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An Inverse Agonist Selective for α5 Subunit-Containing $GABA_A$ Receptors Enhances Cognition

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ABSTRACT

α5IA is a compound that binds with equivalent subnanomolar affinity to the benzodiazepine (BZ) site of $GABA_A$ receptors containing an α1, α2, α3, or α5 subunit but has inverse agonist efficacy selective for the α5 subtype. As a consequence, the in vitro and in vivo effects of this compound are mediated primarily via $GABA_A$ receptors containing an α5 subunit. In a mouse hippocampal slice model, α5IA significantly enhanced the θ burst-induced long-term potentiation of the excitatory postsynaptic potential 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 α5IA may enhance cognition without being proconvulsant were confirmed in in vivo rodent models. Hence, α5IA 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 α5IA 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, α5IA was not anxiogenic-like in the rat elevated plus maze nor did it impair performance in the mouse rotarod assay. Together, these data suggest that the $GABA_A$ α5-subtype provides a novel target for the development of selective inverse agonists with utility in the treatment of disorders associated with a cognitive deficit.

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 more than 40 years (Argyropoulos and Nutt, 1999). In addition, they have therapeutic utility in inducing not only sedation and muscle relaxation but also amnesia before surgical procedures (Williams and Bowie, 1999). Although the amnesic effects of BZ agonists in animals and humans have been known for some time (Ghoneim and Mewaldt, 1990), the precise nature of the processes underlying these effects are still uncertain. Since the anterograde rather than retrograde amnesia (McNaughton and Morris, 1987) produced by BZ agonists is similar to deficits induced by hippocampal lesions in animals and humans, it has been suggested that these drugs may exert their amnesic effects by modulating hippocampal function.

At present, 19 $GABA_A$ receptor subunits have been identified (α1–α6, β1–β3, γ1–γ3, δ, ε, θ, ρ1–3, and π; Simon et al.,

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ABBREVIATIONS: BZ, benzodiazepine; 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; α5IA, 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methyloxy]-1,2,4-triazolo[3,4-*a*]phthalazine; FG 7142, *N*-methyl-β-carboline-3-carboxamide; MBS, modified Barth's solution; aCSF, artificial cerebrospinal fluid; fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; ANOVA, analysis of variance; PTZ, pentylenetetrazol; L-655,708, ethyl (*S*)-[11,12,13,13*a*-tetrahydro-7-methoxy-9-oxo]-[^{9}H]-imidazo[1,5-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine-1-carboxylate; RY-010, ethyl 8-ethyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; RY-024, *t*-butyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; RY-023, *t*-butyl 8[(trimethylsilyl)ethynyl]-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; RY-080, ethyl 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate.

2004) with the majority of $GABA_A$ receptors in the brain containing 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 $\gamma2$ and either an $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ 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). Nonselective BZ agonists such as diazepam enhance the inhibitory effects of GABA at these four (i.e., $\alpha1$-, $\alpha2$-, $\alpha3$-, or $\alpha5$-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 properties 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 $\alpha1$ subunit mediate the sedative/myorelaxant effects of diazepam, whereas those with an $\alpha2$ or $\alpha3$ 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 $\alpha5$ subunit are less well defined. Nevertheless, $\alpha5$-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 $\alpha5$-containing $GABA_A$ receptors may be found at synapses (Brünig et al., 2002), and the presence of a population of $\alpha5$-containing receptors within the synapse is consistent with the observations that inhibitory postsynaptic current amplitude is decreased and the decay time is slower in mice lacking the $\alpha5$ subunit ($\alpha5^{-/-}$ mice; Collinson et al., 2002).

Whereas BZ site agonists such as diazepam increase the GABA-induced chloride flux through $GABA_A$ receptors containing an $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subunit, nonselective inverse agonists, such as the β-carbolines DMCM and FG 7142, decrease chloride flux at these same receptor subtypes, resulting in a membrane depolarization 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 behaviorally 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 nonselective inverse agonists prevents their use in humans (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 associated with 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 for $\alpha5$-containing $GABA_A$ receptors might enhance hippocampally mediated cognitive function (Maubach, 2003) and accordingly such a compound, $\alpha5$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 also is devoid of anxiogenic-like behavior 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. *N*-Methyl-β-carboline-3-carboxamide (FG 7142), methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), diazepam, and flumazenil (Ro 15-1788) were purchased from Sigma-Aldrich (Gillingham, UK), and bretazenil was a gift from F. Hoffman-La Roche (Basel, Switzerland). [^{3}H]Flumazenil ([^{3}H]Ro 15-1788; 70–87 Ci/mmol) and [^{3}H]Ro 15-4513 (20–40 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). 3-(5-Methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methyloxy]-1,2,4-triazolo[3,4-*a*]phthalazine ($\alpha5$IA) (Fig. 1) was synthesized as described previously (Sternfeld et al., 2004).

In Vitro Radioligand Binding Studies. Mouse fibroblast L(tk$^-$) cells expressing human recombinant $GABA_A$ receptors containing $\beta3$ and the $\gamma2$ short isoform in combination with various α subunits were harvested, and binding was performed as described previously (Hadingham et al., 1993, 1996). The choice of $\beta3$ rather than $\beta1$ or $\beta2$ is of little consequence to the BZ site pharmacology since this recognition site occurs at the interface of a $\gamma2$ (but not $\gamma1$ or $\gamma3$) and either an $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subunit (Sieghart, 1995). The inhibition of 1.8 nM [^{3}H]flumazenil binding by $\alpha5$IA was measured in $GABA_A$ receptors containing either an $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subunit and from the IC_{50} 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 [^{3}H]flumazenil binding of 0.92, 1.05, 0.58, and 0.45 nM at the $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subtypes. The affinity of $\alpha5$IA for $GABA_A$ receptors containing $\alpha4$ and $\alpha6$ subunits was measured using 8.0 nM [^{3}H]Ro 15-4513, and from the IC_{50} the K_i was calculated using K_D values of 4.0 and 6.5 nM for [^{3}H]Ro 15-4513 binding at $\alpha4\beta3\gamma2$ and $\alpha6\beta3\gamma2$ receptors, respectively. Nonspecific binding was defined by the inclusion of 10 μM flunitrazepam for the $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subtypes and 10 μM Ro 15-4513 for $\alpha4$ and $\alpha6$ subtypes. The percentage of inhibition of [^{3}H]flumazenil or [^{3}H]Ro 15-4513 binding and the IC_{50} and the K_i values were calculated using ActivityBase (IDBS, Guildford, Surrey, UK).

In Vivo [^{3}H]Flumazenil Binding. The occupancy of brain BZ binding sites by $\alpha5$IA or FG 7142 was assessed using a [^{3}H]flumazenil in vivo binding assay (Atack et al., 1999). In brief, animals were pretreated with compound or corresponding vehicle, and 3 min before killing, [^{3}H]flumazenil (diluted 1:150 with saline) was injected via a tail vein (injection volume 1 and 5 μl/g for rats and mice,

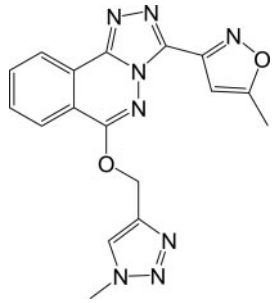

Fig. 1. Structure of $\alpha5$IA (Sternfeld et al., 2004).

respectively). Animals were killed, and the brains were rapidly removed, homogenized in 10 volumes of ice-cold buffer (10 mM phosphate buffer and 100 mM KCl, pH 7.4), and 300-μl aliquots were filtered and washed over Whatman GF/B glass fiber filters (Whatman, Maidstone, UK). 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 nonspecific binding. The extent by which α5IA 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]flumazenil by 50% (ID_{50}) were estimated using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Two-Electrode Voltage-Clamp of *Xenopus laevis* Oocytes. *X. laevis* oocytes were removed from anesthetized 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 to 20 nl of injection buffer (88 mM NaCl, 1 mM KCl, and 15 mM HEPES, pH 7.0, nitrocellulose filtered) containing rat or human α1, α2, α3, or α5 $GABA_A$ subunit cDNAs (6 ng/ml) engineered into the expression vector pCDM8 as well as cDNA for the β3 and γ2 $GABA_A$ receptor subunits. These cDNAs were injected in a 1:1:1 concentration ratio.

The oocytes were stored in an incubator until use with recordings being made 2 to 4 days after injection. Oocytes were placed in a 50-μl bath and perfused with modified Barth's solution (MBS) consisting of 88 mM NaCl, 1.0 mM KCl, 0.91 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, 0.33 mM $Ca(NO_3)_2$, and 2.4 mM $NaHCO_3$, pH 7.5 with 10 M NaOH. Cells were impaled with two 1- to 3-MΩ electrodes containing 2 M KCl and voltage-clamped at -60 mV using a GeneClamp amplifier (Axon Instruments Inc., Union City, CA). Oocytes were continuously perfused with MBS at 4 to 6 ml/min, and drugs were applied to the perfusate. For each oocyte, the expression of receptors was first checked by a 30-s application of a maximal concentration of GABA (300 μM) in MBS, and GABA currents ranged typically from 0.3 to 3 μA. Cells with currents <0.3 μA were rejected. α5IA (30 nM) was preapplied for 30 s before the addition of GABA, which was applied until the peak response was observed, usually within 30 s. To prevent desensitization, at least 3-min washing was allowed between each GABA application. Stable responses to a concentration of GABA giving a current that was 20% of the maximum (EC_{20}) were obtained for each individual oocyte (1–6 μM for α1-, α2-, and α3- and 3 to 10 μM for α5-containing $GABA_A$ receptors), and then the percentage of modulation of this response by α5IA (30 nM) was determined.

Whole Cell Patch-Clamp of L(tk$^-$) Cells. Experiments were performed on L(tk$^-$) cells stably expressing a combination of human β3 and γ2 and either α1, α2, α3, or α5 subunits (Hadingham et al., 1993). Glass coverslips 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 124 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.2, and observed using phase contrast optics. Patch pipettes were pulled with an approximate tip diameter of 2 μm and a resistance of 4 MΩ with borosilicate glass and filled with 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, and 3 mM Mg^+-ATP, with 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, Uckfield, East Sussex, UK) that enabled rapid equilibration around the cell, applied drug solutions. Increasing GABA concentrations were applied for 5-s pulses with a 30-s interval between applications. Curves were fitted using a nonlinear square-fitting program to the equation $f(x) = B_{MAX}/[1 + (EC_{50}/x)^n]$, 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 126 mM NaCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 2.5 mM KCl, 26 mM$NaHCO_3$, and 10 mM D-glucose, which was bubbled with 95% O_2 plus 5% CO_2. Parasagittal sections (350 μm) were cut on a Vibratome, with the brain being submerged in ice-cold aCSF, and subsequent recordings were performed at 33°C. The fibers of the Schaffer collateral-commissural path were stimulated with tungsten bipolar electrodes, and field excitatory postsynaptic 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 version 2.24 script language. The stimulus intensity was set to give an fEPSP with a 20% maximal slope determined from the input-output curve. Test stimuli were applied every 30 s; slopes were calculated on-line and allowed to stabilize so that baseline values varied by no more than 5% for a minimum of 30 min. Long-term potentiation (LTP) was induced by a brief tetanus (10 stimuli at 100 Hz) or a θ burst protocol (four pulses at 100 Hz repeated 10 times at an interval of 200 ms). Regression analysis plus two-way analysis of variance with repeated measures (treatment and time) were performed. The number of positive transients comprising the fEPSP was recorded following single afferent stimuli, and the effect of α5IA (30 nM) on these waveforms was compared with slices treated with vehicle (0.1% dimethyl sulfoxide), 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 = 17–18/group) of male Sprague-Dawley rats (250–300 g; B&K Universal) were given either vehicle (0.5% methyl cellulose p.o.; dose volume 1 ml/kg), one of three doses of α5IA (1, 3, or 10 mg/kg p.o.), or as a positive control and for comparison purposes, the nonselective partial inverse agonist FG 7142 (30 mg/kg i.p. in a 70% polyethylene glycol vehicle). Thirty minutes later, rats were placed on the elevated plus maze for 5 min. A videocamera fitted with a polarizing lens was mounted above the plus maze, connected to a television monitor, and the rat's movement was tracked and analyzed using a VP118 tracking unit (HVS Image Ltd., Leatherhead, Surrey, UK). The open and closed arms (each 10 $\times$ 50 cm) and the central area (10 $\times$ 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 traveled during the 5-min trial were calculated using Flexible Maze Software (HVS Image Ltd.). Following completion of the 5-min test period, a subpopulation of rats (n = 7–8/group) was taken, and the occupancy of α5IA was determined using the in vivo binding of [^{3}H]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 (model 7600; Ugo Basile, Comerio, Italy), which can be used to assess motor performance. Male BTKO mice (22–26 g; B&K Universal) were trained to walk on the rotarod until they could complete three consecutive 120-s sessions without falling off. Mice (n = 8/group) were then given p.o. either vehicle (0.5% methylcellulose) or various doses of either α5IA (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-s trial was then recorded. If the mouse did not fall from the rotarod during the trial, the latency was recorded as 120 s. Immediately following completion of their rotarod trial, mice were taken and occupancy of α5IA was determined as described above.

Proconvulsant Liability. Male Swiss-Webster mice (25–30 g; B&K Universal) were dosed (n = 12–13/group) with either vehicle 70% polyethylene glycol 300/30% water; 10 ml/kg i.p.), α5IA (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 was measured and from this the dose administered was calculated. Separate groups of animals (n = 6–9/group) received vehicle or drug and were used to measure the extent of α5IA 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 subthreshold 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 25–32 g at the beginning of the experiment; Charles River UK Ltd., Margate, Kent, UK) 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.), α5IA (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; consequently, on days 16 to 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 dose of either FG 7142 (40 mg/kg i.p.) or α5IA (10 mg/kg i.p.) for various pretreatment 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 10-day period, hooded Lister rats (300–360 g; Charles River UK Ltd.) were trained to find a submerged platform (13 cm in diameter) in a 2-m-diameter tank filled with opaque water and surrounded by various visual cues. The platform position remained constant during the day but was changed from day to day (Steele and Morris, 1999), and the movements of the animals were tracked using an HVS image and software system (HVS Image Ltd.). Each animal received four trials per day with each trial lasting 60 s. If an animal failed to find the platform within this time, it was guided to the platform by the experimenter. The animal spent 30 s on the platform before being removed prior to its next trial.

Following the 10-day training period, the effects of α5IA were examined on five to eight successive days with drug being administered once a day (n = 9–10/group). The dose-dependent effects of α5IA 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, 0.1, or 0.3 mg/kg α5IA for 5 days; and a second, high-dose experiment in which animals were given either vehicle or 0.3, 1, or 3 mg/kg α5IA 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, α5IA was given 30 min before 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 α5IA, and occupancy was determined 0.5 and 4.5 h after drug administration (corresponding to the times of trials 1 and 2, respectively).

In an additional experiment, and to confirm that the effects observed with α5IA were mediated via the $GABA_A$ receptor BZ site, the performance produced by α5IA (3 mg/kg p.o. given 5 h before trial 1) was assessed on five separate testing days (n = 9–10/group) following a second injection, given 15 min before 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 h with the internal and between trials 2 to 3 and 3 to 4 remaining at 30 s. The trial 1 latencies, which were generally 30 to 45 s, did not differ significantly between groups, indicating the lack of nonspecific effects of α5IA. 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 five to eight successive testing days), and mean difference scores for each group were calculated.

Statistical Analyses. Data are presented as mean ± S.E.M., and comparisons between groups were made using parametric or nonparametric (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 [^{3}H]flumazenil binding showed that α5IA binds with equivalent subnanomolar affinity (0.58–0.88 nM) to the BZ binding site in recombinant human $GABA_A$ receptors containing α1, α2, α3, or α5 subunits in conjunction with β3 and γ2 subunits (Table 1). A comparable affinity was also observed for the BZ site of native rat cortex and hippocampus $GABA_A$ receptors (0.90 and 1.2 nM; Table 1). In contrast, α5IA has much lower affinity for $GABA_A$ receptors containing either α4 or α6 subunits, the so-called diazepam-insensitive $GABA_A$ receptor subtypes.

Intrinsic Efficacy. The intrinsic efficacy of α5IA was measured at human or rat recombinant $GABA_A$ receptors transiently expressed in *X. laevis* oocytes using two-electrode voltage-clamp recording. At α5-containing $GABA_A$ receptors, α5IA attenuated the current produced by a submaximal concentration of GABA concentration (EC_{20}) (Fig. 2A), −29%, was the same against the human and rat receptors (Fig. 2B). This inverse agonism was completely blocked by the prototypic BZ antagonist flumazenil (1 μM), confirming that α5IA mediates this effect via the BZ site (data not shown). In contrast, α5IA 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 α5IA was also measured using whole cell patch-clamp recording from L(tk$^-$) cells stably expressing the same human $GABA_A$ receptor subtypes

TABLE 1

Affinity of α5IA for human recombinant and native rat brain $GABA_A$ receptors

Affinity at α1-, α2-, α3-, and α5-containing recombinant as well as native rat brain $GABA_A$ receptor was measured using a [^{3}H]flumazenil binding assay, whereas affinity for receptors containing either an α4 or α6 subunit was measured using [^{3}H]Ro 15-4513. Data are the mean ± S.E.M. (n = 3–6 separate determinations).

Human Recombinant $GABA_A$ Receptors Containing β3, γ2 Plus						Native Rat Brain Receptor	
α1	α2	α3	α4	α5	α6	Cortex	Hippocampus
K_i, nM							
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

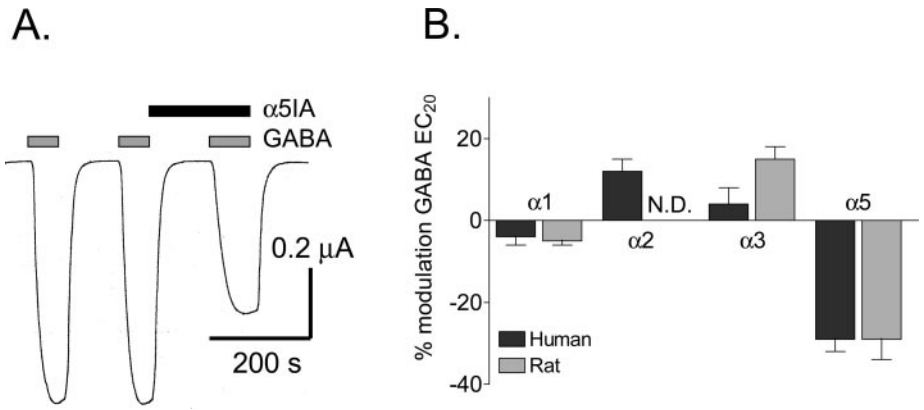

Fig. 2. Efficacy of α5IA at recombinant human and rat $GABA_A$ receptors expressed in *X. laevis* oocytes. A, representative whole cell membrane current recording from a *Xenopus* oocyte transfected with human α5β3γ2 receptors using two-electrode voltage-clamp. A submaximally effective concentration of GABA (equivalent to an EC_{20}) was repetitively applied in the absence and then presence of α5IA during which the efficacy of GABA was reduced by −29 ± 3%. B, inverse agonism of α5IA was selective for the α5 subtype of rat and human $GABA_A$ receptors. On oocytes transfected with α1, α2, or α3 plus β3γ2 subunits, α5IA (30 nM) acted either as a BZ site antagonist at the α1 subtype (−4 to −5%) or very weak partial agonist at the α2 and α3 subtypes (+4 to +15%). Data shown are mean ± S.E.M. of efficacy in four to seven oocytes. N.D., not determined.

TABLE 2

Modulation of the GABA EC_{20}-evoked current in different subtypes of recombinant human and rat $GABA_A$ receptors

Values shown are mean ± S.E.M. of four to seven separate determinations.

Species	Expression System	Recombinant $GABA_A$ Receptors Containing β3, γ2 Plus			
		α1	α2	α3	α5
		%			
Human	*Xenopus* oocytes	−4 ± 2[a]	+12 ± 3[b]	+4 ± 4	−29 ± 3
Rat	*Xenopus* oocytes	−5 ± 1	N.D.	+15 ± 3	−29 ± 5
Human	L(tk⁻) cells	−18 ± 3	+13 ± 3	−7 ± 4	−40 ± 1

N.D., not determined.

[a] Negative values represent an attenuation of the current produced by an EC_{20}-equivalent concentration of GABA.

[b] Positive values represent a potentiation of the current produced by an EC_{20}-equivalent concentration of GABA.

(Fig. 3). For comparative purpose, the nonselective 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 α5IA at the different subtypes of stably transfected human $GABA_A$ receptors was comparable with that seen for human and rat receptors transiently transfected in *Xenopus* oocytes (Table 3). Hence, α5IA had much lower efficacy at the α1, α2, and α3 compared with the α5 subtypes.

The α5 inverse agonism of α5IA of −40% was marginally higher than that of the nonselective partial inverse agonist FG 7142 (−35%), but it was lower than the α5 inverse agonism of DMCM (−57%). However, the most striking feature of α5IA compared with FG 7142 and DMCM was its preferential α5 inverse agonist efficacy (Fig. 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), α5IA had much lower efficacy at the α1, α2, and α3 compared with α5 subtypes (−18, +13, −7%, and −40%, respectively).

Long-Term Potentiation. The physiological mechanism underlying hippocampally mediated cognitive processes may

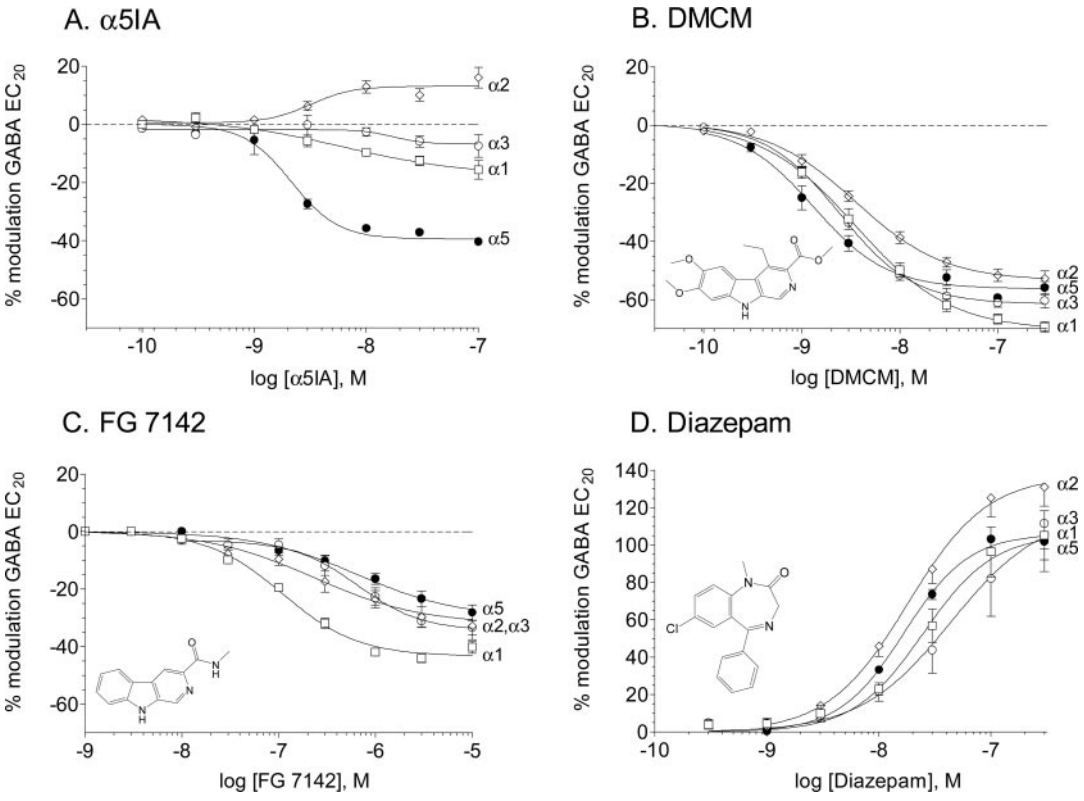

Fig. 3. Efficacy of α5IA compared with the nonselective full inverse agonist DMCM, nonselective partial inverse agonist FG 7142, and nonselective full agonist diazepam. Human recombinant $GABA_A$ receptors were stably expressed in mouse fibroblast L(tk⁻) cells. and the modulation of a GABA concentration equivalent to an EC_{20} 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 EC_{20} current (inverse agonism). Values shown are mean ± S.E.M. (n = 4–9/data point).

TABLE 3

Comparison of the intrinsic efficacy profiles of α5IA, DMCM, FG 7142, and diazepam

Values shown are mean ± S.E.M. of four to nine separate determinations.

		Human Recombinant $GABA_A$ Receptors Containing β3, γ2 Plus			
		α1	α2	α3	α5
α5IA	% modulation[a]	−18 ± 3	+13 ± 3	−7 ± 4	−40 ± 1
	EC_{50}, nM[b]	N.C.	N.C.	N.C.	2.2 ± 0.2
DMCM	% modulation	−71 ± 1	−53 ± 3	−62 ± 2	−57 ± 1
	EC_{50}, nM	3.9	3.7	2.4	1.2
FG 7142	% modulation	−47 ± 2	−38 ± 6	−40 ± 5	−35 ± 4
	EC_{50}, nM	137	5.7	1020	1439
Diazepam	% modulation	103 ± 14	135 ± 11	118 ± 28	106 ± 3
	EC_{50}, nM	27	19	48	17

N.C., not calculated because of small responses producing poorly defined concentration-effect curves.

[a] Values for % modulation are the maximum effects of compound on the current produced by an EC_{20}-equivalent concentration of GABA with negative and positive values representing an attenuation (inverse agonism) or potentiation (agonism), respectively.

[b] The EC_{50} represents the concentration at which compound produces an effect that is 50% of the maximum at that particular subtype.

involve long-term changes in synaptic efficacy, such as LTP (Bliss and Collingridge, 1993). We have shown previously that nonselective BZ site agonists can suppress and inverse agonists potentiate the formation of LTP (Seabrook et al., 1997). Consequently, we investigated whether α5IA could potentiate LTP in mouse hippocampal slices. At low stimulus frequencies (0.033 Hz) α5IA had no direct effect on fEPSP slope (105 ± 6%; $P = 0.47$), amplitude (103 ± 6%; $P = 0.50$), or decay (98 ± 4%; $P = 0.33$). However, LTP was significantly increased from 220 ± 25% in control slices to 340 ± 47% in the presence of α5IA ($P = 0.05$; Fig. 4A). This ability of α5IA to augment LTP induction was occluded in disinhibited slices (data not shown). As with nonselective inverse agonists (Seabrook et al., 1997), these α5IA-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 ± 15% in control slices to 245 ± 19% in the presence of α5IA. 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 nonselective 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 α5IA 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 α5IA had no effect (data not shown), suggesting that α5IA does not affect spontaneous locomotor activity.

The occupancy of the BZ site of Sprague-Dawley rat brain $GABA_A$ receptors by α5IA was dose-dependent (Fig. 5B) with doses of 1, 3, and 10 mg/kg giving occupancies of 32, 54, and 79%, respectively, and an estimated ID_{50} of 2.4 mg/kg. In comparison, FG 7142 (30 mg/kg i.p.) occupied 66% of BZ binding sites.

Motor Impairment. Nonselective BZ site 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 s before falling ($p < 0.05$) and at 30 mg/kg the time spent on the rotarod was only 7 ± 3 s ($p < 0.001$; Fig. 6A). On the other hand, α5IA was without any effect (Fig. 6A), even at a dose of 10 mg/kg.

When occupancy is taken into account (Fig. 6B), α5IA 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 that was

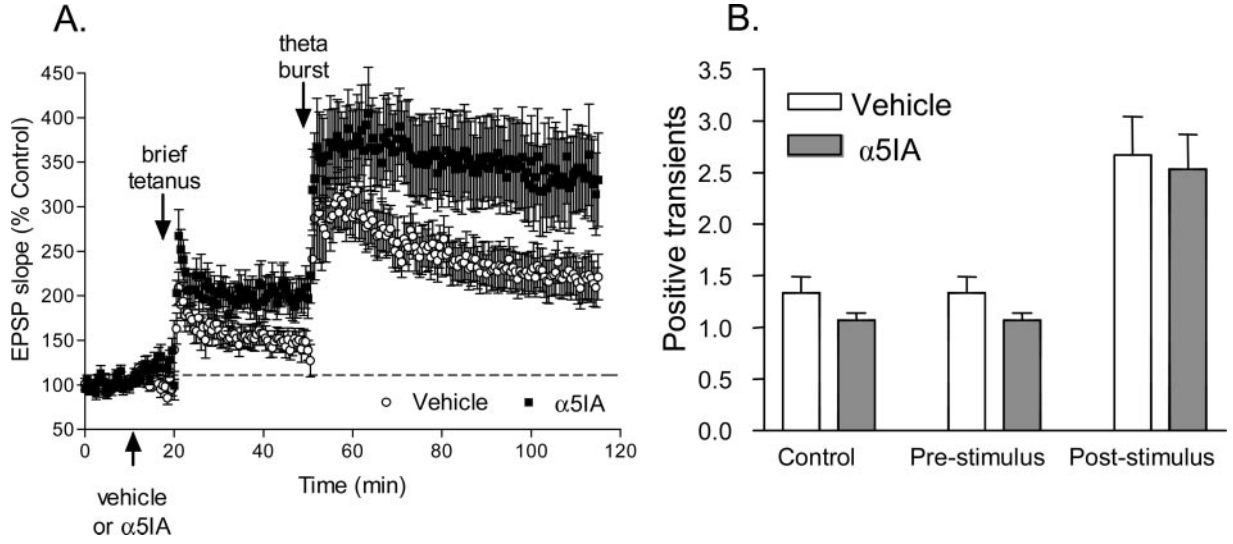

Fig. 4. α5IA enhances LTP but has no effect on paroxysmal discharges recorded in the CA1 region of hippocampal brain slices. A, LTP induced by a brief tetanus (10 stimuli at 100 Hz), and subsequent θ burst stimulus (four stimuli at 100 Hz repeated 10 times every 20 ms) was significantly potentiated by α5IA (30 nM; applied continuously from $t = 10$ min). LTP measured 1 h after the θ burst was significantly increased from 220 ± 25% in vehicle-treated slices to 340 ± 47% in the presence of α5IA; $F = 4.29$ with 1,27 degrees of freedom using two-way analysis of variance with repeated measures, $p < 0.05$. Values shown are mean ± S.E.M. ($n = 15$/group). B, histogram comparing the number of positive transients in response to a single stimulus (at 0.033 Hz) before vehicle or α5IA treatment (control; $t = 1$–10 min), after treatment (prestimulus; $t = 11$–20 min), and after a θ burst stimulus (poststimulus; $t = 85$–95 min). Values shown are mean ± S.E.M. ($n = 15$/group).

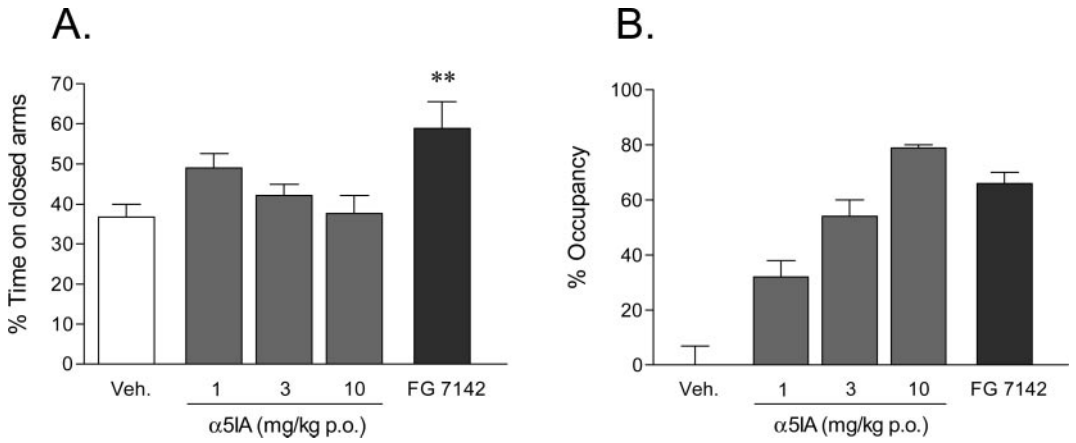

Fig. 5. α5IA does not have anxiogenic-like activity in the elevated plus maze. A, FG 7142 (30 mg/kg i.p.) but not α5IA (1–10 mg/kg p.o.) significantly increased the time spent by rats in the closed arms. Values shown are mean ± S.E.M. (n = 17–18/group). **, $p < 0.01$ versus vehicle control. B, inhibition of the in vivo binding of [^{3}H]flumazenil by α5IA (i.e., α5IA occupancy) was dose-dependent. Values shown are mean ± S.E.M. (n = 7–8/group).

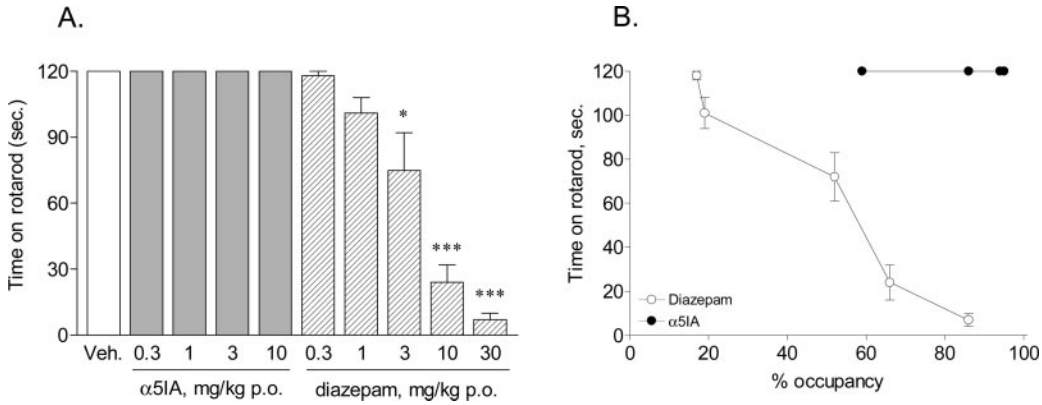

Fig. 6. Lack of effect of α5IA on mouse rotarod performance. A, diazepam dose dependently and significantly impaired rotarod performance such that at a dose of 30 mg/kg p.o., mice only lasted for 7 s before falling off. In contrast, α5IA was without effect up to the highest dose tested (10 mg/kg p.o.). B, following completion of the rotarod trial., animals were taken, and α5IA and diazepam occupancy was measured. The plot of rotarod performance versus percentage of occupancy clearly shows the lack of effect of α5IA even at high levels of occupancy (95%), whereas at lower levels of occupancy, diazepam produced a marked impairment. Values shown are mean ± S.E.M. (n = 8/group). * and ***, $p < 0.05$ and 0.001, respectively, Kruskal-Wallis nonparametric one-way ANOVA followed by Dunn's multiple comparison test.

in marked contrast to α5IA, which at similar levels of occupancy did not alter rotarod performance.

Proconvulsant Activity. When administered alone, α5IA did not induce convulsions in mice at a dose of 10 mg/kg i.p. Likewise, α5IA did not alter the threshold for pentylenetetrazol (PTZ)-induced clonic or tonic seizures (Fig. 7, A and 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 to 25 mg/kg and from 54 to 38 mg/kg, respectively.

The lack of effect of α5IA was not because of a lack of occupancy since a dose of 10 mg/kg corresponded to an occupancy of 98% (Fig. 7C). In contrast, FG 7142 (40 mg/kg) produced much lower levels of occupancy, but despite this, it 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 to 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 α5IA showed no incidence of any type of convulsions (Fig. 7, D and E). Pharmacodynamic experiments not only confirmed that the occupancy of α5IA 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 with α5IA, which gave sustained and high receptor occupancy, such that occupancy 8 h 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 with trial 1 was similar in the two separate experiments (savings of 10.3 ± 2.7 and 10.4 ± 2.2 s in the low- and high-dose experiments, respectively, relative to a trial 1 latency of around 35 s; Fig. 8A). One-way ANOVA showed a significant effect of drug on the savings time in both experiments, with more specific analyses showed that the savings time in α5IA-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 s, respectively; $p < 0.05$). There was no significant difference between drug groups in the time taken to locate the platform on trial 1, and swim speed and path length also did not differ between the vehicle- and α5IA-treated rats (data not shown).

Occupancy of rat brain BZ binding sites by α5IA was

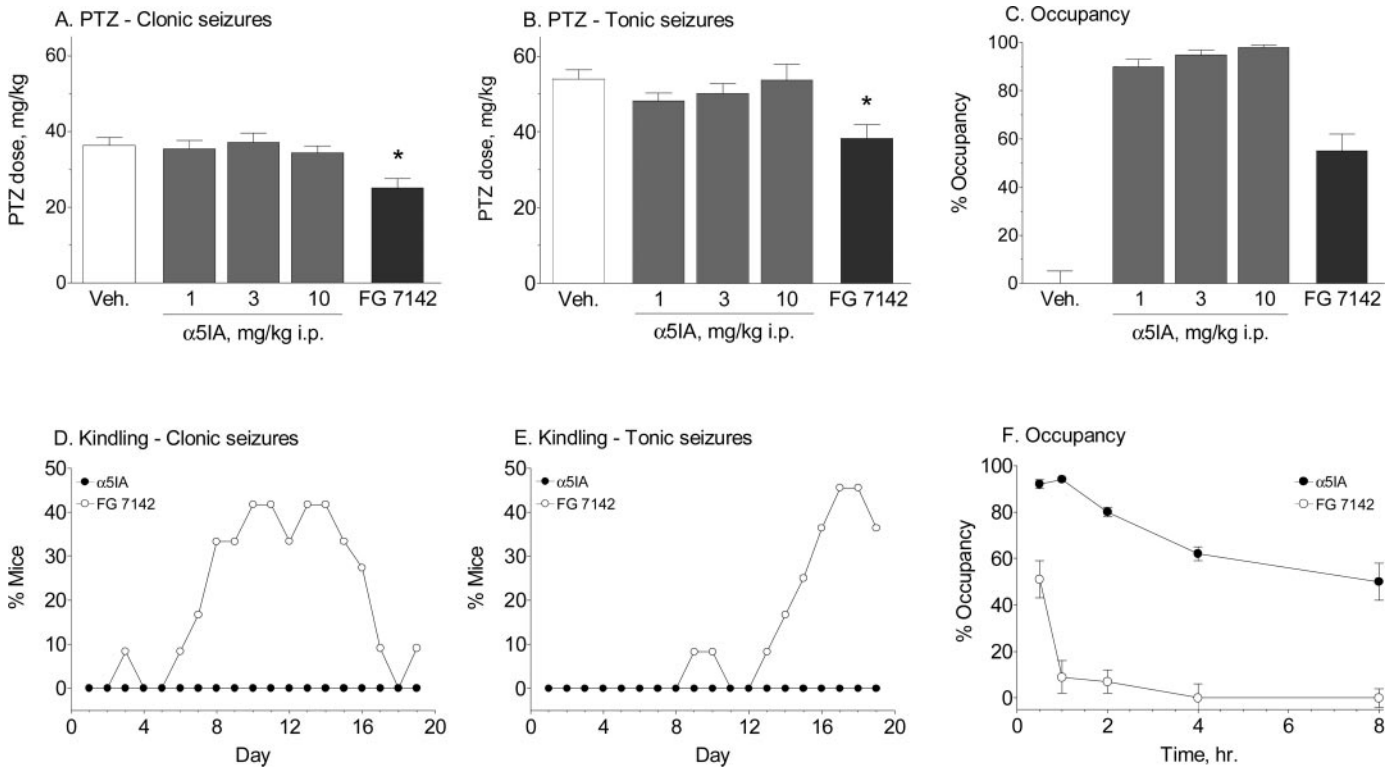

Fig. 7. α5IA was not proconvulsant nor did it produce kindling. A to C, pretreatment of mice for 30 min with α5IA (1–10 mg/kg i.p.) did not reduce the dose of PTZ (i.v. infusion) required to induce either clonic (A) or tonic (B) convulsions. Values shown are mean ± S.E.M. (n = 12–13/group). C, satellite groups of mice received α5IA or FG 7142, and occupancy was measured 30 min after dosing. Values are mean ± S.E.M. (n = 6–9/group). D to F, potential for subthreshold doses of α5IA (10 mg/kg i.p. in 0.5% Methocel) and FG 7142 (40 mg/kg i.p. in 0.2% Tween 80) to produce clonic (D) or tonic (E) 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 α5IA 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 α5IA or either vehicle. F, duration of occupancy of α5IA (10 mg/kg i.p.) in mice compared with the nonselective partial inverse agonist FG 7142 (40 mg/kg i.p.). Maximal occupancy with α5IA (94%) occurred after 1 h and was sustained such that at 8 h, 50% occupancy remained. In contrast, the maximum occupancy of FG 7142 was much less (51%) and short lived. Values shown are mean ± S.E.M. (n = 6/group).

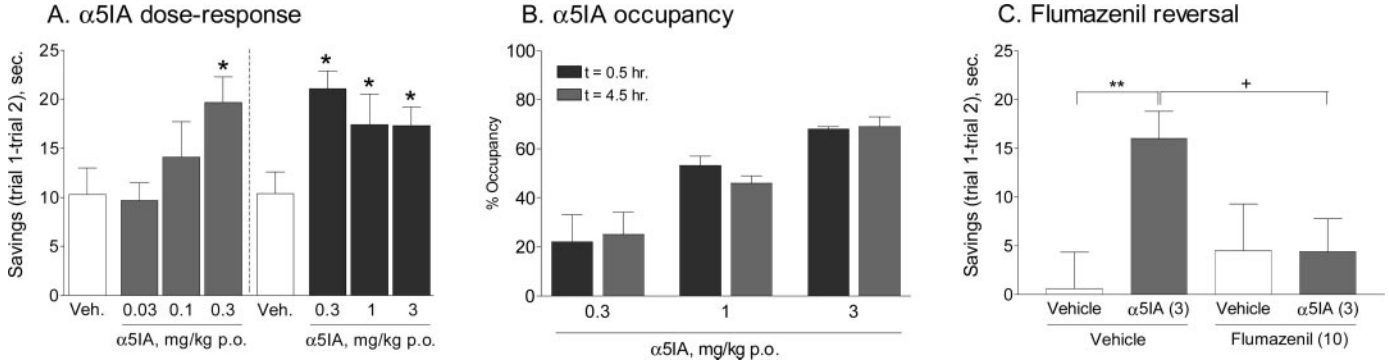

Fig. 8. α5IA enhances performance in the delayed-matching-to-position version of the Morris water maze. A, in two separate experiments, α5IA produced a significant enhancement in performance in the delayed-matching-to-position water maze as measured by the increase in the difference in time, averaged over 5–8 test days, between finding the hidden platform location in trial 2 compared with trial 1 (intertrial interval 4 h). In the low-dose experiment, α5IA had a minimal effective dose of 0.3 mg/kg, whereas in the high-dose experiment, the compound was effective at all three doses tested (0.3, 1, and 3 mg/kg). *, $p < 0.05$ versus vehicle-treated animals. B, occupancy of rat brain BZ sites by α5IA was measured 0.5 and 4.5 h postdosing (corresponding to the times of trials 1 and 2, respectively). Occupancy was dose-dependent but was not appreciably different at these time points. C, ability of α5IA (3 mg/kg p.o.) to increase the mean savings was prevented by the nonselective BZ antagonist flumazenil (10 mg/kg i.p.) given 15 min before trial 1, indicating that the effects of α5IA are mediated via the BZ site of $GABA_A$ receptors (presumably of the α5 subtype). Values shown are mean ± S.E.M. (n = 9–10 group). **, $p < 0.01$ compared with vehicle-vehicle group; +, $p < 0.05$ compared with α5IA-vehicle group.

measured in separate groups of animals. These data (Fig. 8B) showed that α5IA occupancy was dose-dependent but that dose for dose it was comparable at 0.5 and 4.5 h after administration (times that correspond to trials 1 and 2, respectively). Hence, the ID_{50} values at times of 0.5 and 4.5 h were 1.1 and 1.2 mg/kg with the minimal effective dose of 0.3 mg/kg corresponding to an occupancy of 22 to 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 α5IA (3 mg/kg p.o.)-treated rats. In this experiment, α5IA again en-

hanced performance in the delayed-matching-to-position water maze, and this effect was blocked by flumazenil (Fig. 8C). Analysis of the mean savings between trials 1 and 2 on the factors of drug (vehicle or α5IA) and antagonist (vehicle or flumazenil) showed a significant drug × 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 α5IA and vehicle, compared with vehicle-vehicle treated rats [$F(1,34) = 9.02$; $p < 0.01$]. Rats treated with α5IA and vehicle showed significantly greater savings compared with α5IA-treated rats who received flumazenil as the antagonist [$F(1,34) = 4.88$; $p < 0.05$].

Discussion

Comparison of Efficacy- versus Binding-Selective Compounds. The use of compounds that selectively interact with the α5 subtype should help define the functions of this receptor subtype, especially the possibility that an α5-selective inverse agonist might enhance cognition (Maubach, 2003). In this regard, structurally related imidazobenzodiazepines have been described that bind with higher affinity for the α5- compared with α1-, α2- and α3-containing subtypes. Such "binding-selective" compounds, for example L-655,708 (FG 8094), RY-023, and RY-024 have inverse agonist efficacy at the α5 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 α1, α2, and α3 subtypes has not been systematically examined. In the absence of such data, it is clearly not possible to attribute the in vivo effects of α5 binding-selective compounds, such as the effects of RY-010 and RY-024, on contextual memory or fear-related behavior (DeLorey et al., 2001; Bailey et al., 2002), or the convulsant activity of RY-023, RY-024, and RY-080 (Liu et al., 1996), solely to α5-containing GABA$_A$ receptors and at the least highlight the need to characterize the intrinsic efficacy of such compounds at the α1, α2, and α3 as well as α5 subtypes.

In Vitro Properties of α5IA. Given the potential drawbacks of α5 binding-selective drugs (see above), we identified α5IA as an α5 "efficacy-selective" compound (Sternfeld et al., 2004). Although α5IA binds with equivalent subnanomolar affinity to the α1-, α2-, α3-, and α5-subtypes, it has negligible activity (<50% activity at 10 μM) in 127 other receptor and enzymes assays (MDS Pharma Services, Bothwell, WA; data not shown).

α5IA demonstrated essentially the same α5-selective inverse agonist profile against rat and human GABA$_A$ receptors transiently expressed in oocytes (Fig. 2), and the efficacy profile observed in oocytes was also observed with stably expressed human receptors (Figs. 2 and 3). Hence, α5IA has inverse agonism at the α5 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 α1 inverse agonism (−4 to −18%, depending on expression system) that could produce in vivo effects, especially given the greater abundance of the α1 compared with α5 subtype. However, the α1 subtype mediates, at least in part, the proconvulsant effects of PTZ (Rudolph et al., 1999); yet, α5IA was clearly not proconvulsant (Fig. 6), suggesting that the weak α1 inverse agonism does not manifest itself in vivo. Similarly the very weak efficacy at the α2 and α3 subtypes (−7 to +15%) did not seem to have an effect in vivo since these subtypes are associated with anxiolytic-like activity (Atack et al., 2006); yet, α5IA had no obvious effect on anxiety as measured using the rat elevated plus maze (Fig. 5). Overall, the most parsimonious explanation for the in vivo effects of α5IA is that they are mediated primarily via the α5 subtype.

In the mouse hippocampal slice model, α5IA robustly enhanced LTP (Fig. 4A). Since nonselective inverse agonists such as DMCM also produce an increase in LTP (Seabrook et al., 1997), it is tempting to conclude that the α5 subtype is solely responsibly for the enhanced LTP observed with nonselective compounds. However, although α5-containing receptors are enriched within the hippocampus, they are still outnumbered by the combined population of α1-, α2-, and α3-containing GABA$_A$ receptors, which nonselective inverse agonists will also affect.

It is important to emphasize that although the α5 subtype may indeed play a significant role in the *pharmacological* enhancement of LTP, it may play a lesser role in *physiological* LTP. Hence, in α5$^{-/-}$ mice, LTP was not significantly affected whether induced by a brief tetanus followed by a θ burst or a θ 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 α5$^{-/-}$ mice and was specific to the CA1 region of the hippocampus, which is consistent with the distribution of the GABA$_A$ α5 subunit-containing receptors in the brain (Quirk et al., 1996).

The majority of hippocampal α5-containing receptors are localized extrasynaptically (Brünig et al., 2002) where they mediate tonic currents (Caraiscos et al., 2004). Consequently, the mechanism whereby α5IA 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 to 80 Hz (γ) oscillations in hippocampal slices of α5$^{-/-}$ mice suggests that α5-containing GABA$_A$ receptors are associated with hippocampal γ 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 α5 subunit-containing GABA$_A$ receptors might affect the dynamic response of such rhythms to changes in network drive that presumably underlie the procognitive effects of α5IA. Accordingly, it would be interesting to evaluate the effects of α5IA on hippocampal γ frequency oscillations.

In Vivo Properties of α5IA. In vivo, the effects of selective disinhibition of predominantly hippocampally localized α5-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 nonselective 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 α5$^{-/-}$ mice, which do not have a convulsant or

TABLE 4
Summary of in vivo properties of α5IA

Behavior	Drug	Assay	Observation	Route	Dose	Occupancy
					mg/kg	%
Cognition	α5IA	Rat Morris water maze	Enhanced performance	p.o.	0.3	25
Anxiety	α5IA	Rat elevated plus maze	Not anxiogenic	p.o.	10	79
	FG 7142		Anxiogenic	i.p.	30	66
Sedation	α5IA	Mouse rotarod	No effect	p.o.	10	95
	Diazepam		Motor impairment	p.o.	3	52
Proconvulsant liability	α5IA	Mouse PTZ seizure threshold	No effect	i.p.	10	98
	FG 7142		Proconvulsant	i.p.	40	55
Sensitization to seizures (kindling)	α5IA	Mouse seizure activity	No effect	i.p.	10	94[a]
	FG 7142		Development of clonic/tonic seizures	i.p.	40	51[a]

[a] Values refer to maximum occupancy (T_{max} = 1 and 0.5 h for α5IA and FG 7142, respectively).

anxiogenic phenotype, showing that knocking out the α5 $GABA_A$ receptor does not render mice susceptible to spontaneous seizures (Collinson et al., 2002). Furthermore, $\alpha5^{-/-}$ mice demonstrated superior performance in the delayed-matching-to-position water maze task compared with wild-type controls (Collinson et al., 2002).

The absence of an overt convulsant or proconvulsant effect of α5IA in mice is consistent with the lack of an increased frequency of paroxysmal burst discharges (Fig. 4B). In contrast, however, the α5 binding-selective compound RY-080 has been described as being convulsant (Liu et al., 1996). Likewise, whereas α5IA is not anxiogenic in the rats tested on the elevated plus maze, consistent with $\alpha5^{-/-}$ data (Collinson et al., 2002), the α5 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 α5IA is not a consequence of poor pharmacokinetics or brain penetration since we selected doses of α5IA that gave high levels of receptor occupancy. The discrepancies between the in vivo effects of efficacy-selective and binding-selective compounds could be because of the fact that, as discussed above, at higher concentrations or doses an efficacy-selective compound (such as α5IA) maintains its preferential modulation of α5-containing receptors, whereas for a binding-selective compound (for example, RY-080 or L-655,708), significant inverse agonism at the more abundant α1, α2, and α3 subtypes occurs because of appreciable occupancy at these subtypes.

The measurement of receptor occupancy confirms that the lack of effect of α5IA in assays of anxiety-like, proconvulsant, kindling, and motor behaviors is not merely because of compound not occupying BZ binding sites. Thus, high levels of α5IA 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, α5IA 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 α5-containing receptors but rather has equivalent affinity for the different subtypes. However, since α5IA also has comparable affinity across the different subtypes, the inhibition of [^{3}H]flumazenil binding to this combined receptor population by α5IA is because of comparable inhibition of binding (i.e., occupancy) at each different subtype. Hence, α5IA produced an enhancement in cognitive performance by occupying 25% of the α5 subtype.

The observation that α5IA enhances performance in the hippocampal-dependent delayed-matching-to-position in the water maze (Steele and Morris, 1999) suggests that α5-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 nonselective BZs are mediated via distinct $GABA_A$ receptor populations (Rudolph and Möhler, 2004).

Although nonselective BZ site inverse agonists enhance cognition in nonhuman species, they are unsuitable for use in the clinic because of their anxiogenic and proconvulsant, convulsant, or kindling effects (Dorow et al., 1983). However, compared with nonselective full and partial inverse agonists, α5IA has a behaviorally benign profile in rodents with no anxiogenic-like, sedative, motor impairment liabilities. Together, these data suggest that compounds with inverse agonist activity selective for the α5 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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