6	4
$\alpha_4\beta_3\delta$					
GABA	2.7	5.57 ± 0.09	0.75 ± 0.08	100	10
Thio-4-PIOL	2.9	5.54 ± 0.18	1.19 ± 0.29	28 ± 2	6
$\alpha_6\beta_2\delta$					
GABA	0.69	6.16 ± 0.03	0.85 ± 0.10	100	4
Thio-4-PIOL	21	4.68 ± 0.04	1.48 ± 0.67	12 ± 2	5
$\alpha_6\beta_3\delta$					
GABA	0.14	6.85 ± 0.21	1.03 ± 0.05	100	4
Thio-4-PIOL[a]	2.1	5.67 ± 0.15	0.88 ± 0.11	32 ± 3[a]	7
				9.2 ± 1.6 [a]	11

ND, not determinable.
Due to the minute response evoked by Thio-4-PIOL through this receptor, pEC_{50}, n_H and R_{max} values could not be determined.
[a]As outlined in Results, two different $\alpha_5\beta_3\gamma_{2S}$ and $\alpha_6\beta_3\delta$ populations seemed to be formed in oocytes from different batches in terms of the maximal responses elicited by Thio-4-PIOL.

Correction added on 15 October 2013, after first online publication: order of text in table legend amended.

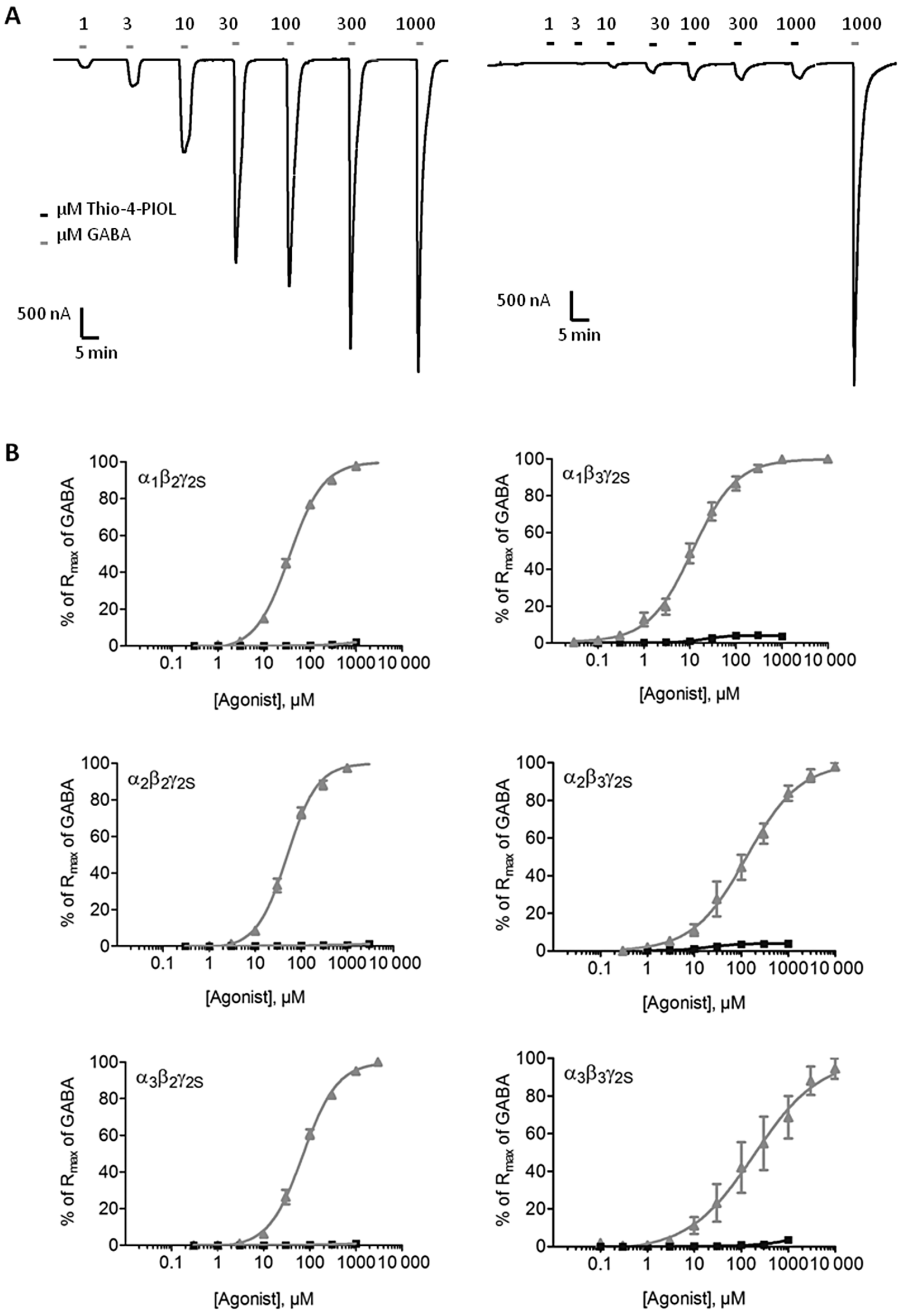


Figure 3

Functional properties of Thio-4-PIOL at synaptic GABA$_A$Rs expressed in *Xenopus* oocytes. (A) Representative traces of the responses elicited GABA (left) and Thio-4-PIOL (right) in oocytes expressing the $\alpha_1\beta_3\gamma_{2s}$ GABA$_A$R. (B) Concentration-response curves for GABA (▲) and Thio-4-PIOL (■) at human $\alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_3\gamma_{2s}$, $\alpha_2\beta_2\gamma_{2s}$, $\alpha_2\beta_3\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$ and $\alpha_3\beta_3\gamma_{2s}$ GABA$_A$Rs expressed in *Xenopus* oocytes. Each data point represents the mean ± SEM values for 4–10 oocytes.

suggests that Thio-4-PIOL targets the orthosteric site in the GABA$_A$R exclusively.

Functional characterization of Thio-4-PIOL at recombinant GABA$_A$Rs in HEK293 cells

The functional properties of Thio-4-PIOL were also determined at human $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ GABA$_A$Rs transiently expressed in HEK293 cells by patch-clamp recordings (Figure 4). Thio-4-PIOL displayed an EC_{50} value of 39 µM [95% confidence interval (CI): 19–80 µM], a Hill slope of 2.3 and a maximal response of 28% of that of GABA (95% CI: 21–35%) at $\alpha_5\beta_2\gamma_2$ ($n = 6$) and an EC_{50} value of 34 µM (95% CI: 8.1–140 µM), a Hill Slope of 1.4 and a maximal response of 5.6% of that of GABA (95% CI: 3.1–8.1%) at $\alpha_1\beta_2\gamma_2$ ($n = 5$; Figure 4).

The very low efficacy exhibited by Thio-4-PIOL at the $\alpha_1\beta_2\gamma_{2S}$ $GABA_AR$ in the patch-clamp recordings is in agreement with previous patch-clamp recordings at the receptor expressed in HEK293 cells as well as with the negligible response evoked by the compound in $\alpha_1\beta_2\gamma_{2S}$-expressing oocytes (Figure 3; Mortensen *et al.*, 2004). In contrast, the relative efficacy displayed by Thio-4-PIOL compared with GABA at $\alpha_5\beta_2\gamma_{2S}$ in HEK293 cells and in oocytes differed considerably (28 and 3.9% respectively). In the absence of a specific explanation for this difference, we can only ascribe it to the fundamental differences in the two recording set-ups or to the putative presence of a cofactor in one of the two cell types and not in the other. Changes in the elastic properties of the membrane lipid bilayer have been shown to induce significantly different gating characteristics of $GABA_ARs$ in both oocytes and HEK293 cells (Søgaard *et al.*, 2006; Chisari *et al.*, 2011), and thus, the difference in the cellular membranes in oocytes and HEK293 cells could be speculated to contribute to the difference. Interestingly, the $\alpha_3\beta_4$ nicotinic acetylcholine receptor, another member of the Cys-loop receptor superfamily, has been proposed to assemble into $(\alpha_3)_2(\beta_4)_3$ and $(\alpha_3)_3(\beta_4)_2$ stoichiometries in oocytes and in HEK293 cells respectively (Krashia *et al.*, 2010). However, considering the invariable subunit arrangement of the $\alpha\beta\gamma_{2S}$ complex, the two cell types are unlikely to express different $\alpha_5\beta_2\gamma_{2S}$ stoichiometries. The routinely use of diazepam to verify incorporation of γ_{2S} into the assembled receptors does not exclude the possibility that pure $\alpha_5\beta_2$ complexes may have been expressed in the oocytes and HEK293 cells. However, the efficacy difference exhibited by Thio-4-PIOL cannot be ascribed to the putative presence of these receptors, as they most likely constitute small fractions of the total receptor populations in the two cell types.

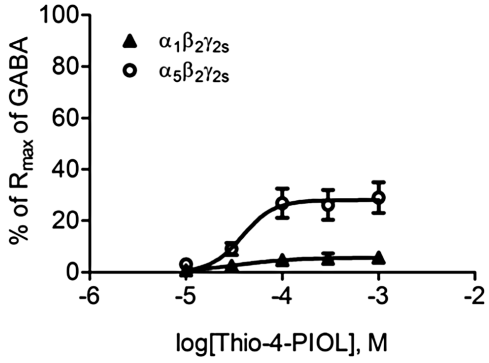


Figure 4

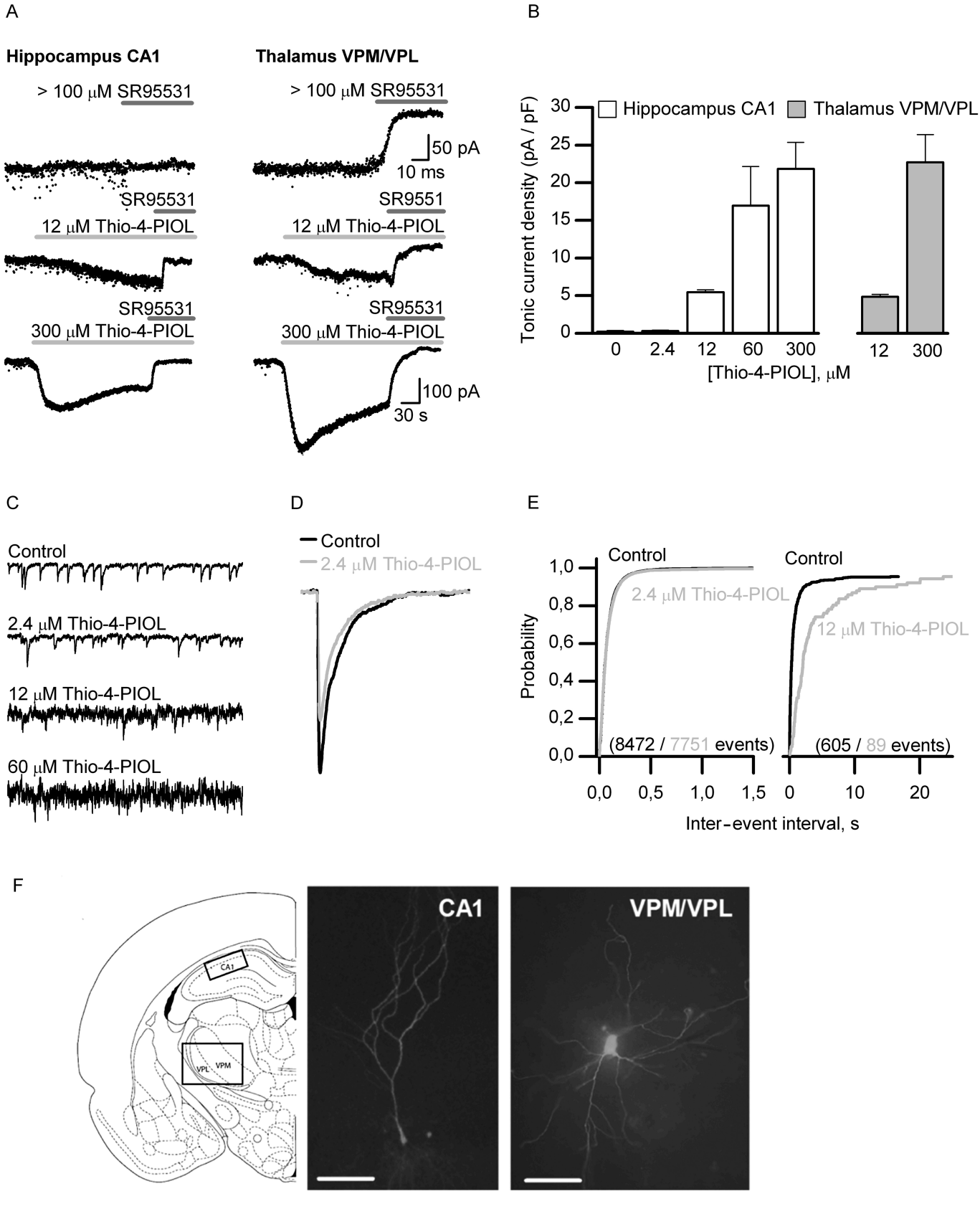
Concentration-response curves for Thio-4-PIOL at human $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_5\beta_2\gamma_{2s}$ $GABA_ARs$ transiently expressed in HEK293 cells and assayed by patch-clamp electrophysiology.

Functional characterization of Thio-4-PIOL at native $GABA_ARs$ by slice electrophysiology

The ability of Thio-4-PIOL to induce tonic current was investigated in slices from rat hippocampus and thalamus. The approximate location and morphology of recorded and stained neurons are shown in Figure 5F. In CA1 hippocampal neurons, Thio-4-PIOL induced a concentration-dependent slowly desensitizing tonic current of 0.2 ± 0.2 pA/pF (baseline; $n = 6$), 0.3 ± 0.1 pA/pF (2.4 μM; $n = 6$), 5.4 ± 0.3 pA/pF (12 μM; $n = 5$), 17 ± 5.2 pA/pF (60 μM; $n = 6$) and 22 ± 3.5 pA/pF (300 μM; $n = 5$) (Figure 5A and B). The compound also induced a pronounced tonic current in thalamic ventral posteromedial thalamic nucleus/ventral posterolateral thalamic nucleus neurons (Figure 5A), which, when normalized to cell size, did not differ significantly from that observed in the hippocampal neurons (4.5 ± 0.3 pA/pF and 21 ± 3.4 pA/pF at 12 and 300 μM Thio-4-PIOL respectively; Figure 5B). A significant tonic current was present in the thalamic neurons in the control situation (Figure 5A). In both regions, the tonic current was completely eliminated by co-application of the competitive $GABA_AR$ antagonist SR95531 (Figure 5A). Representative traces of 1 s recordings of mIPSCs recorded in CA1 hippocampal neurons during control and perfusion of Thio-4-PIOL are shown in Figure 5C. The noise induced by 60 and 300 μM Thio-4-PIOL precluded detection of the mIPSCs in these experiments, and thus, event characteristics is only given for the lowest dose. The averaged mIPSC peak in six cells was significantly decreased from 9.4 ± 0.6 pA in the control situation to 7.9 ± 0.1 pA in the presence of 2.4 μM Thio-4-PIOL (paired *t*-test, $P = 0.003$) (Figure 5D). In contrast, rise-time was unchanged (RT_{10-90}: 0.77 ± 0.04 in control vs. 0.76 ± 0.04 at 2.4 μM Thio-4-PIOL, paired *t*-test, $P = 0.7$), and decay time was also unaffected (7.75 ± 0.30 in control vs. 7.83 ± 0.20 at 2.4 μM Thio-4-PIOL, paired *t*-test, $P = 0.8$). The mean inter-event interval of the mIPSCs in control was 0.1 ± 0.04 s, and the cumulative distribution for six cells is shown in Figure 5E. At 2.4 μM Thio-4-PIOL, no significant difference was observed in the mean inter-event interval (paired *t*-test, $P = 0.39$, $n = 6$). At 12 μM Thio-4-PIOL, the mean inter-event interval increased from 1.2 ± 0.4 s in the control period to 7.2 ± 1.2 s (paired *t*-test, $P = 0.003$, $n = 5$). The cumulative distribution for

Figure 5

Functional properties of Thio-4-PIOL at native $GABA_ARs$ in rat hippocampal and thalamic neurons. (A) Induction of tonic current by 12 μM and 300 μM Thio-4-PIOL in CA1 hippocampal pyramidal neurons and in thalamic ventral posteromedial thalamic nucleus/ventral posterolateral thalamic nucleus (VPM/VPL) neurons. (B) Tonic current induced by different Thio-4-PIOL concentrations in CA1 hippocampal pyramidal neurons and in thalamic VPM/VPL neurons. The tonic current is normalized to the cell capacitance (tonic current density, pA/pF). (C) Representative traces of 1 s duration of CA1 hippocampal pyramidal neuron recordings in control and in the presence of 2.4, 12 and 60 μM Thio-4-PIOL. (D) The average non-contaminated waveform of the mIPSC in control and 2.4 μM Thio-4-PIOL. (E) Cumulative distribution of inter-event interval of mIPSCs in CA1 hippocampal pyramidal neurons in the control situation and in the presence of 2.4 and 12 μM Thio-4-PIOL. The detection level for the mIPSC was set relative to baseline root mean square. (F) Coronal diagram of a map of the rat brain, modified from (Paxinos and Watson, 1998). Whole-cell patch-clamp recordings were made on neurons in coronal brain slices from adult Lister hooded male rats either from CA1 or VPM/VPL in thalamus. Cells were filled with green fluorescent protein, and post-recording, a histological examination was made for each neuron. Pyramidal neuron from CA1 in hippocampus. Scale bar = 100 μm. Neuron from VPM/VPL in thalamus. Scale bar = 20 μm.


A
Hippocampus CA1
Thalamus VPM/VPL
> 100 μM SR95531
50 pA
10 ms
SR95531
12 μM Thio-4-PIOL
SR9551
SR95531
300 μM Thio-4-PIOL
100 pA
30 s
B
Hippocampus CA1
Thalamus VPM/VPL
Tonic current density (pA / pF)
[Thio-4-PIOL], μM
C
Control
2.4 μM Thio-4-PIOL
12 μM Thio-4-PIOL
60 μM Thio-4-PIOL
D
Control
2.4 μM Thio-4-PIOL
E
Control
2.4 μM Thio-4-PIOL
12 μM Thio-4-PIOL
Probability
(8472 / 7751 events)
(605 / 89 events)
Inter-event interval, s
F
CA1
VPM/VPL
VPL
VPM

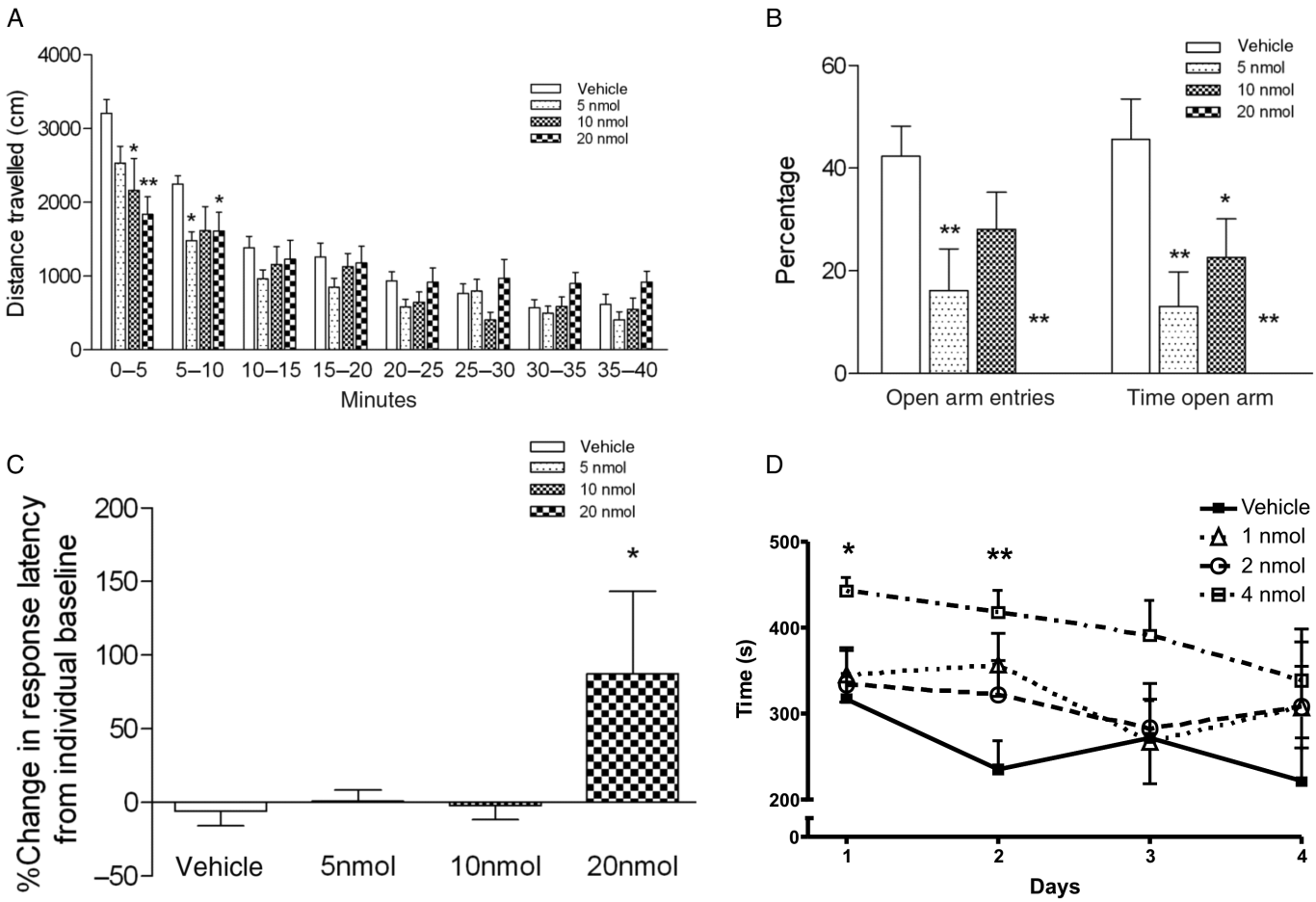


Figure 6

Behavioural effects of Thio-4-PIOL in animal models of locomotion, anxiety, nociception and spatial memory. All graphs show mean values ± SEM. (A) Distance travelled in individual 5 min bins in the open field model. Vehicle (n = 9), 5 nmol (n = 10), 10 nmol (n = 8), 20 nmol (n = 10). (B) Percentage of open arm entries and time spent on the open arm in the EPM. Vehicle (n = 10), 5 nmol (n = 7), 10 nmol (n = 8), 20 nmol (n = 4). (C) Percentage change in individual responses to a nociceptive stimulus using the hot-plate test. (D) Daily cumulative trial times in the Morris water maze following intrahippocampal administration. Vehicle (n = 12), 1 nmol (n = 10), 2 nmol (n = 9), 4 nmol (n = 8).

inter-event interval at control and 12 μM Thio-4-PIOL for five cells is shown in Figure 5E.

Effects of Thio-4-PIOL in rat models for locomotion, anxiety, nociception and spatial memory

Open field. Administration of Thio-4-PIOL influenced locomotor activity differentially over the course of 40 min in the open field test (time × drug $F(21,231) = 2.816$; $P < 0.001$), with an overall significant reduction in the distance travelled in rats dosed with 5 nmol ($P = 0.037$). Reduced locomotion in the other treatment groups was only pronounced in the first individual 5 min time bins, with a strong impact on animals from the 20 nmol group for 10 min ($P = 0.001$, $P = 0.043$) and for rats from the 10 nmol group for 5 min ($P = 0.015$). Interestingly, a trend of increased locomotor activity could be observed in animals from the 20 nmol group towards the end of the test session (Figure 6A).

Elevated plus maze. Increased levels of anxiety-like behaviour were observed after administration of Thio-4-PIOL across all dosage groups, seen in the percentage of time on the open arm [$F(3,25) = 6.084$; $P = 0.003$] (Figure 6B), the number of open arm entries [$F(3,25) = 6.615$; $P = 0.002$, data not shown], and the latency to first enter the open arm [$F(3,25) = 7.881$; $P = 0.001$; data not shown]. As observed in first 5 min of open field testing, administration of Thio-4-PIOL caused a significant decrease in locomotor activity on the EPM [number of closed arm entries: $F(3,25) = 4.340$; $P = 0.014$, data not shown], again only affecting animals from the 10 nmol and 20 nmol but not the 5 nmol group within this time frame.

Hot plate test. Effects of Thio-4-PIOL on pain sensitivity were assessed in the hot plate test and expressed as percentage change in latency to withdraw the paw from the individual baseline. Thio-4-PIOL significantly affected pain responsiveness [overall effect: $F(3,21) = 3.104$; $P = 0.049$], attributed to the strong increase in pain threshold in the 20 nmol group (Figure 6C).

Morris water maze. A repeated measures ANOVA revealed a significant effect of time [$F(3, 105) = 2.72$, $P < 0.05$] and

treatment [$F(3, 35) = 3.81$, $P < 0.05$] but no interaction effect [$F(9, 105) = 0.76$ $P > 0.05$] on performance during the training portion of the experiment. The group treated with the highest dose of Thio-4-PIOL (4 nmol) displayed impaired spatial learning compared with the vehicle-treated group as revealed by *post hoc* testing ($P < 0.01$; Figure 6D). A one-way ANOVA revealed a significant effect of treatment [$F(3, 35) = 3.9$, $P < 0.05$] on time spent in the target quadrant during the probe trial. A *post hoc* test revealed the group treated Thio-4-PIOL (4 nmol) spent significantly less time in the target quadrant compared to animals treated with vehicle alone ($P < 0.05$; data not shown). Swim speed or distance travelled in the probe trial was unaffected by any of the doses of Thio-4-PIOL tested [one-way ANOVA $F(3, 35) = 0.82$, $P < 0.05$ and $F(3, 35) = 0.82$, $P < 0.05$] (data not shown).

Discussion and conclusions

The realization that the same abundance making $GABA_ARs$ attractive drug targets in a wide range of disorders also seems to be the origin of many of the side effects associated with the GABAergic drugs has prompted the search for new drugs with specific activity at selected subtypes. This is perhaps best illustrated by the achievements in the benzodiazepine field. Here, it has proven difficult to develop subtype-selective benzodiazepine derivatives in terms of binding affinity, whereas several functionally selective modulators displaying very different intrinsic activities at the $\alpha_{1,2,3,5}\beta\gamma_2$ subtypes have emerged (Atack, 2011a,b; Ebert *et al.*, 2006). In contrast to the extensive research into allosteric modulators of $GABA_ARs$, medicinal chemistry efforts focused on orthosteric ligands have been sparse, and only a few of these ligands have been characterized functionally at more than one subtype (Frølund *et al.*, 2002). While the pronounced conservation of the orthosteric sites in the $GABA_AR$ subtypes may seem discouraging for the prospects of developing subtype-selective orthosteric ligands, the recent 'rediscovery' of THIP as an $\alpha_4\beta\delta/\alpha_6\beta\delta$-selective agonist has demonstrated that indeed, it is possible to obtain functionally selective orthosteric ligands (Storustovu and Ebert, 2006). In the present study, we present another such ligand, Thio-4-PIOL.

The functional profile exhibited by Thio-4-PIOL at recombinant $GABA_ARs$ in this study is generally in concordance with observations made in previous studies, where Thio-4-PIOL has been shown to exhibit low micromolar binding affinities to native and recombinant $GABA_ARs$ (Frølund *et al.*, 1995; Ebert *et al.*, 1997), to be a very low-efficacious agonist at $\alpha_1\beta_2\gamma_{2S}$ and at native $GABA_ARs$ in cortical neurons (Frølund *et al.*, 1995; Mortensen *et al.*, 2002; 2004) and to be a competitive antagonist at 10 $\alpha_{1,2,3,6}/\beta_{1,2,3}/\gamma_2$ combinations (Ebert *et al.*, 1997). However, the compound has also been reported to be a competitive antagonist at $\alpha_5\beta_3\gamma_2$ $GABA_AR$ (Ebert *et al.*, 1997), which contrasts with its partial agonism at the receptor in the present study (Figure 2). Thus, it seems that the system used by Ebert *et al.* may have underestimated the apparent efficacy of partial agonists (Ebert *et al.*, 1997). This discrepancy may either be due to relatively low expression levels or a relatively slow equilibrium rate, and this could also explain the lower agonist efficacy exhibited by Thio-4-PIOL at $\alpha_4\beta_3\delta$-expressing oocytes in a 2006 study (Storustovu and Ebert, 2006) compared with this study (4.4 vs. 28%; Table 1). Thus, the key finding in this study is that Thio-4-PIOL in addition to its very low agonist efficacy (*de facto* antagonism) at synaptic $GABA_ARs$ exhibits pronounced agonist efficacy at some of the major extrasynaptic $GABA_ARs$, more specifically the β_3-containing $\alpha_5\beta\gamma_2$, $\alpha_4\beta\delta$ and $\alpha_6\beta\delta$ subtypes.

The functional properties of Thio-4-PIOL are quite remarkable from a molecular perspective. Analogously to THIP, the compound is a more efficacious agonist at the extrasynaptic δ-containing subtypes (in particular $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$) than at the synaptic receptors. Compared with the 'superagonism' and full/partial agonism displayed by THIP at $\alpha_{4/6}\beta\delta$ and $\alpha_{1/2/3}\beta\gamma_{2S}$ receptors, respectively (Ebert *et al.*, 2006; Storustovu and Ebert, 2006), the profile of Thio-4-PIOL can be considered as a parallel shift to partial agonism and *de facto* antagonism at the two respective receptor classes. The higher relative agonist efficacies displayed by Thio-4-PIOL at the $\alpha_4\beta_3\delta$, $\alpha_6\beta_3\delta$ and $\alpha_5\beta_3\gamma_2$ receptors than at the corresponding β_2-containing subtypes is also interesting, although the concomitant expression of 'low efficacy' $\alpha_6\beta_3\delta$ and $\alpha_5\beta_3\gamma_2$ populations in other oocytes should be noted (Table 1). In any case, this is the first example of an orthosteric ligand evoking a differential response through $\alpha_4\beta_2\delta$ and $\alpha_4\beta_3\delta$ $GABA_ARs$, even if the functional difference between the two receptors in this case is rather small. It remains to be investigated whether these differences can be ascribed to specific molecular determinants in the receptor subunits or arise from different kinetic activation thresholds in the receptors.

The functional properties displayed by Thio-4-PIOL at recombinant human $GABA_ARs$ are mirrored by its activity in rat CA1 hippocampus and ventrobasal thalamus neurons. The bulk of the massive tonic current elicited by Thio-4-PIOL in the hippocampus is likely to arise from activation of $\alpha_5\beta\gamma_2$ receptors, although non-α_5/δ-containing ($\alpha_4\beta\delta$) subtypes and 'pure' αβ combinations also have been proposed to contribute to tonic inhibition here (Glykys and Mody, 2006; Mortensen and Smart, 2006; Glykys *et al.*, 2008). In contrast, the tonic current produced by Thio-4-PIOL in the thalamus most likely originates from activation of $\alpha_4\beta\delta$ receptors (Pirker *et al.*, 2000; Belelli *et al.*, 2005; Chandra *et al.*, 2006). Considering the agonism of Thio-4-PIOL at $\alpha_6\beta\delta$ $GABA_ARs$ (Figure 2), Thio-4-PIOL is also likely to induce tonic inhibition in cerebellar granule cells and other neurons expressing this third major extrasynaptic receptor but this remains to be investigated (Pirker *et al.*, 2000; Olsen and Sieghart, 2009). As for the antagonistic effects of Thio-4-PIOL on phasic current in CA1 hippocampal neurons, the reduction in amplitude and mIPSC frequency observed upon application of increasing concentrations of Thio-4-PIOL (Figure 5C–E) correlates well with its negligible agonist activity and *de facto* antagonism at $\alpha_{1,2,3}\beta_{2,3}\gamma_{2S}$ receptors (Figure 3).

Just as the activity of Thio-4-PIOL in the slice recordings seem to reflect its functional profile *in vitro*, so do the behavioural effects of the compound in preclinical behavioural tests of anxiety, locomotion, nociception and spatial learning (Figure 6). These processes were investigated due to the large influence the GABAergic system has on emotion (Cryan and Kaupmann, 2005; Möhler, 2012), pain signalling (Neto *et al.*, 2006; Mirza and Munro, 2010) and cognition (Maubach, 2003; Möhler, 2009). Thio-4-PIOL produces an anxiogenic-like effect in the EPM at doses of 5, 10 and 20 nmol

(Figure 6B). However, a clear dissociation between the effects of Thio-4-PIOL on locomotor activity during the first 5 min and on anxiety-like behaviour is present and furthermore, swimming activity in the Morris water maze revealed no effect on locomotion. The reduction in locomotor activity observed at the 10 and 20 nmol doses in the open field test confounds interpretation of the behavioural effects in the EPM, and thus, we refrain from concluding on its anxiogenic effects at these higher doses (Figure 6A and B). We interpret the anxiogenic effects of Thio-4-PIOL at the 5 nmol dose to arise as a consequence of its antagonism of the synaptic $GABA_A$Rs, as they are consistent with the anxiolytic effects of $GABA_A$R agonists and PAMs (Krogsgaard-Larsen *et al.*, 2004; Korpi and Sinkkonen, 2006; Atack, 2011a; Smith and Rudolph, 2012; Smith *et al.*, 2012) and with the anxiogenic effects of $GABA_A$R antagonists (Miller *et al.*, 2010). The antagonism of synaptic $GABA_A$Rs is likely to be a contributing factor to the reduced locomotor activity observed upon Thio-4-PIOL administration, just as the seizures observed in some rats (not included in the studies) could arise from the inhibition of a1bg and other synaptic subtypes (not included in the studies) (Huang *et al.*, 2001; Elsen *et al.*, 2006; Korpi and Sinkkonen, 2006). On the other hand, the significantly increased pain threshold in rats in the hot-plate test upon injection of 20 nmol Thio-4-PIOL (Figure 6C) cannot necessarily be ascribed to its inhibition of synaptic $GABA_A$Rs. Even though bicuculline has displayed analgesic effects in animal tests (Hasanein *et al.*, 2008), so have a wide range of $GABA_A$R agonists and PAMs (Enna and McCarson, 2006; Munro *et al.*, 2008).

A large corpus of data has been collected, which places the GABAergic system as a key modulator of cognitive processes, with α_5-containing $GABA_A$Rs emerging as one of the primary regulators (Möhler, 2009). Mice displaying a genetic reduction to α_5-containing $GABA_A$Rs have displayed improved spatial memory in a previous study (Collinson *et al.*, 2002), and RO4938581, an inverse agonist at α_5-containing $GABA_A$Rs, improves spatial memory in rats (Chambers *et al.*, 2004; Ballard *et al.*, 2009). In contrast, genetic reduction in α_5-containing $GABA_A$Rs has also been reported to impair memory associated with locating objects (Prut *et al.*, 2010). Interestingly, in the present study, intrahippocampal Thio-4-PIOL (4 nmol), a partial agonist at α_5-containing $GABA_A$Rs, impaired the acquisition of spatial memory independently of any effect on swimming ability.

In conclusion, Thio-4-PIOL is only the second orthosteric $GABA_A$R ligand to have been subjected to an elaborate characterization at recombinant and native receptors, and in relevant animal models. The behavioural effects of Thio-4-PIOL are quite illustrative of a fundamental difference between a functionally selective orthosteric ligand and the new generations of functionally subtype-selective PAMs and negative allosteric modulators: an allosteric modulator will not influence the signalling through those subtypes at which it has insignificant efficacy, whereas an orthosteric ligand with negligible efficacy, being a competitive antagonist, certainly will do so. While Thio-4-PIOL is an unlikely candidate to be a future therapeutic agent, the distinct intrinsic activities of the agonist at extrasynaptic and synaptic $GABA_A$Rs and its ability to concomitantly induce tonic inhibition and antagonize phasic current in the GABA system make for a unique ligand and a potentially valuable pharmacological tool for explorations of the physiological roles of the respective subtypes. The examples of THIP and Thio-4-PIOL underline the possibility of obtaining functional selectivity in orthosteric $GABA_A$R ligands and are in line with observations made in the nicotinic ACh receptor field, where several subtype-selective agonists rooted in differential efficacies at the respective subtypes have been identified (Jensen *et al.*, 2005). Thus, the results of this study call for a functional characterization of other orthosteric $GABA_A$R ligands in a similar elaborate manner.

Acknowledgements

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Correction added on 15 October 2013, after first online publication: The sentence beginning 'Even though bicuculline' at the end of the first paragraph has been corrected from 'it has a wide range of' to read 'so have a wide range of' and Ach has been corrected to ACh in the final paragraph.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 The functional properties of THIP at extrasynaptic $\alpha_4\beta\delta$ $GABA_A$ receptors. Concentration-response curves for THIP at human $\alpha_4\beta_2\delta$ (n = 2) and $\alpha_4\beta_3\delta$ (n = 3) $GABA_A$ receptors expressed in *Xenopus* oocytes.