

An inverse agonist selective for $\alpha 5$ subunit-containing GABA_A receptors enhances cognition

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Abbreviations: α 5IA, 3-(5-Methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-*a*]phthalazine; FG 7142, *N*-methyl- β -carboline-3-carboxamide; DMCM, methyl-6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate; Flumazenil (Ro 15-1788), 8-fluoro 5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester; Ro 15-4513, 8-azido 5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester.

ABSTRACT

$\alpha 5$ IA is a compound which binds with equivalent subnanomolar affinity to the benzodiazepine (BZ) site of GABA_A receptors containing an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit but has inverse agonist efficacy selective for the $\alpha 5$ subtype. As a consequence, the *in vitro* and *in vivo* effects of this compound are mediated primarily via GABA_A receptors containing an $\alpha 5$ subunit. In a mouse hippocampal slice model, $\alpha 5$ IA significantly enhanced the theta burst-induced long-term potentiation (LTP) of the fEPSP in the CA1 region but did not cause an increase in the paroxysmal burst discharges that are characteristic of convulsant and proconvulsant drugs. These *in vitro* data suggesting that $\alpha 5$ IA may enhance cognition without being proconvulsant were confirmed in *in vivo* rodent models. Hence, $\alpha 5$ IA significantly enhanced performance in a rat hippocampal-dependent test of learning and memory, the delayed matching-to-position version of the Morris water maze, with a minimum effective oral dose of 0.3 mg/kg which corresponded to a BZ site occupancy of 25%. However, in mice $\alpha 5$ IA was not convulsant in its own right nor did it potentiate the effects of pentylenetetrazole acutely or produce kindling upon chronic dosing even at doses producing greater than 90% occupancy. Finally, $\alpha 5$ IA was not anxiogenic-like in the rat elevated plus maze nor did it impair performance in the mouse rotarod assay. Taken together, these data suggest that the GABA_A $\alpha 5$ -subtype provides a novel target for the development of selective inverse agonists with utility in the treatment of disorders associated with a cognitive deficit.

Introduction

Agonists at the benzodiazepine (BZ) binding site of the GABA_A receptor, such as diazepam, enhance the inhibitory effects of GABA and have been used as anxiolytics and hypnotics for over 40 years (Argyropoulos and Nutt, 1999). In addition, they have therapeutic utility in inducing not only sedation and muscle relaxation but also amnesia prior to surgical procedures (Williams and Bowie, 1999). Although the amnesic effects of BZ agonists in animals and man have been known for sometime (Ghoneim and Mewaldt, 1990), the precise nature of the processes underlying these effects are still unknown. Since the anterograde rather than retrograde amnesia (McNaughton and Morris, 1991) is similar to deficits induced by hippocampal lesions in animals and man, it has been suggested that BZ agonists may exert their amnesic effects by modulating hippocampal function.

At present 19 GABA_A receptor subunits have been identified (α 1- α 6, β 1- β 3, γ 1- γ 3, δ , ϵ , θ , ρ 1-3 and π ; Simon et al., 2004) with the majority of GABA_A receptors in the brain contain α , β and γ subunits in a 2:2:1 stoichiometry (Sieghart and Sperk, 2002). The BZ binding site occurs at the interface of a γ 2 and either an α 1, α 2, α 3 or α 5 subunit with the α subunit being of particular importance in determining the pharmacology of the BZ binding site of native GABA_A receptors (Sieghart, 1995). Non-selective BZ agonists such as diazepam enhance the inhibitory effects of GABA at these four (i.e. α 1-, α 2-, α 3- or α 5-containing) GABA_A receptor subtypes and thereby increase GABA-mediated chloride flux (Sieghart, 1995). These effects translate into the anxiolytic, anticonvulsant, myorelaxant, sedative and cognitive impairing effects observed clinically. Recently, studies using molecular genetic (α subunit point-mutation mice) or pharmacological (subtype-selective compound) approaches suggest that GABA_A receptors containing an α 1 subunit mediate the sedative/muscle relaxant effects of diazepam whereas those with an α 2 or α 3 subunit account

for the anxiolytic/anticonvulsant effects (Rudolph et al., 1999; McKernan et al., 2000; Rudolph and Möhler, 2004; Atack et al., 2005). In contrast, the functions of GABA_A receptors containing an $\alpha 5$ subunit are less well defined. Nevertheless, $\alpha 5$ -containing GABA_A receptors are preferentially expressed in the hippocampus (Quirk et al., 1996) suggesting that they play a key role in hippocampal functions such as learning and memory (Maubach, 2003). Furthermore, these receptors also have a distinct extrasynaptic localization (Brünig et al., 2002) and play a role in tonic inhibition of CA1 pyramidal neurons (Caraiscos et al., 2004). However, these observations do not preclude the possibility that certain $\alpha 5$ -containing GABA_A receptors may be found at synapses (Brünig et al., 2002) and the presence of a population of $\alpha 5$ -containing receptors within the synapse is consistent with the observations that IPSC amplitude is decreased and the decay time is slower in mice lacking the $\alpha 5$ subunit ($\alpha 5^{-/-}$ mice; Collinson et al., 2002).

Whilst BZ site agonists such as diazepam increase the GABA-induced chloride flux through GABA_A receptors containing an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit, non-selective inverse agonists, such as the β -carbolines DMCM and FG 7142 decrease chloride flux at these same receptor subtypes, resulting in a membrane depolarisation and increased neuronal excitability (Haefely et al., 1993). In contrast, BZ site antagonists do not alter the efficacy of GABA (Haefely et al., 1993) with flumazenil (Ro 15-1788) being the prototypic compound of this class, although it may, nevertheless, possess a slight degree of intrinsic efficacy (Malizia and Nutt, 1995). The opposing effects of BZ site agonists and inverse agonists at the molecular level are reflected behaviourally in that inverse agonists are anxiogenic-like, increase vigilance and are either convulsant or proconvulsant (Haefely et al., 1993). Although the increased vigilance of these compounds could be beneficial clinically in terms of enhancing cognition, the anxiogenic and convulsant/proconvulsant liabilities of the non-selective inverse agonists prevents their use in man (Dorow et al., 1983). Clearly, however, a compound

possessing inverse agonism at the GABA_A subtype responsible for the cognition enhancing effects but devoid of efficacy at those subtypes responsible for the anxiogenic and convulsant/proconvulsant properties would be of clinical utility.

Based upon its preferential hippocampal location, it was hypothesized that a compound with inverse agonism selective $\alpha 5$ -containing GABA_A receptors might enhance hippocampally-mediated cognitive function (Maubach, 2003) and accordingly such a compound, $\alpha 5$ IA, was identified (Sternfeld et al., 2004). We now show that in rodents this compound not only enhances the performance in a hippocampus-dependent cognitive test but is devoid of anxiogenic-like behaviour, and convulsant, proconvulsant, kindling or motor impairing activities.

Materials and Methods

Animal experiments. All procedures involving animals were conducted within the remit of project and personal licenses and in accordance with the UK Animals (Scientific Procedures) Act 1986.

Drugs. FG 7142 (*N*-Methyl- β -carboline-3-carboxamide), DMCM (methyl-6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate) diazepam and flumazenil (Ro 15-1788) were purchased from Sigma-Aldrich (Gillingham, UK) and bretazenil was a gift from Hoffman-La Roche (Basel, Switzerland). [³H]flumazenil ([³H]Ro 15-1788; 70-87 Ci/mmol) and [³H]Ro 15-4513 (20-40 Ci/mmol) were purchased from NEN (PerkinElmer Life Sciences, Boston, MA). $\alpha 5$ IA (3-(5-Methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-*a*]phthalazine), Figure 1) was synthesized as described previously (Sternfeld et al., 2004).

In vitro radioligand binding studies. L(tk⁻) cells expressing human recombinant GABA_A receptors containing $\beta 3$ and the $\gamma 2$ short isoform in combination with various α subunits were harvested and binding performed as described elsewhere (Hadingham et al., 1993; 1996). The choice of $\beta 3$ rather than $\beta 1$ or $\beta 2$ is of little consequence to the BZ site pharmacology since this recognition site occurs at the interface of a $\gamma 2$ (but not $\gamma 1$ or $\gamma 3$) and either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit (Sieghart, 1995). The inhibition of 1.8 nM [³H]flumazenil ([³H]Ro15-1788) binding by $\alpha 5$ IA was measured in GABA_A receptors containing either an $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunit and from the IC₅₀ the K_i was calculated using the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + ([\text{radioligand}]/K_D))$; Cheng and Prusoff, 1973) calculated with respective K_D values for [³H]flumazenil binding of 0.92, 1.05, 0.58 and 0.45 nM at the $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subtypes. The affinity of $\alpha 5$ IA for GABA_A receptors containing $\alpha 4$ and $\alpha 6$ subunits was measured using 8.0 nM [³H]Ro 15-4513 and from the IC₅₀ the K_i was calculated using K_D values of 4.0 and 6.5 nM for [³H]Ro 15-4513 binding at $\alpha 4\beta 3\gamma 2$ and $\alpha 6\beta 3\gamma 2$ receptors, respectively. Non-specific binding was defined by the inclusion of 10 μ M flunitrazepam for the $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$ subtypes and 10 μ M Ro 15-4513 for $\alpha 4$ and $\alpha 6$ subtypes. The percentage inhibition of [³H]flumazenil or [³H]Ro 15-4513 binding, the IC₅₀ and the K_i values were calculated using ActivityBase (IDBS; Guildford, Surrey, UK).

In vivo [³H]flumazenil binding. The occupancy of brain BZ binding sites by $\alpha 5$ IA or FG 7142 was assessed using a [³H]flumazenil in vivo binding assay (Atack et al., 1999). In brief, animals were pretreated with compound or corresponding vehicle and 3 min prior to killing, [³H]flumazenil (70-87 Ci/mmol diluted 1:150 with saline; PerkinElmer Life Sciences, Boston, MA) was injected via a tail vein (injection volume = 1 and 5 μ L/g for rats and mice, respectively). Animals were killed and the brains rapidly removed, homogenized in 10

volumes of ice-cold buffer (10 mM phosphate buffer/100 mM KCl, pH 7.4) and 300 μ L aliquots were filtered and washed over Whatman GF/B glass fibre filters. For each experiment, a separate group of animals was dosed with 5 mg/kg bretazenil (i.p. in 100% polyethylene glycol vehicle with a pretreatment time of 30 min.) to define the level of non-specific binding. The extent by which α 5IA reduced the specific in vivo binding of [3 H]flumazenil relative to the binding in vehicle-treated animals was defined as the occupancy. Doses that inhibit the in vivo binding of [3 H]Ro 15-1788 by 50% (ID₅₀) were estimated using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Two-electrode voltage-clamp of *Xenopus* oocytes. *Xenopus laevis* oocytes were removed from anaesthetized frogs and manually defolliculated with fine forceps. After mild collagenase treatment to remove follicle cells (0.5 mg/mL for 6 min), the oocyte nuclei were then directly injected with 10-20 nL of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7.0, nitrocellulose filtered) containing rat or human α 1, α 2, α 3 or α 5 GABA_A subunit cDNAs (6 ng/mL) engineered into the expression vector pCDM8. These subunits were expressed along with β 3 and γ 2 GABA_A receptor subunits in a 1:1:1 concentration ratio.

For recording, the oocytes were stored in an incubator until use with recordings being made 2-4 days after injection. Oocytes were placed in 50 μ L bath and perfused with modified Barth's medium (MBS) consisting of (in mM): NaCl, 88; KCl, 1.0; CaCl₂, 0.91; MgSO₄, 0.82; HEPES, 10; Ca(NO₃)₂, 0.33; NaHCO₃, 2.4; pH 7.5 with 10 M NaOH. Cells were impaled with two 1-3 M Ω electrodes containing KCl (2M) and voltage clamped at -60mV using a Geneclamp amplifier (Axon Instruments). The oocyte was continuously perfused with MBS at 4-6 mL/min, and drugs were applied to the perfusate. For each oocyte the expression of receptors was first checked by a 30 sec. application of a maximal concentration of GABA

(300 μ M) in MBS and GABA currents ranged typically from 0.3 to 3 μ A, and cells with currents <0.3 μ A were rejected. α 5IA (30 nM) was preapplied for 30 sec. before the addition of GABA, which was applied until the peak response was observed, usually within 30 sec. To prevent desensitization, at least 3 min washing was allowed between each GABA application. Stable responses to a concentration of GABA that gave a current that is 20% of maximum (EC_{20}) were obtained for each individual oocyte (1-6 μ M for α 1-, α 2- and α 3-, and 3-10 μ M for α 5-containing GABA_A receptors) and then the % modulation of this response by α 5IA (30 nM) was determined.

Whole cell patch-clamp of L(tk⁻) cells. Experiments were performed on L(tk⁻) cells stably expressing human β 3, γ 2 and either α 1, α 2, α 3 or α 5 subunit combinations (Hadingham et al., 1993). Glass cover-slips containing the cells in a monolayer culture were transferred to a Perspex chamber on the stage of Nikon Diaphot inverted microscope. Cells were continuously perfused with a solution consisting of (in mM): NaCl, 124; KCl, 2; CaCl₂, 2; MgCl₂, 1; KH₂PO₄, 1.25; NaHCO₃, 25; D-glucose, 11; at pH 7.2, and observed using phase-contrast optics. Patch-pipettes were pulled with an approximate tip diameter of 2 μ m and a resistance of 4 M Ω with borosilicate glass and filled with 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, 3 mM Mg⁺-ATP, pH adjusted to 7.3 with CsOH. Cells were patch-clamped in whole-cell mode using an Axopatch-200B patch-clamp amplifier. A double-barreled pipette assembly, controlled by a stepping motor attached to a Burleigh manipulator (Scientifica) which enabled rapid equilibration around the cell, applied drug solutions. Increasing GABA concentrations were applied for 5 sec. pulses with a 30 sec. interval between applications. Curves were fitted using a non-linear square-fitting program to the equation $f(x) =$

$B_{\text{MAX}}/[1+(EC_{50}/x)^n]$ where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response and n is the Hill coefficient.

Brain slice electrophysiology. Brain slices were prepared from 6 to 9 month old C57 mice (B&K Universal, Hull, UK). The brain was quickly removed and rinsed with ice cold aCSF consisting of (in mM): NaCl, 126; NaH_2PO_4 , 1.2; MgCl_2 , 1.3; CaCl_2 , 2.4; KCl, 2.5; NaHCO_3 , 26; D-glucose, 10 which was bubbled with 95% O_2 plus 5% CO_2 . Parasagittal sections (350 μm) were cut on a Vibratome whilst the brain was submerged in ice cold aCSF with subsequent recordings being performed at 33°C with slices submerged in aCSF. The fibres of the Schaffer collateral-commissural path were stimulated with tungsten bipolar electrodes and field excitatory post-synaptic potentials (fEPSPs) in the stratum radiatum of the CA1 region were recorded using glass microelectrodes filled with 2 M NaCl. The signals were filtered (8 kHz high pass and 1.5 kHz low pass) and recorded using a CED 1401plus A/D interface and a program written in Spike2 v2.24 script language. The stimulus intensity was set to give a fEPSP with a 20% maximal slope determined from the input-output curve. Test stimuli were applied every 30 sec., slopes were calculated on-line and allowed to stabilise so that baseline values varied by no more than 5% for a minimum of 30 min. LTP was induced by a brief tetanus (10 stimuli at 100 Hz) or a theta-burst protocol (4 pulses at 100 Hz repeated 10 times at an interval of 200 msec.). Regression analysis plus two-way analysis of variance with repeated measures (treatment and time) were performed. The number of negative transients comprising the fEPSP were recorded following single afferent stimuli and the effect of $\alpha 5\text{IA}$ (30 nM) on these waveforms was compared with that of treating slices with vehicle (0.1% DMSO), before and after the induction of LTP using a blinded protocol.

Elevated plus maze. The elevated plus maze assay is an unconditioned, ethologically based animal model of fear and has been shown to be sensitive to a variety of agonists and inverse agonists acting at the BZ site of the GABA_A receptor (Pellow and File, 1986). Five groups (n=18/group) of male Sprague Dawley rats (250-300g; B&K Universal) were given either vehicle (0.5% methyl cellulose p.o.; dose volume = 1 mL/kg), one of three doses of α 5IA (1, 3 or 10 mg/kg p.o.), or, as a positive control and for comparison purposes, the non-selective partial inverse agonist FG 7142 (30 mg/kg i.p. in a 70% polyethylene glycol vehicle). Thirty min later rats were placed on the elevated plus maze for 5 min. A video camera fitted with a polarising lens was mounted above the plus maze, connected to a television monitor and the rat's movement was tracked and analysed using a VP118 tracking unit (HVS Image Ltd., UK). The open and closed arms (each 10 * 50 cm) and the central area (10 * 10 cm) of the plus maze were defined in the tracking system. The time spent in the closed arms of the maze and the total distance travelled during the 5 min. trial were calculated using Flexible Maze Software (HVS Image Ltd., UK). Following completion of the 5 min. test period, a subpopulation of rats (n=7-8/group) were taken and the occupancy of α 5IA determined using the in vivo binding of [³H]flumazenil as described above.

Mouse rotarod. The rotarod consists of a 4 cm diameter rod rotating at a fixed speed of 16 revolutions per minute (Ugo Basile model 7600, Comerio, Italy), which can be utilized to assess motor performance. Male BTKO mice (22-26g; B&K Universal) mice were trained to walk on the rotarod until they could complete three consecutive 120 sec. sessions without falling off. Mice (n=8/group) were then given p.o. either vehicle (0.5% methylcellulose), or various doses of either α 5IA (0.3 - 10 mg/kg), or diazepam (0.3-30 mg/kg), 30 min before

being placed on the rotarod. The latency to fall from the rotarod during a 120 sec. trial was then recorded. If the mouse did not fall from the rotarod during the trial, the latency was recorded as 120 sec. Immediately following completion of their rotarod trial, mice were taken and occupancy of α 5IA determined as described above.

Proconvulsant liability. Male Swiss Webster mice (25-30g; B&K Universal) were dosed (n=12-13/group) with either vehicle 70% PEG300: 30% water; 10 mL/kg i.p.), α 5IA (1, 3 or 10 mg/kg i.p.) or FG 7142 (40 mg/kg i.p. in 0.2% Tween 80). Thirty minutes later the mice were infused with pentylenetetrazole (15 mg/mL solution, infusion rate = 0.2 mL/min) and the time taken to clonic and full tonic seizures determined. Separate groups of animals (n=6-9/group) received vehicle or drug and were used to measure the extent of α 5IA or FG 7142 occupancy using the in vivo [3 H]flumazenil binding assay.

Kindling. Kindling is the process by which repeated neuronal activation leads to a long lasting increase in transmission efficiency. The resulting hyperexcitability allows a previously ineffective or sub-threshold stimulus to provoke seizure activity and may be produced either by repeated electrical stimulation or by repeated administration of subconvulsant doses of drugs such as pentylenetetrazole or the proconvulsant β -carboline, FG 7142 (Stephens and Turski, 1993). Male CD1 mice (n=12/group weighing 25-32g at the beginning of the experiment; Charles River UK Ltd., Margate, Kent) were dosed daily (i.p., dose volume = 10 mL/kg) for a period of 19 days with drug vehicle (0.2% Tween 80 or 0.5 % methyl cellulose i.p.), α 5IA (10 mg/kg in 0.5% methyl cellulose) or FG 7142 (40 mg/kg in 0.2% Tween 80). Six mice were injected at a time and were then observed for 45 min. During the observation period, the behavior of the mice (e.g. hypolocomotion, Straub tail, slit eyes and flattened ears) was recorded in addition to the incidence of myoclonic jerks and

generalized seizures (characterized by clonic or tonic contraction of the limbs including loss of righting reflex i.e. the mice fell onto their side or back). After the 45 min. observation period, mice were returned to their home cage. One animal in the FG 7142 group experienced full tonic seizures and was killed on Day 15 and consequently on days 16-19 percentages in the FG 7142 group are expressed relative to a group size of 11 rather than 12.

Separate groups of mice (n=6/group) received a single, acute dose of either FG 7142 (40 mg/kg i.p.) or α 5IA (10 mg/kg i.p.) for various pre-treatment times after which occupancy was measured using the [3 H]flumazenil in vivo binding assay described above.

Delayed matching to position in the water maze. Over a ten day period, hooded Lister rats (300-360 g; Charles River UK Ltd.) were trained to find a submerged platform (13 cm diameter) in a 2 m diameter tank filled with opaque water and surrounded by various different visual cues. The platform position remained constant during the day but was changed from day to day (Steele and Morris, 1999) and the animal's movements were tracked using a HVS image and software system (HVS Ltd., UK). Each animal received four trials per day with each trial lasting 60 sec. If an animal failed to find the platform within this time period, it was guided to the platform by the experimenter. The animal spent 30 sec on the platform before being removed prior to its next trial began.

Following the ten day training period, the effects of α 5IA were examined on 5 to 8 successive days with drug being administered once a day. The dose-dependent effects of α 5IA were examined in two separate experiments, a first, low-dose experiment in which rats received (p.o.) vehicle (0.5% methyl cellulose) or 0.03-0.3 mg/kg α 5IA for 5 days and a second, high-dose experiment in which animals were given either vehicle or 0.3-3 mg/kg α 5IA for 8 days (this latter experiment had more inherent variability and therefore was

continued for a longer time than the initial, low-dose experiment). In both experiments, $\alpha 5IA$ was given 30 min. prior to commencement of Trial 1. In parallel with the high dose (0.3-3 mg/kg) study, separate groups of hooded Lister rats received a single dose of $\alpha 5IA$ and occupancy was determined 0.5 and 4.5 hr after drug administration (corresponding to the times of Trials 1 and 2, respectively).

In an additional experiment, and in order to confirm that the effects observed with $\alpha 5IA$ were mediated via the GABA_A receptor BZ site, the performance produced by $\alpha 5IA$ (3 mg/kg p.o. given 5 hr. prior to trial 1) was assessed on 5 separate testing days following a second injection, given 15 min prior to Trial 1, of either vehicle or the prototypic BZ site antagonist flumazenil (10 mg/kg i.p.).

During the drug testing period, the interval between Trials 1 and 2 was 4 hr. with the inter-trial delay and between Trials 2 to 3 and Trials 3 to 4 remaining at 30 sec. (Trials 3 and 4 were primarily to reinforce the rule that within any one day, the platform position remained constant). The Trial 1 latencies, which were generally in the region of 30-45 sec. and did not differ significantly between groups, indicating the lack of non-specific effects of $\alpha 5IA$. The primary measure of recall was the difference score or "savings" between Trial 1 and Trial 2. Difference scores (savings) were calculated for each animal (averaged over 5 to 8 successive testing days) and mean difference scores for each group were calculated.

Statistical Analyses. Data are presented as mean \pm SEM and comparisons between groups were made using parametric or non-parametric (Kruskal-Wallis) ANOVA followed by either Dunnett's, Student Newman-Keuls or Dunn's multiple comparison *post hoc* tests as appropriate.

Results

Binding affinity. Inhibition of [³H]flumazenil binding showed that $\alpha 5$ IA binds with equivalent subnanomolar affinity (0.6-0.9 nM) to the BZ binding site in recombinant human GABA_A receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunits in conjunction with $\beta 3$ and $\gamma 2$ subunits (Table 1). A comparable affinity was also observed for the BZ site of native rat cortex and hippocampus GABA_A receptors (0.9 and 1.2 nM; Table 1). In contrast, $\alpha 5$ IA has much lower affinity for GABA_A receptors containing either $\alpha 4$ or $\alpha 6$ subunits, the so-called diazepam insensitive GABA_A receptor subtypes.

Intrinsic efficacy. The intrinsic efficacy of $\alpha 5$ IA was measured at human or rat recombinant GABA_A receptors transiently expressed in *Xenopus laevis* oocytes using two-electrode voltage clamp recording. At $\alpha 5$ -containing GABA_A receptors, $\alpha 5$ IA attenuated the current produced by a submaximal concentration of GABA concentration (EC₂₀) and this inverse agonist efficacy (Figure 2A), -29%, was the same against the human and rat receptors (Figure 2B). This inverse agonism was completely blocked by the prototypic BZ antagonist flumazenil (1 μ M), confirming that $\alpha 5$ IA mediates this effect via the BZ site (data not shown). In contrast, $\alpha 5$ IA was found to be either a low efficacy partial inverse agonist, antagonist or very weak partial agonist at other α -subtypes (range of efficacies = -5% to +15%; Fig. 2B; Table 2).

In addition to using two-electrode voltage clamp recording from *Xenopus* oocytes, the efficacy of $\alpha 5$ IA was also measured using whole-cell patch-clamp recording from L(tk-) cells stably expressing the same human GABA_A receptor subtypes (Figure 3). For comparative purpose, the non-selective full inverse agonist DMCM and partial inverse agonist FG 7142 as well as diazepam were also evaluated. Using this assay, concentration-effect curves were

constructed and the efficacy profile of $\alpha 5$ IA at the different subtypes of stably transfected human GABA_A receptors was comparable to that seen for human and rat receptors transiently transfected in *Xenopus* oocytes (Table 3). Hence $\alpha 5$ IA had much lower efficacy at the $\alpha 1$, $\alpha 2$ and $\alpha 3$ compared to the $\alpha 5$ subtypes.

The $\alpha 5$ inverse agonism of $\alpha 5$ IA of -40% was marginally greater than that of the non-selective partial inverse agonist FG 7142 (-35%), but was lower than the $\alpha 5$ inverse agonism of DMCM (-57%). However, the most striking feature of $\alpha 5$ IA compared to FG 7142 and DMCM was its preferential $\alpha 5$ inverse agonist efficacy (Figure 3). Thus, whereas FG 7142 and DMCM have comparable inverse agonist efficacy at the different subtypes (respective ranges of -35 to -47% and -53 to -71%; Table 3), $\alpha 5$ IA had much lower efficacy at the $\alpha 1$, $\alpha 2$ and $\alpha 3$ compared to $\alpha 5$ subtypes (-18, +13, -7% and -40%, respectively).

Long-term potentiation. The physiological mechanism underlying hippocampally mediated cognitive processes may involve long-term changes in synaptic efficacy, such as long-term potentiation (LTP), (Bliss and Collingridge, 1993). We have earlier shown that non-selective BZ site agonists can suppress and inverse agonists potentiate the formation of LTP (Seabrook et al., 1997). Consequently we investigated whether $\alpha 5$ IA could potentiate LTP in mouse hippocampal slices. At low stimulus frequencies (0.033 Hz) $\alpha 5$ IA had no direct effect on fEPSP slope ($105 \pm 6\%$, $P=0.47$), amplitude ($103 \pm 6\%$, $P=0.50$) or decay ($98 \pm 4\%$, $P=0.33$). However, LTP was significantly increased from $220 \pm 25\%$ in control slices to $340 \pm 47\%$ in the presence of $\alpha 5$ IA ($P=0.05$, Fig. 4A). This ability of $\alpha 5$ IA to augment LTP induction was occluded in disinhibited slices (data not shown). As with non-selective inverse agonists (Seabrook et al., 1997), these $\alpha 5$ IA-mediated changes in synaptic plasticity were associated with an enhancement of post-tetanic potentiation measured 1.5 min after the

application of the brief, high-frequency stimulus from $190 \pm 15\%$ in control slices to $245 \pm 19\%$ in the presence of $\alpha 5IA$. The increase in synaptic plasticity was not associated with the appearance of paroxysmal burst discharges (Fig. 4B).

Elevated plus maze. An ANOVA showed a significant effect of treatment on the time rats spent in the closed arms of the elevated plus maze ($F(4,84) = 4.85$, $p=0.002$). The non-selective partial inverse agonist FG 7142 significantly increased the amount of time spent in the closed arms of the maze suggesting an anxiogenic-like effect ($p<0.01$) whereas $\alpha 5IA$ had no effect on the time spent in the closed arms (Fig. 5A). In addition, FG 7142 reduced the total distance moved in this assay whereas again $\alpha 5IA$ had no effect (data not shown), suggesting that $\alpha 5IA$ does not affect spontaneous locomotor activity.

The occupancy of the BZ site of Sprague-Dawley rat brain GABA_A receptors by $\alpha 5IA$ was dose-dependent (Figure 5B) with doses of 1, 3 and 10 mg/kg giving occupancies of 32, 54 and 79%, respectively and an estimated ID₅₀ of 2.4 mg/kg. In comparison, FG 7142 (30 mg/kg i.p.) occupied 66% of BZ binding sites.

Motor impairment. Non-selective BZ receptor agonists induce sedation and motor impairment and this can be demonstrated in mice using a 'rotarod'. In this experiment, there was a significant effect of treatment with, more specifically, diazepam dose-dependently reducing the latency to fall from the rod such that at a dose of 3 mg/kg mice remained on the rotarod for 72 ± 11 sec. before falling ($p< 0.05$) and at 30 mg/kg the time spent on the rotarod was only 7 ± 3 sec. ($p<0.001$; Fig. 6A) On the other hand, $\alpha 5IA$ was without any effect (Fig. 6A), even at a dose of 10 mg/kg.

When occupancy is taken into account (Figure 6B), α 5IA did not impair performance even at a dose (10 mg/kg) that produced 95% occupancy. On the other hand, diazepam produced a significant impairment at a dose (3 mg/kg) corresponding to 52% occupancy. Moreover, at 86% occupancy (30 mg/kg), diazepam produced a profound impairment which was in marked contrast to α 5IA which at similar levels of occupancy did not alter rotarod performance.

Proconvulsant activity. When dosed alone, α 5IA did not induce convulsions in mice at doses (10 mg/kg i.p.). Similarly, α 5IA did not alter the threshold for PTZ-induced clonic or tonic seizures (Figure 7A,B), suggesting it did not have proconvulsant activity. In contrast, when FG 7142 was administered to mice it reduced the dose of PTZ required to produce clonic and tonic convulsions from 36.3 to 25.1 mg/kg and from 53.9 to 38.2 mg/kg, respectively.

The lack of effect of α 5IA was not due to a lack of occupancy since a dose of 10 mg/kg corresponded to an occupancy of 98% (Figure 7C). In contrast, FG 7142 (40 mg/kg) produced much lower levels of occupancy but despite this still possessed a robust proconvulsant activity.

Kindling. Although on days 1 and 2, FG 7142 (40 mg/kg i.p.) produced no overt effects, upon more repeated administration animals gradually developed kindled seizures with increasing severity as the experiment progressed. Thus, by days 10-14, around 40% of mice displayed clonic convulsions (Fig. 7D), the incidence of which decreased after day 14 as mice converted to tonic seizure activity (Fig. 7E). Throughout the experiment, animals treated with vehicle or α 5IA showed no incidence of any type of convulsions (Fig. 7D,E).

Pharmacodynamic experiments not only confirmed that the occupancy of $\alpha 5$ IA was much greater than that of FG 7142 30 min after dosing (respective occupancies of 94 and 51%) but also demonstrated that occupancy of FG 7142 was short-lived (Fig. 7F) in comparison to $\alpha 5$ IA which gave sustained and high receptor occupancy, such that occupancy 8 hr. after dosing was 50%.

Water maze. In the water maze delayed-matching-to-position task, the increase in performance of vehicle-treated rats in Trial 2 compared to Trial 1 was similar in the two separate experiments (savings of 10.3 ± 2.7 and 10.4 ± 2.2 sec. in the low-dose and high-dose experiments, respectively, relative to a Trial 1 latency of around 35 sec.; Fig. 8A). Whilst one-way ANOVA showed a significant effect of drug on the savings time in both experiments, more specific analyses showed that the savings time in $\alpha 5$ IA-treated animals was significantly greater than vehicle at doses of 0.3, 1 and 3 mg/kg ($p < 0.05$), with the improvement in performance at 0.3 mg/kg being comparable in the separate low- and high-dose experiments (savings = 19.7 ± 2.6 and 21.1 ± 1.8 sec., respectively; $p < 0.05$). There was no significant difference between drug groups in the time taken to locate the platform on Trial 1 and neither did the swim speed nor path length differ between the vehicle- and $\alpha 5$ IA-treated rats (data not shown).

Occupancy of rat brain BZ binding sites by $\alpha 5$ IA was measured in separate groups of animals. These data (Figure 8B) showed that $\alpha 5$ IA occupancy was dose-dependent but dose-for-dose was comparable at 0.5 and 4.5 hr after administration (times that correspond to Trial 1 and Trial 2, respectively). Hence, the ID_{50} values at times of 0.5 and 4.5 hr were 1.1 and 1.2 mg/kg with the minimal effective dose of 0.3 mg/kg corresponding to an occupancy of 22-25%.

An additional experiment was performed in which the prototypic BZ antagonist flumazenil was given 15 min. before Trial 1 (10 mg/kg i.p.) in vehicle- and $\alpha 5$ IA (3 mg/kg p.o.)-treated rats. In this experiment, $\alpha 5$ IA again enhanced performance in the delayed-matching to position water maze, and this effect was blocked by flumazenil (Fig. 8C). Analysis of the mean savings between Trial 1 and Trial 2 on the factors of drug (vehicle or $\alpha 5$ IA) and antagonist (vehicle or flumazenil) showed a significant drug x antagonist interaction ($F(1,34) = 4.35$, $p = 0.04$). This was followed up with simple main effects tests which showed a significant increase in savings by rats treated with $\alpha 5$ IA and vehicle, compared to vehicle-vehicle treated rats ($F(1,34) = 9.02$, $p < 0.01$). Rats treated with $\alpha 5$ IA and vehicle, showed significantly greater savings compared to $\alpha 5$ IA-treated rats who received flumazenil as the antagonist ($F(1,34) = 4.88$, $p < 0.05$).

Discussion

Comparison of efficacy- versus binding-selective subtype selective compounds. The use of compounds which selectively interact with the $\alpha 5$ subtype should help define the functions of this receptor subtype, especially the possibility that an $\alpha 5$ -selective inverse agonist might enhance cognition (Maubach, 2003). In this regard, structurally-related imidazobenzodiazepines have been described which bind with higher affinity for the $\alpha 5$ - compared to $\alpha 1$ -, $\alpha 2$ - and $\alpha 3$ -containing subtypes. Such “binding-selective” compounds, for example L-655,708 (FG 8094), RY-023 and RY-024 have inverse agonist efficacy at the $\alpha 5$ subtype (Liu et al., 1995, 1996; Quirk et al., 1996; Kelly et al., 2002) and are therefore presumed to exert their in vivo actions via this receptor population. However, the efficacy of these compounds at the $\alpha 1$, $\alpha 2$ and $\alpha 3$ subtypes have not been systematically examined. In the

absence of such data, it is clearly not possible to attribute the *in vivo* effects of $\alpha 5$ binding selective compounds, such as the effects of RY-010 and -024 on contextual memory or fear-related behaviour (DeLorey et al., 2001; Bailey et al., 2002), or the convulsant activity of RY-024, -024 and -080 (Liu et al., 1996), solely to the $\alpha 5$ -containing GABA_A receptors and at the least highlight the need to characterize the intrinsic efficacy of such compounds at the $\alpha 1$, $\alpha 2$, $\alpha 3$ as well as $\alpha 5$ subtypes.

In vitro properties of $\alpha 5$ IA. Given the potential drawbacks of $\alpha 5$ binding selective drugs (see above), we identified $\alpha 5$ IA as an $\alpha 5$ “efficacy selective” compound (Sternfeld et al., 2004). Although $\alpha 5$ IA binds with equivalent subnanomolar affinity to the $\alpha 1$ - $\alpha 2$ -, $\alpha 3$ - and $\alpha 5$ -subtypes, it has negligible activity (<50% activity at 10 μ M) in 127 other receptor and enzymes assays (MDS Pharma Services, data not shown).

$\alpha 5$ IA demonstrated essentially the same, $\alpha 5$ -selective inverse agonist profile against rat and human GABA_A receptors transiently expressed in oocytes (Figure 2) and the efficacy profile observed in oocytes was also observed with stably expressed human receptors (Figures 2 and 3). Hence, $\alpha 5$ IA has inverse agonism at the $\alpha 5$ subtype of -29% and -40% (at GABA_A receptors expressed in oocyte and L(tk⁻) cells, respectively), the latter of which lies between the efficacy of FG 7142 and DMCM (-35% and -57%) when tested against GABA_A receptors expressed in a comparable system (i.e. L(tk⁻) cells). There is a modest degree of inverse agonism (-4% to -18%, depending on expression system) that could produce *in vivo* effects, especially given the greater abundance of the $\alpha 1$ compared to $\alpha 5$ subtype. However, the $\alpha 1$ subtype mediates, at least in part, the proconvulsant effects of PTZ (Rudolph et al., 1999) yet $\alpha 5$ IA was clearly not proconvulsant (Figure 6) suggesting that the weak $\alpha 1$ inverse agonism does not manifest itself *in vivo*. Similarly the very weak efficacy at the $\alpha 2$ and $\alpha 3$ subtypes (-7% to +15%) did not appear to have an effect *in vivo* since these subtypes are associated with

anxiolytic-like activity (Atack et al., 2005b) yet $\alpha 5$ IA had no obvious effect on anxiety as measured using the rat elevated plus maze (Figure 5). Overall, the most parsimonious explanation for the in vivo effects of $\alpha 5$ IA is that they are mediated primarily via the $\alpha 5$ subtype.

In the mouse hippocampal slice model, $\alpha 5$ IA robustly enhanced LTP (Fig. 4A). Since non-selective inverse agonists such as DMCM also produce an increase in LTP (Seabrook et al., 1997), it is tempting to conclude that the $\alpha 5$ subtype is solely responsible for the enhanced LTP observed with non-selective compounds. However, although $\alpha 5$ -containing receptors are enriched within the hippocampus, they are still outnumbered by the combined population of $\alpha 1$ -, $\alpha 2$ and $\alpha 3$ -containing GABA_A receptors which non-selective inverse agonists will also affect.

It is important to emphasize that whilst the $\alpha 5$ subtype may indeed play a significant role in the *pharmacological* enhancement of LTP, it may play a lesser role in *physiological* LTP. Hence, in $\alpha 5^{-/-}$ mice, LTP was not significantly affected whether induced by a brief tetanus followed by a theta burst or a theta burst alone (Collinson et al., 2002). On the other hand, the ability of paired-pulse stimuli to facilitate the amplitude of synaptic potentials was significantly enhanced in $\alpha 5^{-/-}$ mice and was specific to the CA1 region of the hippocampus, which is consistent with the distribution of the GABA_A $\alpha 5$ subunit-containing receptors in the brain (Quirk et al., 1996).

The majority of hippocampal $\alpha 5$ -containing receptors are localized extrasynaptically (Brünig et al., 2002) where they mediate tonic currents (Caraiscos et al., 2004a). Consequently, the mechanism whereby $\alpha 5$ IA enhances LTP via modulation of tonic currents remains unclear. Nevertheless, the fact that there is enhanced power and increased stability in the frequency domain of 20-80 Hz (gamma) oscillations in hippocampal slices of $\alpha 5^{-/-}$ mice

suggests that $\alpha 5$ -containing GABA_A receptors are associated with hippocampal gamma frequency network activity (Towers et al., 2004). Such temporal characteristics of network rhythms have been proposed to underlie the coordination of neuronal activity and more specifically cognitive processes (Mann and Paulsen, 2005). Therefore, it is possible that $\alpha 5$ subunit-containing GABA_A receptors might affect the dynamic response of such rhythms to changes in network drive which presumably underlie the pro-cognitive effects of $\alpha 5$ IA. Accordingly, it would be interesting to evaluate the effects of $\alpha 5$ IA on hippocampal gamma frequency oscillations.

In vivo properties of $\alpha 5$ IA. In vivo, the effects of selective disinhibition of predominantly hippocampally localized $\alpha 5$ -containing GABA_A receptors resulted in improved performance of rats in the delayed matching-to-position version of the Morris water maze, without the anxiogenic-like or convulsant properties associated with non-selective GABA_A receptor inverse agonists (summarized in Table 4), with the caveat of potential species differences between the cognition and anxiety assays (rat) and the proconvulsant, kindling and sedation assays (mice). This is consistent with the behavioral phenotype of $\alpha 5^{-/-}$ mice which do not have a convulsant or anxiogenic phenotype, showing that knocking out the $\alpha 5$ GABA_A receptor does not render mice susceptible to spontaneous seizures (Collinson et al., 2002). Furthermore, $\alpha 5^{-/-}$ mice demonstrated superior performance in the DMTP water maze task compared to wild-type controls (Collinson et al., 2002).

The absence of an overt convulsant or proconvulsant effect of $\alpha 5$ IA in mice is consistent with the lack of an increased frequency of paroxysmal burst discharges (Fig. 4B). In contrast, however, the $\alpha 5$ binding selective compound RY-080 has been described as being convulsant (Liu et al., 1996). Similarly, whereas $\alpha 5$ IA is not anxiogenic in the rats tested on

the elevated plus maze, consistent with $\alpha 5^{-/-}$ data (Collinson et al., 2002), the $\alpha 5$ binding selective compound L-655,708 is reported to be anxiogenic (Navarro et al., 2002). Moreover, the lack of anxiogenic or convulsant or proconvulsant effects of $\alpha 5$ IA is not a consequence of poor pharmacokinetics or brain penetration since we selected doses of $\alpha 5$ IA that gave high levels of receptor occupancy. The discrepancies between the in vivo effects of efficacy-selective and binding-selective compounds could be due to the fact that, as discussed above, at higher concentrations or doses an efficacy-selective compound (such as $\alpha 5$ IA) maintains its preferential modulation of $\alpha 5$ -containing receptors whereas for a binding-selective compound (for example RY-080 or L-655708), significant inverse agonism at the more abundant $\alpha 1$, $\alpha 2$ and $\alpha 3$ subtypes occurs due to appreciable occupancy at these subtypes.

The measurement of $\alpha 5$ IA receptor occupancy confirms that its lack of effect in assays of anxiety-like, proconvulsant, kindling and motor behaviours is not merely due to compound not occupying BZ binding sites. Thus, high levels of $\alpha 5$ IA occupancy were achieved without effects on the elevated plus maze (79% occupancy), PTZ and kindling assays (94-98%) and rotarod (95%) assays (Table 4). In contrast, $\alpha 5$ IA produced cognition enhancing effects at a dose (0.3 mg/kg) that corresponded to an occupancy of 25%. [3 H]Flumazenil does not selectively bind to $\alpha 5$ -containing receptors but rather has equivalent affinity for the different subtypes. However, since $\alpha 5$ IA also has comparable affinity across the different subtypes, the inhibition of [3 H]flumazenil to this combined receptor population by $\alpha 5$ IA is due to comparable inhibition of binding (i.e. occupancy) at each different subtype. Hence, $\alpha 5$ IA produced an enhancement in cognitive performance by occupying 25% of the $\alpha 5$ subtype.

The observation that $\alpha 5$ IA enhances performance in the hippocampal-dependent delayed-matching-to-place (DMTP) in the water maze (Steele and Morris, 1999), suggests that $\alpha 5$ -containing GABA_A receptors play a role in hippocampal dependent cognitive processes (Collinson et al., 2002; Crestani et al., 2002) and further supports the hypothesis

that the multiple effects of non-selective BZs are mediated via distinct GABA_A receptor populations (Rudolph and Möhler, 2004).

Although non-selective BZ-site inverse agonists enhance cognition in non-human species, they are unsuitable for use in the clinic due to their anxiogenic and proconvulsant, convulsant or kindling effects (Dorow et al., 1983). However, compared to non-selective full and partial inverse agonists, $\alpha 5$ IA has a behaviorally benign profile in rodents with no anxiogenic-like, sedative, motor impairment liabilities. Taken together, these data suggest that compounds with inverse agonist activity selective for the $\alpha 5$ subtype of GABA_A receptors may prove useful for the treatment of disorders with an associated cognitive dysfunction such as Alzheimer's disease, especially since this receptor population is relatively spared in the hippocampus of such patients (Howell et al., 2000).

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References

- Argyropoulos SV, and Nutt DJ (1999) The use of benzodiazepines in anxiety and other disorders. *Eur Neuropsychopharmacol* **9(Suppl 6)**:S391-392.
- Attack JR, Smith AJ, Emms F, and McKernan RM (1999) Regional differences in the inhibition of mouse in vivo [^3H]Ro 15-1788 binding reflect selectivity for $\alpha 1$ versus $\alpha 2$ and $\alpha 3$ subunit-containing GABA_A receptors. *Neuropsychopharmacol* **20**:255-262.
- Attack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, Cook SM, Collins I, Wafford K, McKernan RM, and Dawson GR (2005a) Anxiogenic properties of an inverse agonist selective for $\alpha 3$ subunit-containing GABA_A receptors. *Br J Pharmacol* **144**:357-366.
- Attack JR, Wafford KA, Tye SJ, Cook SM, Sohal S, Pike A, Sur C, Melillo D, Bristow L, Bromidge F, Ragan CI, Kerby J, Street L, Carling R, Castro J-L, Whiting P, Dawson GR, and McKernan RM (2005b) TPA023, an agonist selective for $\alpha 2$ - and $\alpha 3$ -containing GABA_A receptors, is a non-sedating anxiolytic in rodents and primates. *J Pharmacol Expt Therap* in press
- Bailey DJ, Tetzlaff JE, Cook JM, He X, and Helmstetter FJ (2002) Effects of hippocampal injections of a novel ligand selective for the $\alpha 5\beta 2\gamma 2$ subunits of the GABA/benzodiazepine receptor on Pavlovian conditioning. *Neurobiol Learn Mem* **78**:1-10.
- Bliss TV, and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**:31-39.
- Brünig I, Scotti E, Sidler C, and Fritschy JM (2002) Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol* **443**:43-55.
- Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford AK, MacDonald JF, and Orser BA (2004) Tonic inhibition in hippocampal pyramidal neurons is regulated by $\alpha 5$ subunit containing GABA_A receptors. *Proc Natl Acad Sci USA* **101**:3662-3667.

- Cheng YC, and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.
- Collinson N, Kuenzi F, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Attack JR, McKernan RM, Seabrook GR, Dawson, GR, Whiting PW, and Rosahl TW (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the $\alpha 5$ subunit of the GABA_A receptor. *J Neurosci* **22**:5572-5580.
- Crestani F, Keist R, Fritschy J-M, Benke D, Vogt K, Prut L, Blüthmann H, Möhler H, and Rudolph U (2002) Trace fear conditioning involves hippocampal $\alpha 5$ GABA_A receptors. *Proc Natl Acad Sci USA* **99**:8980-8985.
- DeLorey TM, Lin RC, McBrady B, He X, Cook JM, Lameh J, and Loew GH (2001) Influence of benzodiazepine binding site ligands on fear-conditioned contextual memory. *Eur J Pharmacol* **426**:45-54.
- Dorow R, Horowski R, Pascheke G, Amin M, and Braestrup C (1983) Severe anxiety induced by FG 7142, a β -carboline ligand for benzodiazepine receptors. *Lancet* **2**:98.
- Ghoneim MM, and Mewaldt SP (1990) Benzodiazepines and human memory: A review. *Anesthesiology* **72**:926-938.
- Hadingham KL, Wingrove P, Le Bourdelles B, Palmer KJ, Ragan CI, and Whiting PJ (1993) Cloning of cDNA sequences encoding human $\alpha 2$ and $\alpha 3$ γ -aminobutyric acidA receptor subunits and characterization of the benzodiazepine pharmacology of recombinant $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ -, and $\alpha 5$ -containing human γ -aminobutyric acidA receptors. *Mol Pharmacol* **43**:970-975.
- Hadingham KL, Garrett EM, Wafford KA, Bain C, Heavens RP, Sirinathsinghji DJ, Whiting PJ (1996) Cloning of cDNAs encoding the human γ -aminobutyric acid type A receptor $\alpha 6$ subunit and characterization of the pharmacology of $\alpha 6$ -containing receptors. *Mol Pharmacol* **49**:253-259.

- Haefely WE, Martin JR, Richards JG, and Schoch P (1993) The multiplicity of actions of benzodiazepine receptor ligands. *Can J Psychiatry* **38**:S102-108.
- Howell O, Attack JR, Dewar D, McKernan RM, and Sur C (2000) Density and pharmacology of $\alpha 5$ subunit-containing GABA_A receptors are preserved in hippocampus of Alzheimer's disease patients. *J Neurosci* **98**:669-675.
- Kelly MD, Smith A, Banks G, Wingrove P, Whiting PW, Attack J, Seabrook GR, and Maubach KA (2002) Role of the histidine residue at position 105 in the human $\alpha 5$ containing GABA_A receptor on the affinity and efficacy of benzodiazepine site ligands. *Br J Pharmacol* **135**:248-256.
- Liu R, Zhang P, McKernan RM, Wafford K, and Cook JM (1995) Synthesis of novel imidazobenzodiazepines selective for the $\alpha 5\beta 3\gamma 2$ (Bz5) GABA_A/benzodiazepine receptor subtype. *Med Chem Res* **5**:700-709.
- Liu R, Hu RJ, Zhang P, Skolnick P, and Cook JM (1996) Synthesis and pharmacological properties of novel 8-substituted imidazobenzodiazepines: High-affinity, selective probes for $\alpha 5$ -containing GABA_A receptors. *J Med Chem* **39**:1928-1934.
- Malizia AL, and Nutt DJ (1995) The effects of flumazenil in neuropsychiatric disorders. *Clin Neuropharmacol* **18**:215-232.
- Mann EO, and Paulsen O (2005) Mechanisms underlying gamma ('40 Hz') network oscillations in the hippocampus-a mini-review. *Prog Biophys Mol Biol* **87**:67-76.
- Maubach K (2003) GABA_A receptor subtype selective cognition enhancers. *Curr Drug Targets – CNS & Neurol Disord* **2**:233-239.
- McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Attack JR, Farrar S, Myers J, Cook G, Ferris P, Garret L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, and Whiting PJ (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA_A receptor $\alpha 1$ subtype. *Nature Neurosci* **3**:587-592.

- McNaughton N, and Morris, RGM (1991) Chlordiazepoxide, an anxiolytic benzodiazepine, impairs place navigation in rats. *Behav Brain Res* **24**:39-46.
- Navarro JF, Buron E, and Martin-Lopez M (2002) Anxiogenic-like activity of L-655,708, a selective ligand for the benzodiazepine site of GABA_A receptors which contain the alpha-5 subunit, in the elevated plus-maze test. *Prog Neuropsychopharmacol Biol Psychiat* **26**:1389-1392.
- Pellow S, and File SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in the elevated plus maze: a novel test of anxiety in the rat. *Pharmacol Biochem Behav* **24**:525-529.
- Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, and McKernan RM (1996) [³H]L-655,708, A novel ligand selective for the benzodiazepine site of GABA_A receptors which contain the α 5-subunit. *Neuropharmacol* **35**:1331-1335.
- Rudolph U, and Möhler H (2004) Analysis of GABA_A receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol* **44**:475-498.
- Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy J-M, Martin JR, Bluethmann H, and Möhler H (1999) Benzodiazepine actions mediated by specific γ -aminobutyric acidA receptor subtypes. *Nature* **401**:796-800.
- Seabrook GR, Easter A, Dawson GR, and Bowery BJ (1997) Modulation of long-term potentiation in CA1 region of mouse hippocampal brain slices by GABA_A receptor benzodiazepine site ligands. *Neuropharmacol* **36**:823-830.
- Sieghart W (1995) Structure and pharmacology of γ -aminobutyric acidA receptor subtypes. *Pharmacol Rev* **47**:181-234.
- Sieghart W, and Sperk G (2002) Subunit composition, distribution and function of GABA_A receptor subtypes. *Curr Top Med Chem* **2**:795-816.
- Simon J, Wakimoto H, Fujita N, Lalande M, and Barnard EA (2004) Analysis of the set of GABA_A receptor genes in the human genome. *J Biol Chem* **279**:41422-41435.

- Steele RJ, and Morris RG (1999) Delay-dependent impairment of a matching-to-place task with chronic and intrahippocampal infusion of the NMDA-antagonist D-AP5. *Hippocampus* **9**:118-136.
- Stephens DN, and Turski L (1993) Kindling to the benzodiazepine receptor inverse agonist, FG 7142: evidence for involvement of NMDA, but not non-NMDA, glutamatergic receptors. *Neuropharmacol* **32**:1011-1017.
- Sternfeld F, Carling RW, Jelley RA, Ladduwahetty T, Merchant KJ, Moore KW, Reeve AJ, Street LJ, O'Connor D, Sohal B, Atack JR, Cook S, Seabrook G, Wafford K, Tattersall FD, Collinson N, Dawson GR, Castro JL, and MacLeod AM. Selective, orally active GABA-A $\alpha 5$ receptor inverse agonists as cognition enhancers. *J Med Chem* **47**:2176-2179.
- Towers SK, Glovelli T, Traub RD, Engel D, Fradley R, Rosahl TW, Maubach KA, Buhl EH, and Whittington MA. (2004) $\alpha 5$ subunit containing GABA_A receptors affect the dynamic range of hippocampal gamma frequency oscillations *in vitro*. *J Physiol* **559**:721-728.
- Williams TJ, and Bowie PE (1999) Midazolam sedation to produce complete amnesia for bronchoscopy: 2 years's experience at a district general hospital. *Respir Med* **93**:361-365.

Figure Legends

Figure 1. Structure of $\alpha 5$ IA (3-(5-Methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-*a*]phthalazine; Sternfeld et al., 2004)

Figure 2. Efficacy of $\alpha 5$ IA at recombinant human and rat GABA_A receptors expressed in *Xenopus laevis* oocytes. **A.** Representative whole cell membrane current recording from a *Xenopus* oocyte transfected with human $\alpha 5\beta 3\gamma 2$ receptors using two-electrode voltage clamp. A submaximally effective concentration of GABA (equivalent to an EC₂₀) was repetitively applied in the absence and then presence of $\alpha 5$ IA during which the efficacy of GABA was reduced by $-29 \pm 3\%$. **B.** The inverse agonism of $\alpha 5$ IA was selective for the $\alpha 5$ subtype. On oocytes transfected with $\alpha 1$, $\alpha 2$, or $\alpha 3$ plus $\beta 3\gamma 2$ subunits, $\alpha 5$ IA (30 nM) acted either as a BZ-site antagonist at the $\alpha 1$ subtype (-4 to -5%) or very weak partial agonist at the $\alpha 2$ and $\alpha 3$ subtypes ($+4$ to $+15\%$). Data shown are mean \pm SEM of efficacy in 4-7 different oocytes. N.D. = not determined.

Figure 3. Efficacy of $\alpha 5$ IA compared to the non-selective full inverse agonist DMCM, non-selective partial inverse agonist FG 7142 and non-selective full agonist diazepam. Human recombinant GABA_A receptors were stably expressed in mouse fibroblast L(tk⁻) cells and the modulation of a GABA concentration equivalent to an EC₂₀ was measured using whole-cell patch clamping. Positive values in this assay indicate potentiation of the GABA-evoked current (i.e. agonism) whereas negative values reflect a reduction in the GABA EC₂₀ current (inverse agonism). Values shown are mean \pm SEM (n=4-9 per data point).

Figure 4. $\alpha 5$ IA enhances LTP but has no effect on paroxysmal discharges recorded in the CA1 region of hippocampal brain slices. **A.** LTP induced by a brief tetanus (10 stimulus at 100Hz) and subsequent theta-burst stimulus (4 stimuli at 100 Hz repeated ten times every 20 msec) was significantly potentiated by $\alpha 5$ IA (30 nM; applied continuously from t=10 min.). LTP measured 1 hr after the theta-burst was significantly increased from $220 \pm 25\%$ in vehicle-treated slices to $340 \pm 47\%$ in the presence of $\alpha 5$ IA; $F = 4.29$ with 1.27 degrees of freedom using two way analysis of variance with repeated measures, $P < 0.05$. Values shown are mean \pm SEM (n=15/group). **B.** Histogram comparing the number of positive transients in response to a single stimulus (at 0.033 Hz) before vehicle or $\alpha 5$ IA treatment (control; t=1-10 min.), after treatment (pre-stimulus; t=11-20 min.), and after a theta burst stimulus (post-stimulus; t=85-95 min.). Values shown are mean \pm SEM (n=15/group).

Figure 5. $\alpha 5$ IA does not have anxiogenic-like activity in the elevated plus maze. **A.** FG 7142 (30 mg/kg i.p.), but not $\alpha 5$ IA (1–10 mg/kg p.o.) significantly increased the time spent by rats in the closed arms. Values shown are mean \pm SEM (n=17-18/group). ** = $p < 0.01$ versus vehicle control. **B.** The inhibition of the in vivo binding of [3 H]flumazenil by $\alpha 5$ IA (i.e. $\alpha 5$ IA occupancy) was dose dependent. Values shown are mean \pm SEM (n=7-8/group).

Figure 6. Lack of effect of $\alpha 5$ IA on mouse rotarod performance. **A.** Diazepam dose-dependently and significantly impaired rotarod performance such that at a dose of 30 mg/kg p.o., mice only lasted for 7 sec before falling off. In contrast, $\alpha 5$ IA was without effect up to the highest dose tested (10 mg/kg p.o.). **B.** Following completion of the rotarod trial., animals were taken and $\alpha 5$ IA and diazepam occupancy measured. The plot of rotarod performance versus % occupancy clearly shows the lack of effect of $\alpha 5$ IA even at high levels of occupancy (95%) whereas at lower levels of occupancy, diazepam produced a marked impairment. Values shown are mean \pm SEM (n=8/group). * and ***, $p < 0.05$ and 0.001, respectively, Kruskal-Wallis non-parametric one-way ANOVA followed by Dunn's multiple comparison test.

Figure 7: $\alpha 5$ IA was not proconvulsant nor did it produce kindling. **A-C.** Pretreatment of mice for 30 min. with $\alpha 5$ IA (1-10 mg/kg i.p.) did not reduce the dose of pentylenetetrazol (PTZ, i.v. infusion) required to induce either **A.** clonic or **B.** tonic convulsions. Values shown are mean \pm SEM (n=12-13/group). **C.** Satellite groups of mice received $\alpha 5$ IA or FG 7142 and occupancy was measured 30 min. after dosing. Values are mean \pm SEM (n=6-9/group). **D-F.** The potential for subthreshold doses of $\alpha 5$ IA (10 mg/kg i.p. in 0.5% Methocel) and FG 7142 (40 mg/kg i.p. in 0.2% Tween 80 i.p.) to produce **D.** clonic or **E.** tonic convulsions upon chronic dosing (i.e. kindling) was evaluated in mice. Animals (n=12/group) received either vehicle (0.2% Tween 80 or 0.5% Methocel), FG 7142 or $\alpha 5$ IA once a day for 19 days and the percentage of animals demonstrating clonic or tonic convulsions was scored during a 45 min observation period after injection. Kindling was observed in animals chronically treated with FG 7142 but not $\alpha 5$ IA. **F.** Duration of occupancy of $\alpha 5$ IA (10 mg/kg i.p.) in mice compared to the non-selective partial inverse agonist FG 7142 (40 mg/kg i.p.). Maximal occupancy with $\alpha 5$ IA (94%) occurred after 1 hr and was sustained such that at 8 hr, 50% occupancy remained. In contrast, the maximum occupancy of FG 7142 was much less (51%) and short-lived. Values shown are mean \pm SEM (n=6/group).

Figure 8. $\alpha 5$ IA enhances performance in the delayed-matching-to-position version of the Morris water maze. **A.** In two separate experiments, $\alpha 5$ IA produced a significant enhancement in performance in the delayed-matching to position water maze as measured by the increase in the difference in time, averaged over 5-8 test days, between finding the hidden platform location in Trial 2 compared to Trial 1 (inter-trial interval = 4 hr). In the low-dose experiment, $\alpha 5$ IA had a minimal effective dose of 0.3 mg/kg whereas in the high-dose experiment, the compound was effective at all 3 doses tested (0.3, 1 and 3 mg/kg). * $p < 0.05$ vs. vehicle-treated animals. **B.** The occupancy of rat brain BZ sites by $\alpha 5$ IA was measured 0.5 and 4.5 hr post-dosing (corresponding to the times of Trials 1 and 2, respectively). Occupancy was dose-dependent but was not appreciably different at these time points. **C.** The ability of $\alpha 5$ IA (3 mg/kg p.o.) to increase the mean savings was prevented by the non-selective BZ antagonist, flumazenil (10 mg/kg i.p.) given 15 min. prior to trial 1, indicating that the effects of $\alpha 5$ IA are mediated via the BZ site of GABA_A receptors (presumably of the $\alpha 5$ subtype). Values shown are mean \pm SEM (n=9-10 group) ** $p < 0.01$ compared to vehicle-vehicle group; + $p < 0.05$ compared to $\alpha 5$ IA-vehicle group.

Table 1: Affinity of $\alpha 5$ IA for human recombinant and native rat brain GABA_A receptors

Ki, nM							
Human recombinant GABA _A receptors containing $\beta 3$, $\gamma 2$ plus						Native rat brain receptor	
$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	Cortex	Hippocampus
0.88 ± 0.19	0.58 ± 0.17	0.61 ± 0.17	60 ± 7	0.66 ± 0.14	418 ± 72	0.90 ± 0.07	1.2 ± 0.2

Affinity at $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and $\alpha 5$ -containing recombinant as well as native rat brain GABA_A receptor were measured using a [³H]flumazenil binding assay whereas affinity for receptors containing either an $\alpha 4$ or $\alpha 6$ subunit was measured using [³H]Ro 15-4513

Data shown are the mean \pm SEM (n=3-6 separate determinations)

Table 2: Modulation of the GABA EC₂₀-evoked current in different subtypes of recombinant human and rat GABA_A receptors

Species	Expression system	Recombinant GABA _A receptors containing β3, γ2 plus			
		α1	α2	α3	α5
Human	<i>Xenopus</i> oocytes	-4 ± 2% ^a	+12 ± 3% ^b	+4 ± 4%	-29 ± 3%
Rat	<i>Xenopus</i> oocytes	-5 ± 1%	N.D.	+15 ± 3%	-29 ± 5%
Human	L(tk-) cells	-18 ± 3%	+13 ± 3%	-7 ± 4%	-40 ± 1%

^{a,b}, negative and positive values represent an attenuation or potentiation, respectively, of the current produced by an EC₂₀-equivalent concentration of GABA.

N.D. = not determined. Values shown are mean ± SEM of 4-7 separate determinations.

Table 3: Comparison of the intrinsic efficacy profiles of $\alpha 5$ IA, DMCM, FG 7142 and diazepam

		Human recombinant GABA _A receptors containing $\beta 3$, $\gamma 2$ plus			
		$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$
$\alpha 5$ IA	% modulation ^a	-18 \pm 3%	+13 \pm 3%	-7 \pm 4%	-40 \pm 1%
	EC ₅₀ , nM ^b	N.C.	N.C.	N.C.	2.2 \pm 0.2
DMCM	% modulation	-71 \pm 1%	-53 \pm 3%	-62 \pm 2%	-57 \pm 1%
	EC ₅₀ , nM	3.9	3.7	2.4	1.2
FG 7142	% modulation	-47 \pm 2%	-38 \pm 6%	-40 \pm 5%	-35 \pm 4%
	EC ₅₀ , nM	137	5.7	1020	1439
Diazepam	% modulation	103 \pm 14%	135 \pm 11%	118 \pm 28%	106 \pm 3%
	EC ₅₀ , nM	27	19	48	17

^a, figures for % modulation are the maximum effects of compound on the current produced by an EC₂₀-equivalent concentration of GABA with negative and positive values representing an attenuation (inverse agonism) or potentiation (agonism), respectively.

^b, The EC₅₀ represents the concentration at which compound produces an effect that is 50% of the maximum at that particular subtype.

N.C. = not calculated due to small responses producing poorly-defined concentration-effect curves.

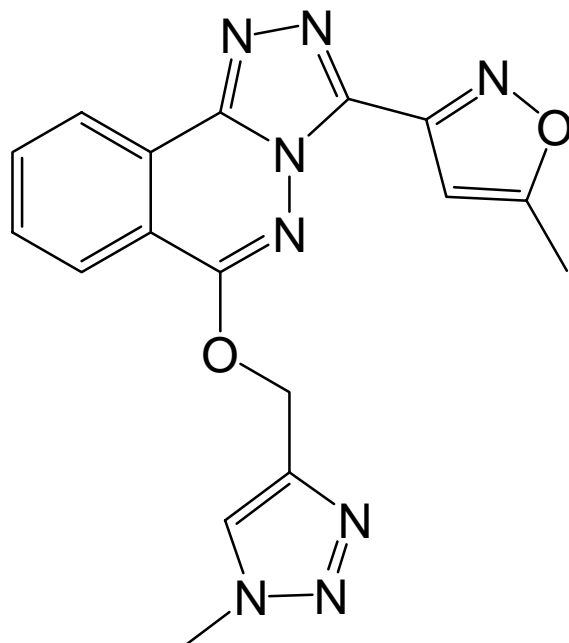
Values shown are mean \pm SEM of 4-9 separate determinations.

Table 4: Summary of in vivo properties of α 5IA

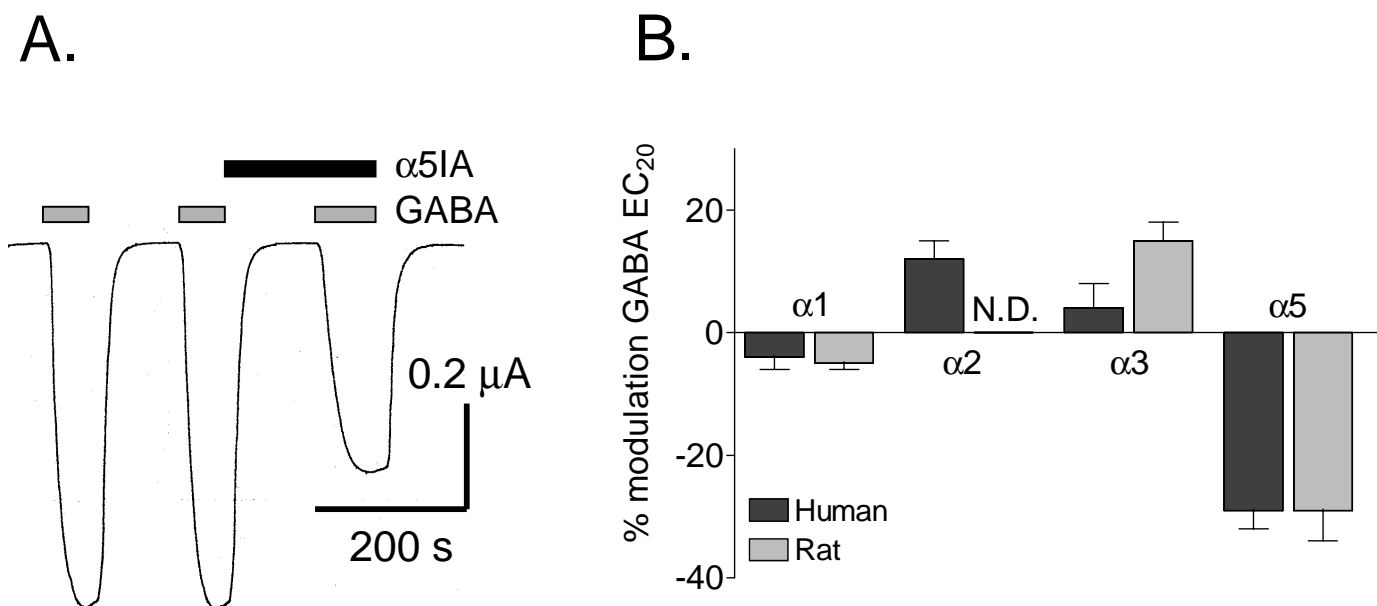
Behaviour	Drug	Assay	Observation	Route	Dose, mg/kg	Occupancy
Cognition	α 5IA	Rat Morris water maze	Enhanced performance	p.o.	0.3	25%
Anxiety	α 5IA	Rat Elevated plus maze	Not anxiogenic	p.o.	10	79%
	FG 7142		Anxiogenic	i.p.	30	66%
Sedation	α 5IA	Mouse rotarod	No effect	p.o.	10	95%
	Diazepam		Motor impairment	p.o.	3	52%
Proconvulsant liability	α 5IA	Mouse PTZ seizure threshold	No effect	i.p.	10	98%
	FG 7142		Proconvulsant	i.p.	40	55%
Sensitization to seizures (kindling)	α 5IA	Mouse seizure activity	No effect	i.p.	10	94% ^a
	FG 7142		Development of clonic/ tonic seizures	i.p.	40	51% ^a

^a, figures refer to maximum occupancy (t_{\max} = 1 hr and 0.5 hr for α 5IA and FG 7142, respectively)

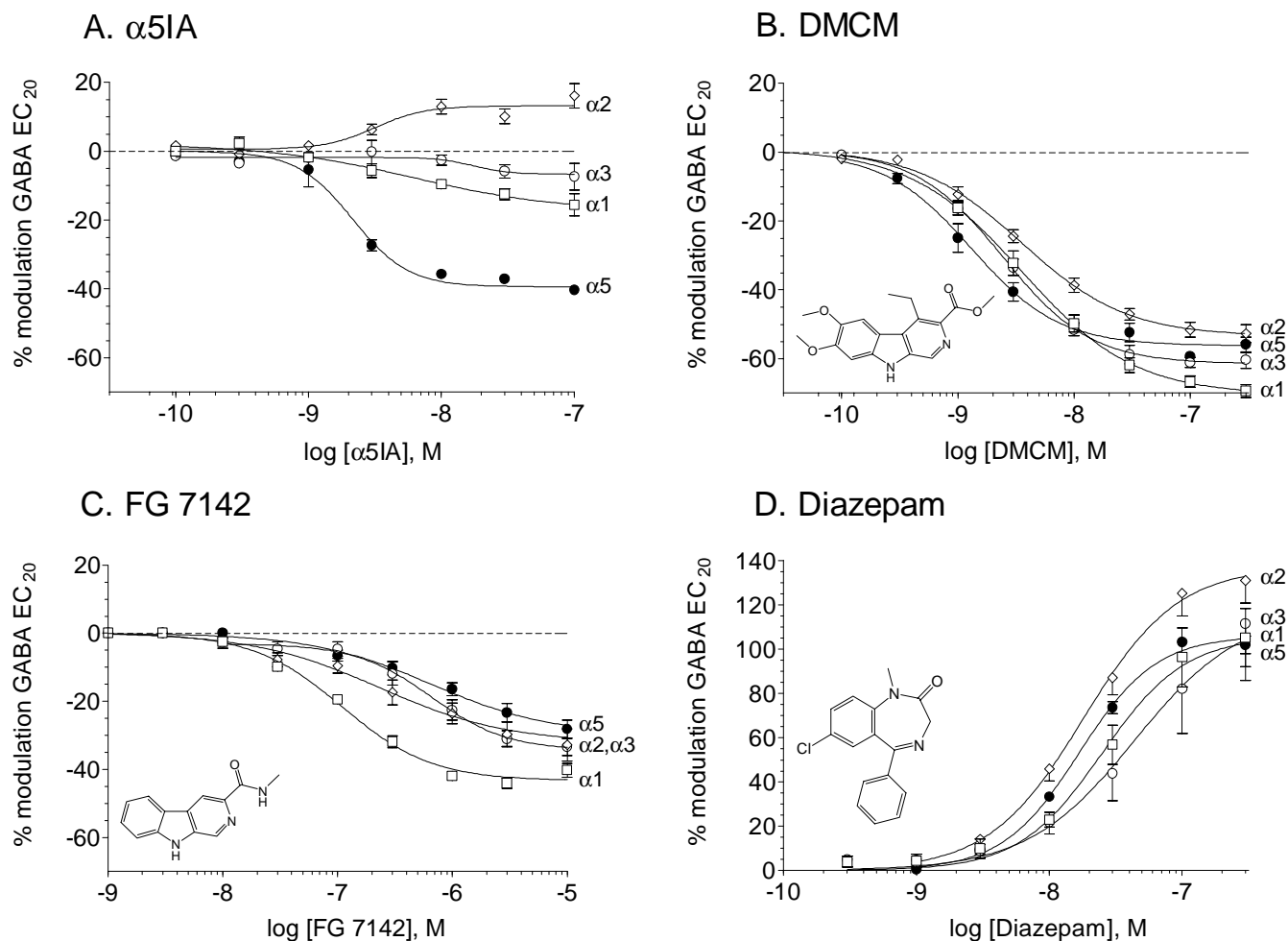
JPET # 92320 Figure 1



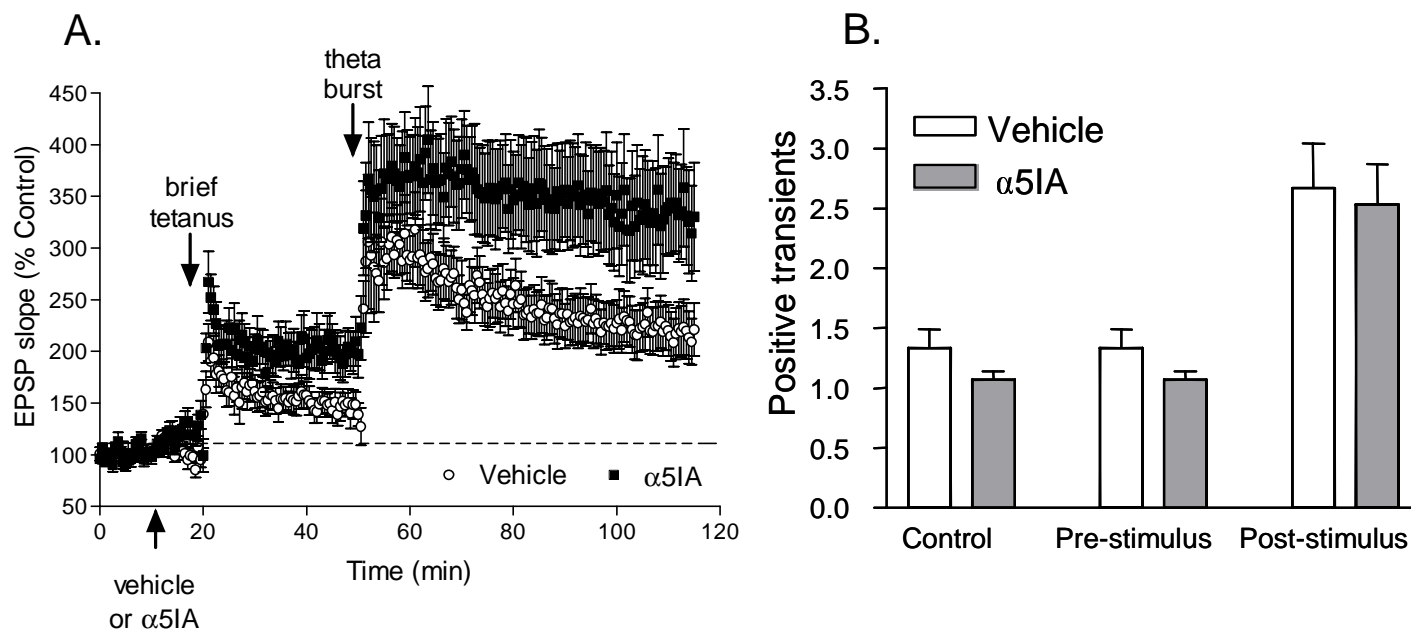
JPET # 92320 Figure 2



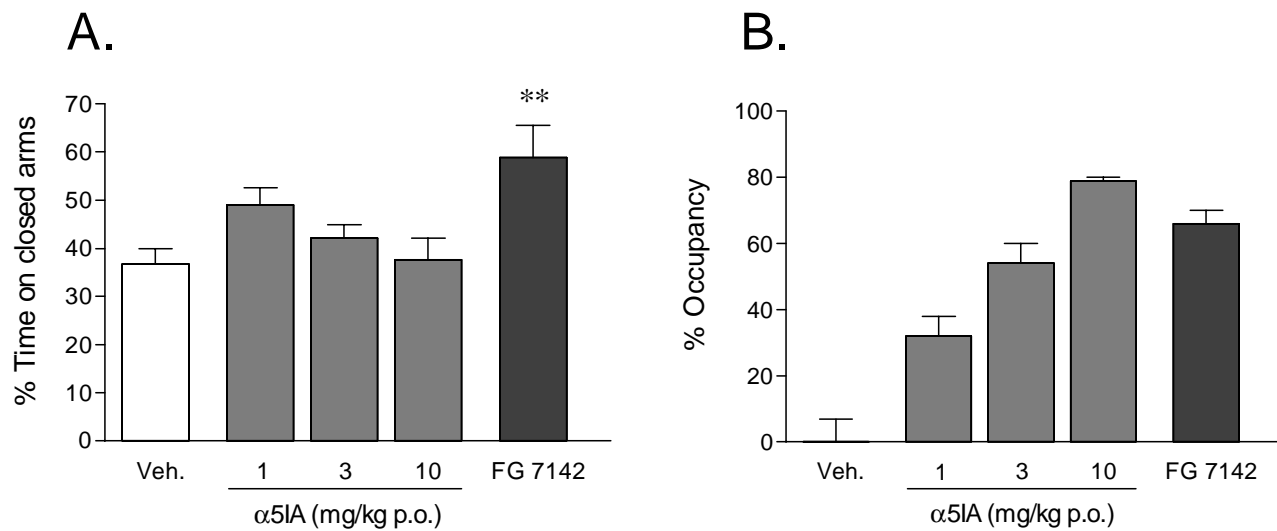
JPET # 92320 Figure 3



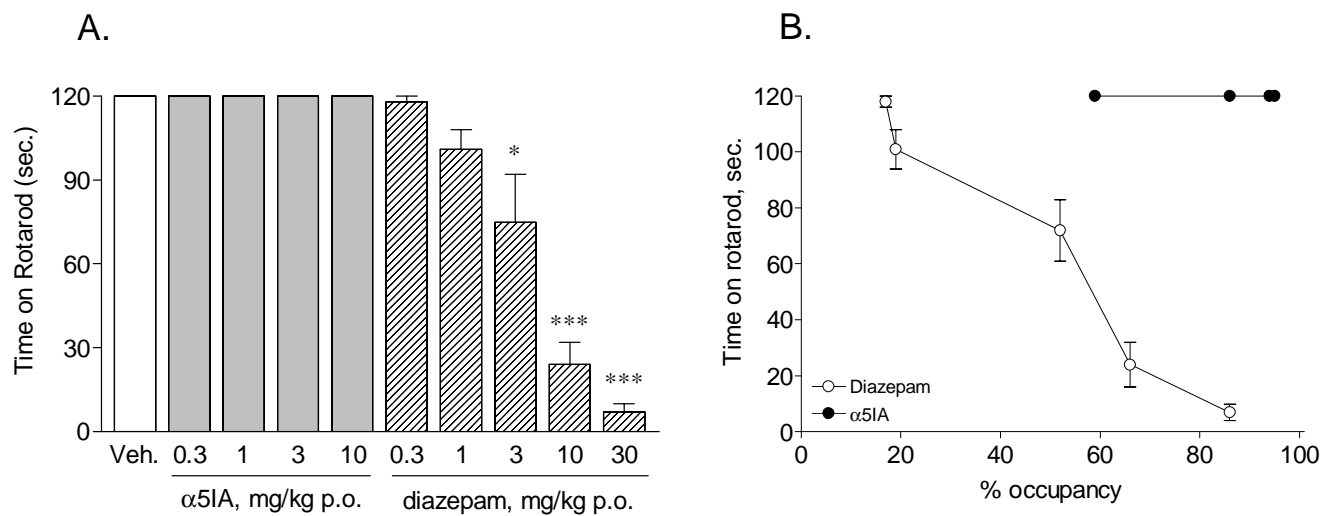
JPET # 92320 Figure 4



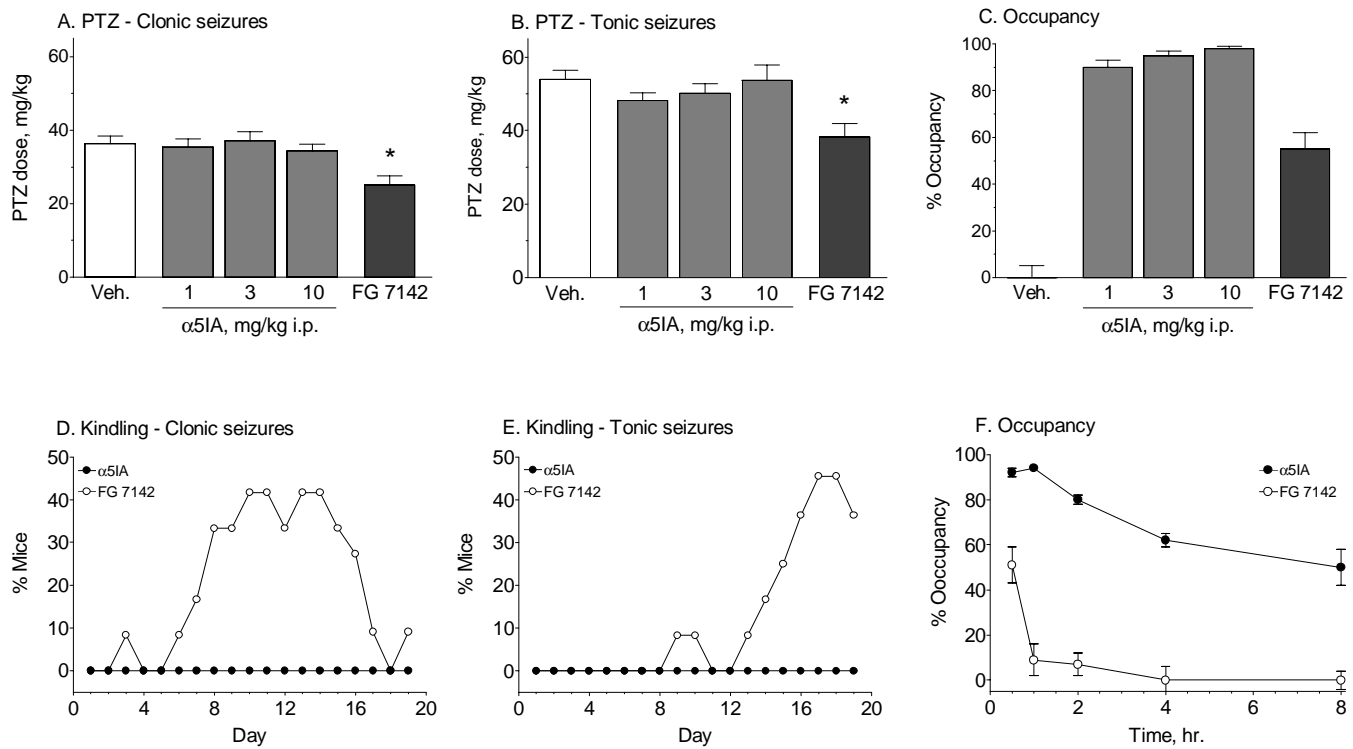
JPET # 92320 Figure 5



JPET # 92320 Figure 6



JPET # 92320 Figure 7



JPET # 92320 Figure 8

