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3-Hydroxy-2′-methoxy-6-methylflavone: A potent anxiolytic with a unique selectivity profile at $GABA_A$ receptor subtypes

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ABSTRACT

Genetic and pharmacological studies have demonstrated that α2- and α4-containing $GABA_A$ receptors mediate the anxiolytic effects of a number of agents. Flavonoids are a class of ligands that act at $GABA_A$ receptors and possess anxiolytic effects *in vivo*. Here we demonstrate that the synthetic flavonoid, 3-hydroxy-2′-methoxy-6-methylflavone (3-OH-2′MeO6MF) potentiates GABA-induced currents at recombinant α1/2β2, α1/2/4/6β1–3γ2L but not α3/5β1–3γ2L receptors expressed in *Xenopus* oocytes. The enhancement was evident at micromolar concentrations (EC_{50} values between 38 and 106 μM) and occurred in a flumazenil-insensitive manner. 3-OH-2′MeO6MF displayed preference for β2/3- over β1-containing receptors with the highest efficacy observed at α2β2/3γ2L, displaying a 4–11-fold increase in efficacy over α2β1γ2L and α1/4/6-containing subtypes. In contrast, 3-OH-2′MeO6MF acted as a potent bicuculline-sensitive activator, devoid of potentiation effects at extrasynaptic α4β2/3δ receptors expressed in oocytes. The affinity of 3-OH-2′MeO6MF for α4β2/3δ receptors (EC_{50} values between 1.4 and 2.5 μM) was 10-fold higher than at α4β1δ $GABA_A$ receptors. 3-OH-2′MeO6MF acted as a full agonist at α4β2/3δ (105% of the maximal GABA response) but as a partial agonist at α4β1δ (61% of the maximum GABA response) receptors. In mice, 3-OH-2′MeO6MF (1–100 mg/kg *i.p.*) induced anxiolytic-like effects in two unconditioned models of anxiety: the elevated plus maze and light/dark paradigms. No sedative or myorelaxant effects were detected using holeboard, actimeter and horizontal wire tests and only weak barbiturate potentiating effects on the loss of righting reflex test. Taken together, these data suggest that 3-OH-2′MeO6MF is an anxiolytic without sedative and myorelaxant effects acting through positive allosteric modulation of the α2β2/3γ2L and direct activation of α4β2/3δ $GABA_A$ receptor subtypes.

1. Introduction

γ-Aminobutyric acid (GABA) mediates fast synaptic, inhibitory neurotransmission in the mammalian brain acting on GABA type A ($GABA_A$) receptors. $GABA_A$ receptors are members of the cys-loop family of ligand-gated ion channels that include the nicotinic acetylcholine, serotonin type 3 and glycine receptors. They assemble from different subunits to form a pentameric structure and chloride ions are conducted through the resulting pore. Sixteen subunits have been cloned: α1–6, β1–3, γ1–3, δ, ε, π, θ [1–5] with the majority of $GABA_A$ receptors in the brain containing two α, two β and one single γ [1] or δ [2] subunit. The distribution of these subunits in the brain is highly distinct, suggesting differential functional roles for different $GABA_A$ receptor subtypes. The subunit composition determines the GABA sensitivity and the pharmacological properties of the receptor.

$GABA_A$ receptors are located in the synapse to mediate phasic inhibition, but are also located extrasynaptically to regulate the resting potential of neurons by activating tonic currents in these locations [2]. Therefore, it is not surprising that $GABA_A$ receptors are implicated in a series of major neurological disorders such as anxiety, epilepsy, sleep and cognitive disorders as well as psychiatric disorders including mood disorders and schizophrenia [6].

Abbreviations: GABAγ-, aminobutyric acid; 3-OH-2′MeO6MF, 3-hydroxy-2′-methoxy-6-methylflavone; PTZ, pentylenetetrazole; *i.p.*, intraperitoneal; BDZs, benzodiazepines.

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Anxiety disorders, including generalized anxiety disorder, panic disorder and social anxiety are common and disabling diseases. Benzodiazepines (BDZs) are the standard treatment for such disorders, but their usefulness is seriously limited by their unfavourable side effects such as sedation, cognitive impairment and dependence [7]. The search for new and improved $GABA_A$ receptor ligands identified a variety of compounds structurally different from the BDZ scaffold, such as β-carbolines [8], trizolopyridazines [9] and quinolines [8,10]. Few agents exhibit specificity for $GABA_A$ receptor subtypes.

Recent studies have demonstrated that, in general, α2/3-containing $GABA_A$ receptors mediate anxiolytic effects, α1-containing $GABA_A$ receptors mediate sedative effects, while learning and memory is attributed to α5-containing $GABA_A$ receptors [11]. Furthermore myorelaxation and motor impairment effects have been shown to involve α2-, α3-, and α5-subunits [12–14]. As a result, the specific BDZ action is a reflection of the differential expression of $GABA_A$ receptor subtypes across brain regions [15]. Given this evidence, the search for new non-sedative anxiolytics targeting α2- or α3-containing $GABA_A$ receptors that do not possess sedative effects has been the subject of a number of drug discovery studies [11,16].

Flavonoids are secondary plant metabolites [17] and display a wide range of biological activities [18] including anxiolytic actions [19–21]. The ability of flavonoids to compete with radiolabelled BDZ's in rat and bovine brain tissues, and to exhibit anxiolytic activity in rodents, suggests that they mediate their activities through the BDZ site at $GABA_A$ receptors. However, numerous studies have demonstrated that flavonoids can also modulate $GABA_A$ receptors at site(s) independent of the classical BDZ binding site [22–26], indicating that this series of compounds may exert their action through novel, yet unidentified, binding site(s).

In this study, we evaluated a previously reported flavonoid, 3-hydroxy-2′-methoxy-6-methylflavone (3-OH-2′MeO6MF; Fig. 1A [27,28]) on 29 human recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes using 2-electrode voltage clamp. 3-OH-2′MeO6MF exhibits a unique selectivity profile for $GABA_A$ receptors, enhancing the action of GABA with high intrinsic activity at α2β2/3γ2L, and acting as a potent allosteric agonist at α4β2/3δ receptors. The effects of 3-OH-2′MeO6MF were not blocked by the BDZ neutralizing modulator, flumazenil indicating it does not work at the classical "high-affinity" BDZ site. When evaluated in mouse models of anxiety, 3-OH-2′MeO6MF had anxiolytic effects that were free from either sedative or myorelaxant effects.

2. Materials and methods

2.1. Compounds

GABA, bicuculline, flumazenil, Tween, theophylline, pyruvate, gentamicin, horse serum, tricaine, 2-methoxybenzaldehyde, and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA) while diazepam was purchased from Apin Chemicals Ltd. (Oxon, UK). [^{3}H]Muscimol (36.6 Ci/mmol) and [^{3}H]flunitrazepam (74.10 Ci/mmol)) were obtained from Perkin Elmer (Boston, MA, USA). Pentylenetetrazole (PTZ) was purchased from Tocris Biosciences (Bristol, UK) and sodium thiopental from Jurox (Rutherford, NSW, Australia). 3-OH-2′MeO6MF was synthesized, in house, according to Scheme 1 (see supplementary data for Scheme 1 and experimental details).

2.2. Synthesis of 3-hydroxy-2′-methoxy-6-methylflavone (3-OH-2′MeO6MF)

The synthesis of 3-OH-2′MeO6MF is depicted in Scheme 1 (supplementary data). The synthesis involves Claisen–Schmidt condensation followed by an Algar–Flynn–Oyamada reaction. 2′-Hydroxy-2-methoxy-5′-methylchalcone was prepared by base-catalyzed aldol condensation of 2′-hydroxy-5′-methylacetophenone with 2-methoxybenzaldehyde. The chalcone was converted to 3-hydroxy-2′-methoxy-6-methylflavone via oxidative cyclization reaction using alkaline hydrogen peroxide. The crude product was treated with activated charcoal, filtered through celite and recrystallized twice from ethanol to afford 3-hydroxy-2′-methoxy-6-methylflavone (purity: ≥98% by NMR and HPLC) as a colorless solid. The assigned structure was in agreement with the ^{1}H (400 MHz) and ^{13}C (100.5 MHz) NMR spectra. Further details concerning synthesis, analytical and spectral characterization data are given in supplementary data.

2.3. Animal use

All procedures involving animals were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes published by the National Health and Medical Research Council of Australia (NH&MRC), and were approved by the Animal Ethics Committee of the University of Sydney.

2.4. GABA receptor subunit constructs

Human α1, α2, α6, β1, β2 and γ2L cDNA subcloned in pcDM8, α3 and β3 in pGEMHE, α5 subcloned in pcDNA3, α4 and δ subcloned in pcDNA1/Amp were linearized with the appropriate restriction endonucleases and capped transcripts were produced from linearized plasmids using the 'mMessage mMachine' T7 transcript kit from Ambion (Austin, TX, USA). The quality of mRNA was determined by 0.5% agarose gel electrophoresis. mRNA concentrations were measured by NanoDrop® ND-1000 UV–vis spectrophotometer, and diluted with nuclease free water and stored at −80 °C.

2.5. Expression of recombinant GABA receptors in Xenopus oocytes

2.5.1. Oocyte preparation

Sexually mature female *Xenopus laevis* were kept under optimal conditions by authorized animal keepers at The University of Sydney Animal House and fed standard frog food. Stage V–VI oocytes were harvested under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anaesthesia. Oocytes were defolliculated by shaking for approximately 1 h at 18 °C in collagenase (2 mg/mL) dissolved in OR2 solution containing (in mmol/L): NaCl 96; KCl 2; $CaCl_2$ 1, $MgCl_2$ 1; HEPES 5, pH 7.4.

Stage V–VI oocytes were sorted and injected (Nanoject, Drummond Scientific Co., Broomali, PA) with cRNA reconstituted in nuclease free water in a ratio of 1α (25 ng): 1β (25 ng): 5 (γ) (125 ng) and 5α (125 ng): 1β (25 ng): 5 (δ) (125 ng). Oocytes were incubated for up to 4–8 days in standard ND96, pH 7.4, supplemented with pyruvate (2.5 mM), theophylline (0.5 mM), gentamycin (50 μg/mL) and 2% horse serum at 18 °C.

2.5.2. Electrophysiology

Currents were recorded using the two-electrode voltage clamp technique as described elsewhere [29]. Glass microelectrodes were filled with 3 M KCl (0.5–2 MΩ). Oocytes were clamped at −60 mV and continuously superfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Current amplitudes were calculated off-line using Chart software v3.6 (ADInstruments, NSW, Australia). Responses to GABA applications were normalized as $I\% = (I/I_{max}) \times 100$, where I is the peak amplitude of current response and I_{max} is the maximum current produced by GABA measured from individual cells.

Direct activation responses of 3-OH-2′MeO6MF were normalized as $\%\ I\ (\text{drug}) = (I_{drug}/I_{max\ GABA}) \times 100$, where I_{drug} is the peak

amplitude of current response of 3-OH-2′MeO6MF and I_{max} is the maximal current produced by GABA measured in each individual cell. Modulation of GABA-elicited currents was tested by co-applying increasing concentrations of the drugs with a concentration of GABA that produced 10% of maximal activation (EC_{10}, determined for each cell). Current responses were recorded and normalized as: % potentiation = $((I_{drug+GABA} - I_{GABA})/I_{GABA}) \times 100$, where $I_{drug+GABA}$ is the control GABA current in the presence of a given concentration of drug, and I_{GABA} is the amplitude of the control GABA current alone. In cases of γ2L-subunit containing receptors, a 3–5 min and for δ-subunit containing receptors 10–15 min washout period was allowed between drug applications to avoid receptor desensitization. Normalized responses were pooled and graphed as mean ± S.E.M. from at least three oocytes from at least two different batches. Responses were fitted to the four-parameter logistic equation: $I = I_{max}/(1 + (EC_{50}/[A])n_H)$; where I is the peak amplitude of the current elicited by a given concentration of agonist $[A]$, I_{max} is the maximum amplitude of the current, EC_{50} is the concentration required for half-maximal response, and n_H is the Hill coefficient (Prism v5 GraphPad software, San Diego, CA). When two groups were compared, the Mann–Whitney test was used to determine significance and when more than two groups were compared, a one-way ANOVA followed by Dunnett's post-hoc test was used.

2.6. Binding studies

2.6.1. Membrane preparations

All binding assays were performed using rat brain synaptic membranes of the cortex and central hemispheres from adult male Wistar rats with tissue preparations as described earlier [30]. On the day of the assay, the membrane preparation was quickly thawed, suspended in 40 vol of ice-cold 50 mM Tris–HCl buffer (pH 7.4) using an UltraTurrax homogenizer, and centrifuged at 48,000 × *g* for 10 min at 4 °C. This washing step was repeated four times. The final pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) for the binding assay.

2.6.2. Binding assays

Membranes (100 μg of protein/aliquot) in Tris–HCl buffer (50 mM, pH 7.4) were incubated with either [^{3}H]muscimol (5 nM) and GABA (1 mM) or [^{3}H]flunitrazepam (1 nM) and diazepam (10 μM) at 0 °C for 60 min in a total volume of 250 μL. GABA (1 mM) and diazepam (10 μM) were used to determine non-specific binding. The binding reaction was terminated by rapid filtration through GF/C unifilters (Perkin Elmer, Boston, MA, USA) using a 24-channel cell harvester (Brandel, Gaithersburg, MD) followed by washing with 3 × 250 μL of ice-cold binding buffer. ULTIMA-GOLD liquid scintillation cocktail (Perkin Elmer, Boston, MA, USA) was added to the dried filters, and the amount of filter-bound radioactivity was quantified in a Packard Top Count microplate scintillation counter (Perkin Elmer, MA, USA). Data were expressed as percentage of control specific binding. EC_{50} values are expressed as mean ± S.E.M. from three independent experiments.

2.7. In vivo studies

2.7.1. Animals

All behavioral tests were performed on male Balb-c mice (Animal Resources Centre, Perth, Australia) aged 8–10 weeks, weighing 25–35 g. Animals were housed in groups of four per cage with free access to food and water, and maintained in a controlled environment (20–23 °C, 45–55% relative humidity) with a 12/12 h light/dark cycle (light on at 06:00 h). Illumination of the experimental room was 80 lx. All animals were experimentally naive. Drugs were dissolved in the vehicle comprising of DMSO (5%), Tween 80 (1%) and saline (94%), and administered by intraperitoneal (*i.p.*) injection in a volume of 5 μL/g. Sodium thiopental was dissolved in saline. All behavioral studies were carried out between 09:00 and 14:00 h.

2.7.2. General behavioral and acute toxicity tests in mice

The overt behavioral effects of 3-OH-2′MeO6MF on mice after *i.p.* injection were assessed using the protocol described by Irwin [31] and described elsewhere [32]. In brief, animals tails were marked with waterproof markers to assign them to one of five groups each consisting of eight animals for random blind allocation to one of five experimental conditions: vehicle, test dose 1, test dose 2, test dose 3, and test dose 4 which were 1, 10, 30 and 100 mg/kg.

Observations were recorded pertaining to the following parameters: spontaneous activity, exophthalmics, piloerection, aggressiveness, writhing, tremors, clonic convulsions, tonic convulsions, gasping, hypersensitivity, docility, position struggle, salivation, cyanosis, vasoconstriction, vasodilatation, finger approach, finger escape, hind limb placing, ataxia, hyperesthesia, straub tail, visual placing, tail pinch, righting reflex, catalepsy, bizarre behavior, prehensile strength and pupil size according to the procedure prescribed by Irwin [31].

2.8. Elevated plus maze

The apparatus consisted of two open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm × 15 cm) made of black Plexiglas connected by an open central platform (5 cm × 5 cm) and elevated 40 cm from ground level. A raised ledge (3 mm high and 1 mm thick) surrounded the perimeter of the open arms. Animals were injected with vehicle or drugs, and 20 min later placed in the center of the apparatus facing an open arm and allowed to explore the maze for 5 min. An arm entry was defined by having all four paws inside the arm. All sessions were videotaped by a camera positioned above the maze, and at the end of the test, the number of arm entries and time spent in arms were recorded. To assess the involvement of the GABAergic system, animals were either pretreated *i.p.* with vehicle, flumazenil (2.5 mg/kg or 5 mg/kg) or PTZ (20 mg/kg) before treating with diazepam (2 mg/kg) or 3-OH-2′MeO6MF (10 mg/kg). After individual trials, the apparatus was carefully cleaned with wet paper towel (mixture of ethanol, detergent and water) to remove any residue or odors. The % of open arm entries and the % of time spent in the open arms were recorded as measure of anxiety state [33].

2.9. Light/dark box test

The light/dark apparatus consists of an acrylic box of dimensions 44 cm × 21 cm × 21 cm, divided into a small dark compartment (one-third) and a large illuminated compartment (two-thirds), the division between zones contains an opening of 6 cm × 3 cm. The box possesses 16 light beams, 11 in the lit area and 5 in the dark area, which detect the movement of the animal. The box is connected to a computer that records the number of transitions between areas, latency to the first transition, time and activity in each zone and total activity in a 5 min session. An increase in the exploration of the lit area is associated with an anxiolytic effect; as such, two parameters were selected as a measure of anxiety: the time spent in the lit compartment and the total number of transitions [34].

2.10. Measurement of locomotor activity

Animals were placed in a box made of clear Plexiglas, with a floor of 30 cm × 15 cm and 15 cm high walls, which possesses 15 optical beams, that allows the measure of animal activity along a single axis

of motion [22]. Spontaneous locomotor activity was measured for 5 min and expressed as total number of light beams crossed.

2.11. Holeboard assay

This assay was conducted in an automatic apparatus described previously [22]. Twenty minutes after injection with vehicle or the drug, animals were placed in the center of the board and allowed to freely explore the arena for 5 min. The number and duration of head dips were recorded. After each trial the apparatus was wiped clean to remove traces of the previous assay. A decrease in the number of head dips and/or the time spent head dipping is associated with a sedative effect [33].

2.12. Thiopental-induced sleep time

A sub-threshold dose of sodium thiopental (40 mg/kg) was injected *i.p.* into mice 20 min after a similar injection of vehicle or drugs. The duration of the loss of the righting reflex was recorded [35].

2.13. Horizontal wire test

Animals were trained in two separate sessions the day before. On the day of the assay, animals were injected with vehicle or drug and the number of animals unable to grasp the wire was recorded as a measure of myorelaxation [36].

2.14. Statistical analysis

Data was expressed as mean ± S.E.M. When several treatments were compared, one-way ANOVA was used and post-hoc comparisons between vehicle and drug treated groups were made using Dunnett's multiple comparison test. When two groups were compared, Student's *t*-test was used. Data from the horizontal wire test were analyzed using Fisher's exact test.

3. Results

3.1. Binding studies

3-OH-2′MeO6MF was initially tested for its ability to inhibit [^{3}H]flunitrazepam and [^{3}H]muscimol binding to rat brain synaptic membranes. Diazepam (10 μM) but not 3-OH-2′MeO6MF (100 μM and 300 μM) inhibited [^{3}H]flunitrazepam (Fig. 1B). However, like diazepam, 3-OH-2′MeO6MF enhanced [^{3}H]muscimol binding in a concentration-dependent manner. The maximum enhancement of muscimol binding (E_{max}) produced by diazepam was 339 ±17% with an EC_{50} value of 27.05 ± 5.65 nM (n = 4 independent experiments), while 3-OH-2′MeO6MF produced an E_{max} of 328 ± 18% with an EC_{50} value of 28.25 ± 6.50 nM (n = 4 independent experiments; Fig. 1C).

3.2. Electrophysiological studies

3.2.1. 3-OH-2′MeO6MF is a flumazenil-insensitive allosteric modulator with high efficacy at α2β2/3γ2L $GABA_A$ receptors expressed in Xenopus oocytes

In order to validate that all tested subunit combinations resulted in GABA-sensitive ion channels, GABA was initially tested on *Xenopus* oocytes expressing α1/2β2, α4/6β3, α1–6β1–3γ2L and α4/6β1–3δ subtypes. The inward whole-cell currents ranged between 200 and 3000 nA.

3-OH-2′MeO6MF (100 μM) was then screened for direct effects at γ2L-containing receptors. 3-OH-2′MeO6MF did not directly activate α1–5β1–3γ2L, α6β1γ2L, α1/2β2 and α4β3 receptors (data not shown). Instead, 3-OH-2′MeO6MF (1–300 μM) enhanced the action of a low concentration of GABA (EC_{10}) at α1/2β2 and α1/2β1–3γ2L receptors (Fig. 2). An example of the enhancement produced by 3-OH-2′MeO6MF at α1β2γ2L and α2β2γ2L $GABA_A$ receptors is shown in Fig. 2A and 2B, respectively. The maximum enhancement observed at α1β2 and α1β1–3γ2L ranged from 167 to 370% (Fig. 2C and Table 1).

In contrast, the enhancement of GABA (EC_{10}) by 3-OH-2′MeO6MF (1–300 μM) at α2β2/3γ2L receptors was considerably higher (Fig. 2B and C; Table 1) than that at α1β1–3γ2L receptors. The maximum enhancement of GABA by 3-OH-2′MeO6MF at α2β2γ2L and α2β3γ2L receptors was 1707 ± 198% and 1345 ± 68%, respectively (Table 1). This enhancement was also significantly higher than what was observed at α2β1γ2L receptors (E_{max} 159% ± 6%; $F_{2,9} = 167.0$ $p < 0.0001$; ANOVA with Dunnett's post-hoc test; Fig. 2C and Table 1). However the potency of 3-OH-2′MeO6MF at all receptors was not significantly different, with EC_{50} values ranging between 38 and 80 μM (Table 1).

3-OH-2′MeO6MF (100 μM) had no significant effect on the GABA concentration–response curve at α2β1γ2L receptors (Fig. 2D). In contrast, 3-OH-2′MeO6MF (100 μM) significantly shifted the GABA concentration–response curve to the left decreasing the EC_{50} value for GABA by 100-fold at α2β2γ2L receptors from 83 μM (95% CI = 61.22–112.5 μM) to 0.83 μM (95% CI = 0.48–1.40 μM; Fig. 2E). The data indicate that the type of β-subunit plays a significant role in the compound's efficacy and to a much lesser extent on potency.

In order to determine the role of the γ2L-subunit, we evaluated the effects of 3-OH-2′MeO6MF on α1/2β2 devoid of the γ2L-subunit. The fact that 3-OH-2′MeO6MF (1–300 μM) also enhanced the response elicited by GABA (EC_{10}) at α1/2β2 receptors indicates that the enhancement is not dependent on the γ2L-subunit, inferring that the binding site is located on α-β subunits, albeit the γ2L-subunit is required for maximum efficacy. The decreasing order of efficacy for 3-OH-2′MeO6MF at α1- and α2-containing receptors is $\alpha2\beta2\gamma2L \geq \alpha2\beta3\gamma2L > \alpha2\beta2 > \alpha1\beta2\gamma2L \approx \alpha1\beta3\gamma2L > \alpha1\beta2 > \alpha1\beta1\gamma2L \approx \alpha2\beta1\gamma2L$ (Table 1).

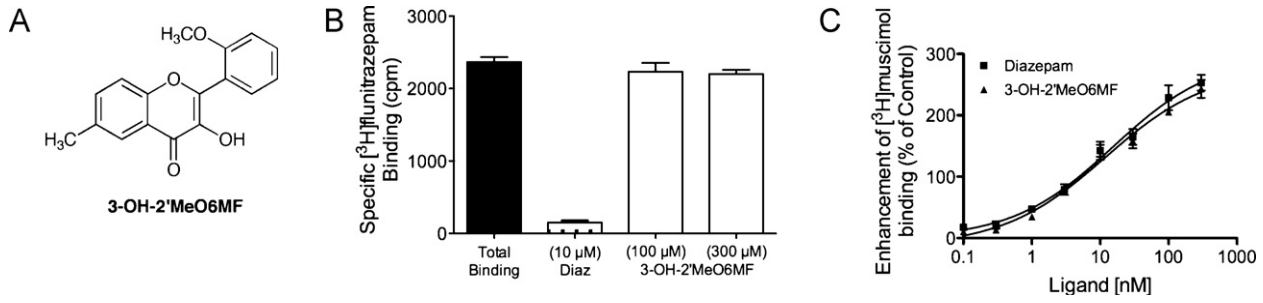

Fig. 1. (A) Structure of 3-OH-2′MeO6MF. (B) Effect of diazepam (10 μM) and 3-OH-2′MeO6MF (100 and 300 μM) on [^{3}H]flunitrazepam binding. Values represent mean ± S.E.M. of three different experiments. (C) Enhancement of [^{3}H]muscimol binding by diazepam and 3-OH-2′MeO6MF. Each data point is the mean ± S.E.M. of four different experiments.

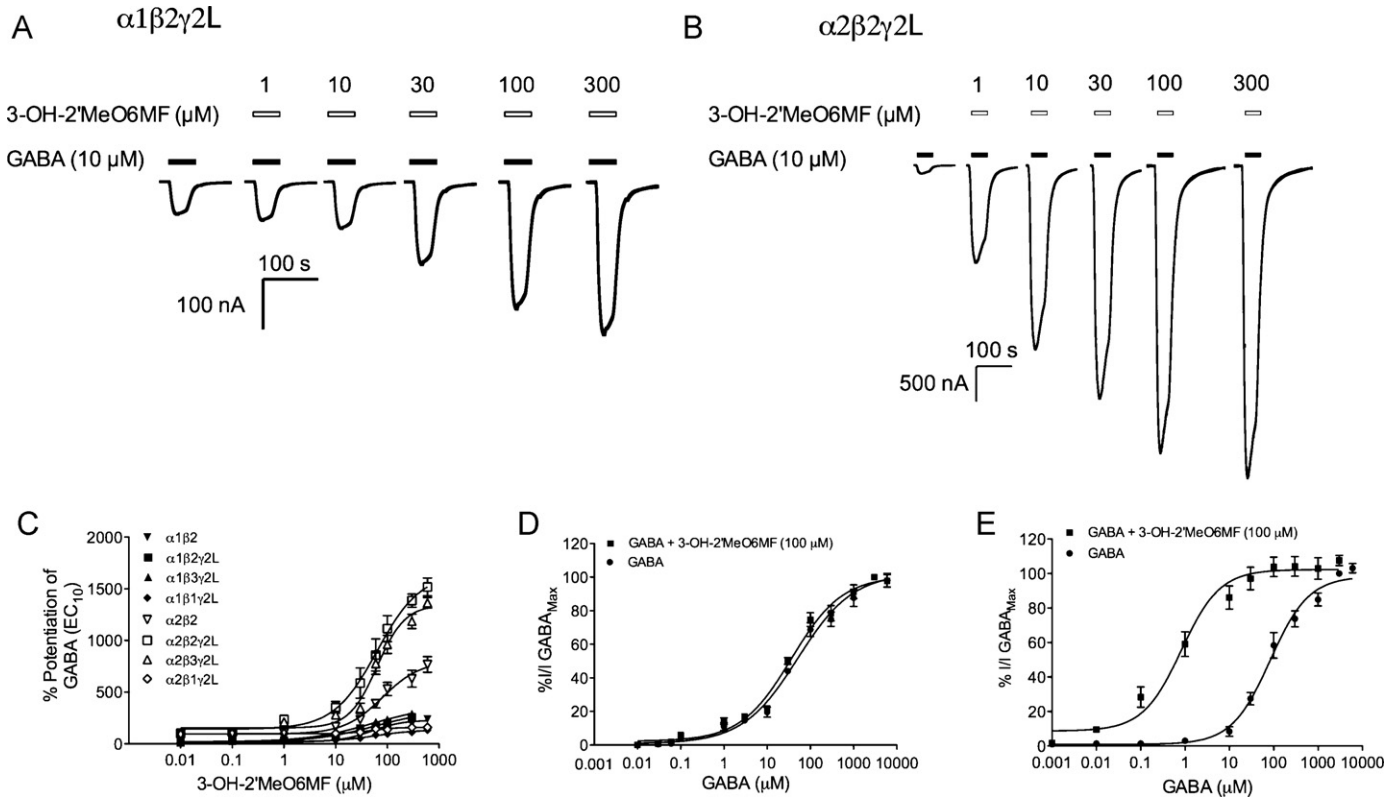

Fig. 2. 3-OH-2′MeO6MF is a positive modulator of α1/2-containing $GABA_A$ receptors expressed in *Xenopus* oocytes. (A) Representative current trace showing the potentiation of GABA (EC_{10}: 10 μM) by various concentrations of 3-OH-2′MeO6MF (1, 10, 30, 100, and 300 μM) at human recombinant α1β2γ2L $GABA_A$ receptors. Horizontal bars show the duration of drug application. (B) Representative current trace showing the potentiation of GABA (EC_{10}: 10 μM) by various concentrations of 3-OH-2′MeO6MF (1, 10, 30, 100, and 300 μM) at human recombinant α2β2γ2L $GABA_A$ receptors. (C) Concentration–response curves for 3-OH-2′MeO6MF in the presence of GABA (EC_{10}: 10 μM) at recombinant α1β2 (▼), α1β2γ2L (■), α1β3γ2L (▲) and α1β1γ2L (◆) α2β2 (▽), α2β2γ2L (□), α2β3γ2L (△) and α2β1γ2L (◇) receptor subtypes. Data are mean ± S.E.M. (n = 3–6 oocytes). (D) Concentration–response curves for GABA alone (●) and GABA in the presence of 3-OH-2′MeO6MF (100 μM (■)) at recombinant α2β1γ2L $GABA_A$ receptors. Data are mean ± S.E.M. (n = 3–6 oocytes). At 100 μM 3-OH-2′MeO6MF has no significant effect on the EC_{50} value for GABA. (E) Concentration–response curves for GABA alone (●) and GABA in the presence of 3-OH-2′MeO6MF (100 μM (■)) at recombinant α2β2γ2L $GABA_A$ receptors. Data are mean ± S.E.M. (n = 3–6 oocytes). 3-OH-2′MeO6MF (100 μM) shifts the concentration–response curve to the left. The EC_{50} value for GABA shifted 100-fold from 83 μM (95% CI = 61–112) down to 0.83 μM (95% CI = 0.48–1.4).

Flumazenil (0.1–10 μM) did not block the 3-OH-2′MeO6MF-induced enhancement of GABA (EC_{10}) at α1β2γ2L or α2β2γ2L $GABA_A$ receptors (Fig. 3A and C, respectively) which supports the binding data which show 3-OH-2′MeO6MF does not inhibit [^{3}H]flunitrazepam binding (Fig. 1B). By contrast, the enhancement of the response of GABA (EC_{10}) produced by diazepam (1 μM) was significantly attenuated by 1–10 μM flumazenil at α1β2γ2L and α2β2γ2L $GABA_A$ receptors (Fig. 3B and D, respectively).

Interestingly, 3-OH-2′MeO6MF (1–300 μM) did not activate or significantly enhance the response to GABA EC_{10} at recombinant

Table 1
Pharmacology of 3-OH-2′MeO6MF at various recombinant ionotropic GABA receptor subtypes expressed in *Xenopus* oocytes.

Receptor subtype	3-OH-2′MeO6MF EC_{50} value (μM) (95% CI)	Fitted % E_{max}	n_H	n
α1β1γ2L	53.2 (80.5–252.7)	167 ± 41	0.7	3
α1β2γ2L	59.6 (11.6–305.5)	338.0 ± 39.2	0.8	8
α1β2	38.0 (10.6–135.8)	280.2 ± 21.4	0.9	6
α1β3γ2L	48.2 (21.4–108.3)	367.2 ± 25.4	0.8	5
α2β1γ2L	49.1 (26.0–92.8)	159.3 ± 6.0	1.1	4
α2β2γ2L	56.9 (28.4–114.2)	1707 ± 198	1.0	6
α2β3γ2L	63.8 (49.2–82.7)	1345 ± 68	1.2	5
α2β2	80.6 (41.8–155.2)	803.4 ± 93.2	1.1	3
α3β1–3γ2L	N.E.	–	–	6
α4β1γ2L	75.0 (38.0–149.6)	181.8 ± 71.9	1.0	5
α4β2γ2L	51.4 (29.8–88.6)	494.7 ± 78.1	1.2	6
α4β3γ2L	46.6 (27.9–78.1)	464.7.5 ± 50.2	1.1	4
α4β3	51.4 (23.7–111.5)	533.3 ± 80.0	0.9	4
α5β1–3γ2L	N.E.	–	–	6
α6β1γ2L	106.0 (17.3–651.9)	238.5 ± 82.8	0.9	3
α6β2γ2L	31.4 (16.0–61.6)	443.2 ± 119.8	1.1	5
α6β3γ2L	43.3 (6.2–301.2)	400.4 ± 45.5	0.8	5
α6β3	45.3 (23.1–88.9)	492.5 ± 63.6	1.1	4
α6β2δ	67.8 (26.3–175.2)	305.3 ± 51.0	1.1	4
α6β3δ	47.75 (25.1–90.9)	348.1 ± 46.8	0.8	4

95% CI: 95% confidence interval. E_{max}: maximum potentiation produced by 3-OH-2′MeO6MF relative to GABA EC_{10}. n_H: Hill coefficient. N.E.: no effect. Data are presented as mean ± S.E.M. and 'n' is the number of individual oocytes tested.

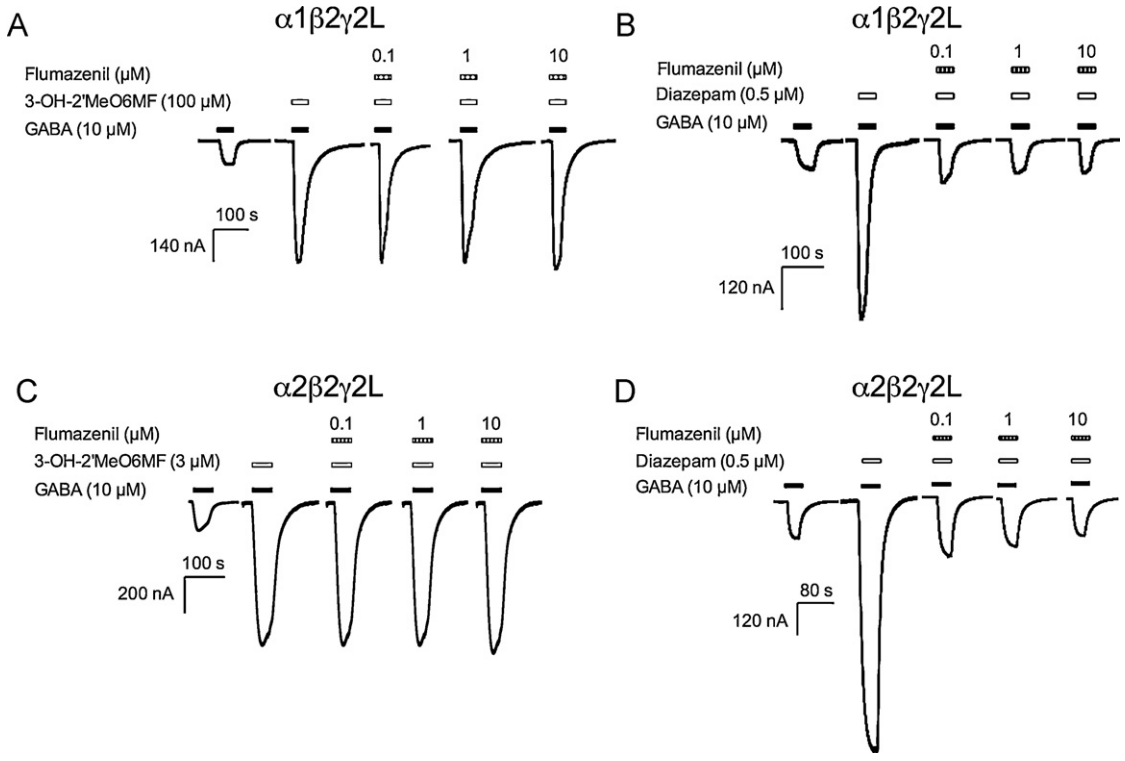

Fig. 3. Current traces from individual oocytes showing the potentiation of GABA (10 μM) at human recombinant α1β2γ2L $GABA_A$ receptors by (A) 3-OH-2′MeO6MF (100 μM) and (B) diazepam (0.5 μM); and α2β2γ2L $GABA_A$ receptors by (C) 3-OH-2′MeO6MF (3 μM) and (D) diazepam (0.5 μM). The potentiating effect of 3-OH-2′MeO6MF was not attenuated by flumazenil (0.1–10 μM) whereas the potentiation by diazepam was inhibited by flumazenil (0.1–10 μM).

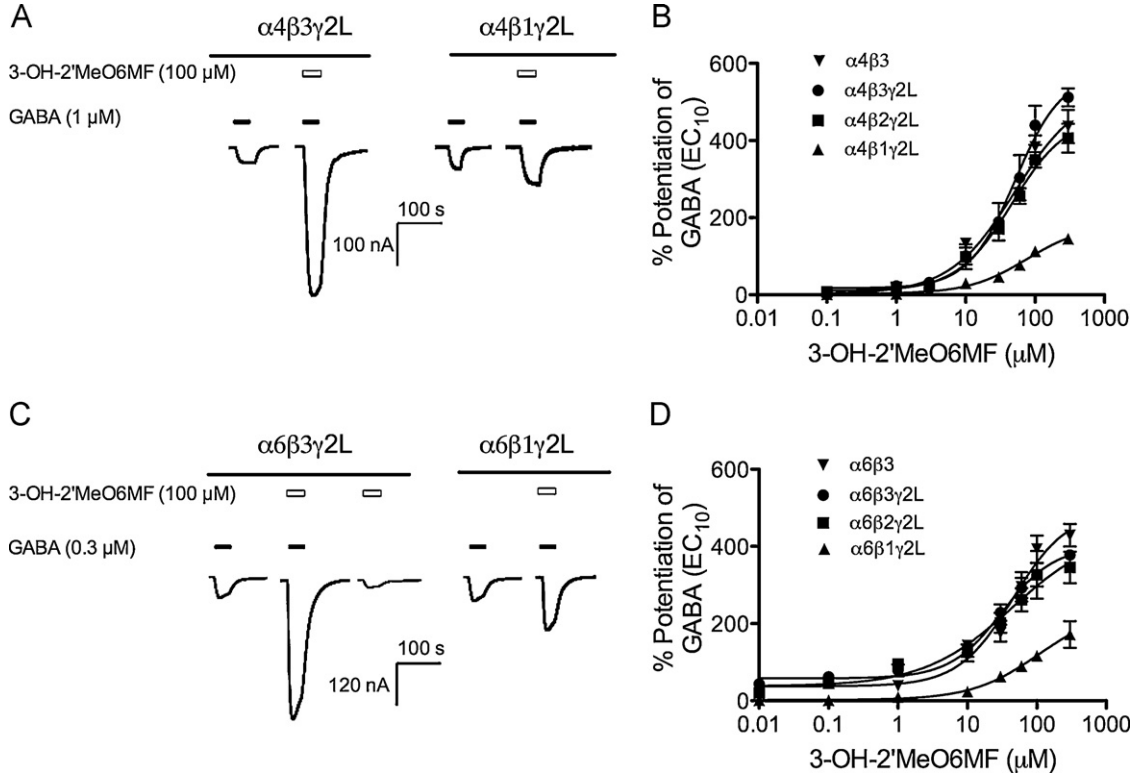

Fig. 4. 3-OH-2′MeO6MF is a positive modulator of α4/6β2/3γ2L $GABA_A$ receptors expressed in *Xenopus* oocytes. (A) Representative trace of oocytes expressing α4β3γ2L (left panel) and α4β1γ2L (right panel). 3-OH-2′MeO6MF (100 μM) potentiates the response to GABA (EC_{10}) by 350% and 150% respectively, indicating a greater efficacy for the α4β3γ2L $GABA_A$ receptor. (B) Concentration–response curves for 3-OH-2′MeO6MF at recombinant α4β3 (▼), α4β3γ2L (●), α4β2γ2L (■) and α4β1γ2L (▲) $GABA_A$ receptors. (C) Representative trace of oocytes expressing α6β3γ2L (left panel) and α6β1γ2L (right panel). 3-OH-2′MeO6MF (100 μM) potentiates the response to GABA (EC_{10}) by 380% and 160% respectively, indicating a greater efficacy for the α6β3γ2L $GABA_A$ receptor. (D) Concentration–response curves for 3-OH-2′MeO6MF at recombinant α6β3 (▼), α6β3γ2L (●), α6β2γ2L (■) and α6β1γ2L (▲) $GABA_A$ receptors. Data are mean ± S.E.M. (n = 4–6 oocytes).

α3/5β1–3γ2L receptors ($n = 4$, Mann–Whitney test: $p > 0.05$; supplementary data Fig. S1), indicating that the type of α-subunit is important for activity. Table 1 summarizes the effects of 3-OH-2′MeO6MF at all receptor combinations tested.

We then evaluated the activity of 3-OH-2′MeO6MF on α4β1–3γ2L and α6β1–3γ2L receptors to explore the role of the α4/6-subunits. α4β3 and α6β3 binary combinations were also included to evaluate the contribution of the γ2L-subunit in the modulation. 3-OH-2′MeO6MF potentiated GABA-induced currents at α4/6β3 and α4/6β2/3γ2L receptors with similar activities and efficacies, indicating that the γ2L-subunit does not affect the modulation (Fig. 4; Table 1).

In contrast, the type of β-subunit played a significant role in the efficacy and to a lesser extent, potency. Like α1- and α2-containing $GABA_A$ receptors, there was a reduction in the efficacy produced by 3-OH-2′MeO6MF at α4- and α6-subunits combined with the β1- and γ2L-subunits. Substituting the β1- for either the β2- or β3-subunit resulted in a significant increase in efficacy (ANOVA followed by Dunnett's multiple comparison test; $F_{2,6} = 54.64$ $p < 0.001$). Fig. 4A shows the effect of 3-OH-2′MeO6MF (100 μM) enhancing the response elicited by a low concentration of GABA (EC_{10}) at α4β3γ2L (left panel) and α4β1γ2L (right panel). 3-OH-2′MeO6MF (100 μM) enhanced the response elicited by GABA by 439 ± 51% and 113 ± 12%, respectively. The concentration–response curves for 3-OH-2′MeO6MF at α4β1–3γ2L-containing receptors are shown in Fig. 4B, and clearly show the differences in efficacy between the β1- and β2/3-containing $GABA_A$ receptors.

A similar result was observed with the α6-containing receptors. Fig. 4C shows the effect of 3-OH-2′MeO6MF (100 μM) enhancing the response elicited by a low concentration of GABA (EC_{10}) at α6β3γ2L (left panel) and α6β1γ2L (right panel). 3-OH-2′MeO6MF (100 μM) enhanced the response elicited by GABA by 326 ± 61% and 110 ± 3.5%, respectively. In addition, the potency of 3-OH-2′MeO6MF was also reduced at the β1-containing receptors (Table 1). The decreasing order of efficacy at α4- and α6-containing receptors is α4β3 > α4β2γ2L > α6β3 ≈ α4β3γ2L ≈ α6β2γ2L ≈ α6β3γ2L > α6β1γ2L ≈ α4β1γ2L. At α6β3γ2L receptors, 3-OH-2′MeO6MF (100 μM) evoked a small current in the absence of GABA that equated to less than 3% of maximum GABA-induced response (Fig. 4C left panel).

3.2.2. 3-OH-2′MeO6MF directly activates extrasynaptic α4β1–3δ and α6β1–3δ $GABA_A$ receptors

$GABA_A$ receptors without either the γ- or δ-subunit are highly sensitive to inhibition by Zn^{2+}, whereas those expressing a γ- or δ subunit are not [37,38]. Thus, in order to evaluate the effect of the δ-subunit on 3-OH-2′MeO6MF activity, oocytes expressing α4β3δ receptors were initially screened with zinc (Zn^{2+}; 100 nM and 1 μM) in the presence of GABA (10 μM) to determine whether the δ-subunit was incorporated. Zn^{2+} (100 nM and 1 μM) attenuated the response elicited by GABA by 60 and 90% of respectively at α4β3 receptors, whereas at α4β3δ receptors, Zn^{2+} (100 nM and 1 μM) inhibited the response elicited by GABA by only 5% and 10% respectively (supplementary data Fig. S2). This finding demonstrates that the δ-subunit is in fact incorporated in to the receptor complex. At α4β1–3δ $GABA_A$ receptors, GABA produced a concentration-dependent increase in the inward whole-cell currents until a maximal response was reached (100–2800 nA).

3-OH-2′MeO6MF had no direct effects at α4β3 and α6β3 receptors but directly activated α4β1–3δ and α6β1–3δ $GABA_A$ receptors indicating that the δ-subunit is required for the activation and provides an indication that the δ-subunit is indeed incorporated (Fig. 5A). 3-OH-2′MeO6MF did not enhance the

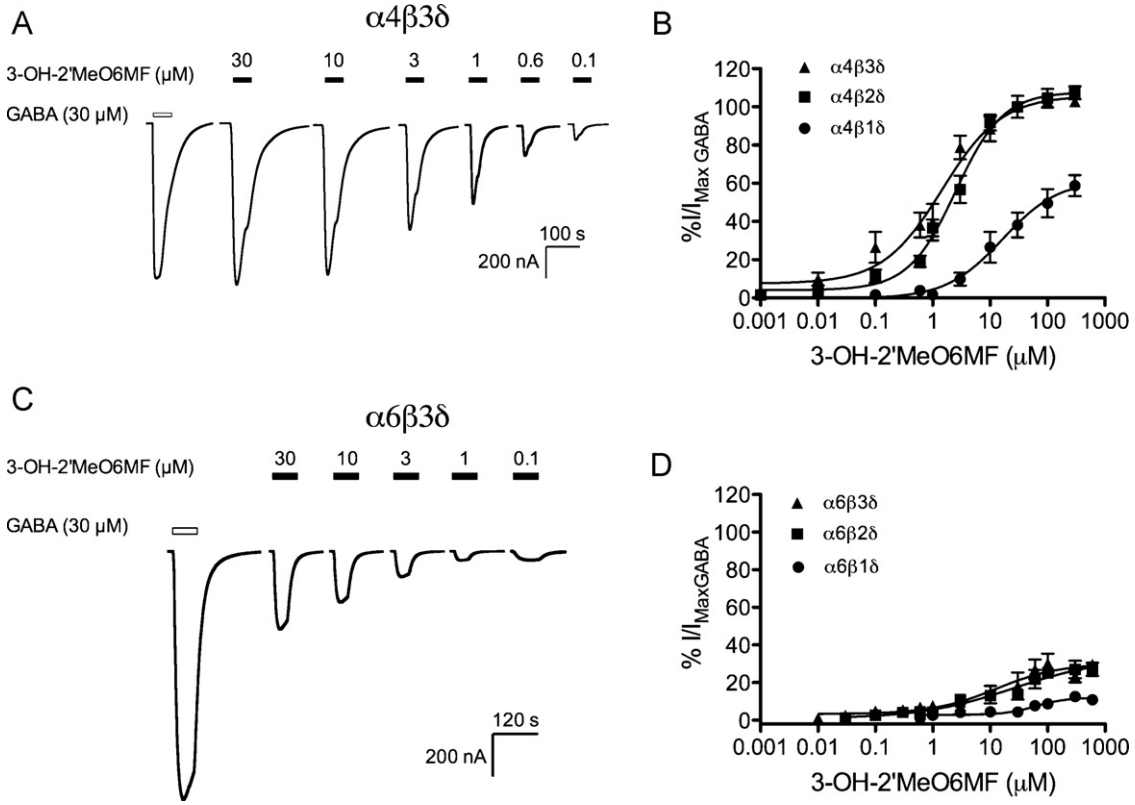

Fig. 5. 3-OH-2′MeO6MF directly activates α4β1–3δ and α6β1–3δ receptors expressed in oocytes. (A) Current trace showing the agonist effect of increasing concentrations of 3-OH-2′MeO6MF on recombinant α4β3δ $GABA_A$ receptors against the maximal effect of GABA (30 μM). (B) Concentration–response curves for 3-OH-2′MeO6MF at α4β1–3δ $GABA_A$ receptors. (C) Current trace showing the agonist effect of increasing concentrations of 3-OH-2′MeO6MF on recombinant α6β3δ $GABA_A$ receptors against the maximal effect of GABA (30 μM). (D) Concentration–response curves for 3-OH-2′MeO6MF at α6β1–3δ $GABA_A$ receptors. Data points represent mean ± S.E.M. ($n = 4$–6 oocytes).

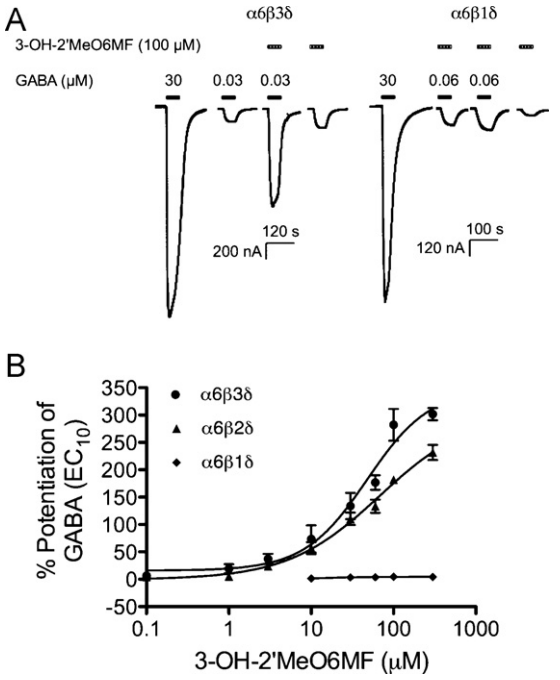

Fig. 6. 3-OH-2′MeO6MF is also a positive modulator of α6β2/3δ $GABA_A$ receptors expressed in *Xenopus* oocytes. (A) Representative trace of oocytes expressing α6β3δ (left panel) and α6β1δ (right panel). 3-OH-2′MeO6MF (100 μM) potentiates the response to GABA (EC_{10}) by 330% and 15% respectively, indicating a greater efficacy for the α6β3δ $GABA_A$ receptor. (B) Concentration–response curves for 3-OH-2′MeO6MF at recombinant α6β3δ (●), α6β2δ (▲) and α6β1δ (◇) $GABA_A$ receptors. Data points represent mean ± S.E.M. (n = 4–6 oocytes).

response of GABA (EC_{10}) at α4β1–3δ receptors (supplementary data Fig. S3). 3-OH-2′MeO6MF activated α4β1–3δ $GABA_A$ receptors in a concentration-dependent manner reaching a maximum response with 30 μM (Fig. 5A and B). The potency and efficacy for 3-OH-2′MeO6MF at α4β2/3δ were 1.4 μM (E_{max} = 106%) and 2.5 μM (E_{max} = 108%), respectively (Table 1). Substituting the β2- or β3- with the β1-subunit resulted in a significant reduction in both potency and efficacy ($F_{2,6} = 50.23$ $p < 0.0001$; ANOVA followed by Dunnett's post-hoc test; Fig. 5B).

Similarly at α6β1–3δ $GABA_A$ receptors, increasing concentrations of GABA produced a concentration-dependent increase in whole-cell currents until a maximal response was reached (current range between 100 and 5000 nA). 3-OH-2′MeO6MF directly evoked currents through α6β1–3δ receptors and reached a maximum response ranging from 12 to 30% (Fig. 5C and D), which were significantly lower than at α4β1–3δ receptors. The potencies for 3-OH-2′MeO6MF ranged between 12.7 μM and 62.2 μM (Table 2). Table 2 summarizes the direct activation exerted by 3-OH-2′MeO6MF extrasynaptic $GABA_A$ receptors.

Table 2
Summary of the direct actions of 3-OH-2′MeO6MF at δ-containing $GABA_A$ receptors expressed in *Xenopus* oocytes.

Receptor subtype	3-OH-2′MeO6MF EC_{50} value (μM) (95% CI)	% I_{max}	n_H	n
α4β1δ	16.4 (6.5–41.7)	62 ± 9	0.9	3
α4β2δ	1.40 (0.88–2.4)	106 ± 5	0.9	4
α4β3δ	2.52 (1.90–3.3)	108 ± 3	1.0	6
α6β1δ	62.2 (28.55–135.6)	12 ± 4	1.5	4
α6β2δ	25.8 (3.25–45.8)	29 ± 9	0.6	4
α6β3δ	12.7 (3.5–45.8)	30 ± 5	0.7	6

95% CI: 95% confidence interval. I_{max}: maximum activation produced by 3-OH-2′MeO6MF relative to GABA I_{max}. n_H: Hill coefficient. Data are presented as mean ± S.E.M. and 'n' is the number of individual oocytes tested

3-OH-2′MeO6MF was also able to potentiate currents evoked by GABA (EC_{10}) at α6β2/3δ receptors. 3-OH-2′MeO6MF maximally potentiated GABA-induced currents by 305 ± 5% (EC_{50} value of 67.8 μM) at α6β2δ and 348 ± 46% (EC_{50} value of 47.75 μM at α6β3δ receptors; Fig. 6A and B).

In order to determine whether 3-OH-2′MeO6MF acted as an orthosteric or allosteric agonist at α4β3δ receptors, we evaluated whether the $GABA_A$ receptor antagonist, bicuculline, could block competitively or non-competitively the currents produced by 3-OH-2′MeO6MF. In the presence of bicuculline (1 μM, 3 μM and 10 μM), the current produced by 3-OH-2′MeO6MF (30 μM) was attenuated in a concentration-dependent manner (Fig. 7A). A decrease in the maximal response produced by 3-OH-2′MeO6MF without affecting its EC_{50} value in the presence of bicuculline (3 and 10 μM). At these concentrations, bicuculline inhibited the maximal effect of 3-OH-2′MeO6MF by 20% and 40% respectively (Fig. 7B). The data infer that bicuculline attenuates the effect of 3-OH-2′MeO6MF in a non-competitive manner.

3.3. Effects of 3-OH-2′MeO6MF on general behavior and acute toxicity in mice

3-OH-2′MeO6MF was tested at doses 1, 10, 30 and 100 mg/kg. Overt behavioral effects were observed for each mouse at 0, 30 and 60 min, 24, 48 and 72 h and 1 week after injection. No overt acute toxicity was observed at any time point as judged from the observed lack of convulsions, respiratory distress (cyanosis, gasping), writhing, changes to reflex activity or mortality. 3-OH-2′MeO6MF produced only mild behavioral effects shortly after injection and 30 min after injection of 30 and 100 mg/kg in two of the eight animals injected at each dose but these effects did not persist when checked at 60 min after injection. The observed effects were included increased hypersensitivity (measured as

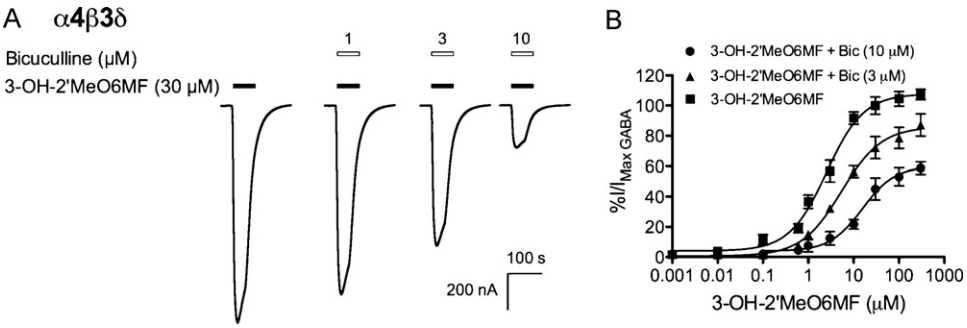

Fig. 7. Bicuculline inhibits 3-OH-2′MeO6MF-induced activation in a non-competitive manner at α4β3δ $GABA_A$ receptors. (A) Representative current trace showing a concentration-dependent inhibition of 3-OH-2′MeO6MF (30 μM) induced activation by bicuculline (1–10 μM). Horizontal bar shows the duration of drug application. (B) Concentration–response curves of 3-OH-2′MeO6MF alone (■) and in the presence of bicuculline (3 μM (▲) and 10 μM (●)). Currents are expressed as a percent of the peak current elicited by 30 μM GABA. Data are mean ± S.E.M. (n = 4–6 oocytes).

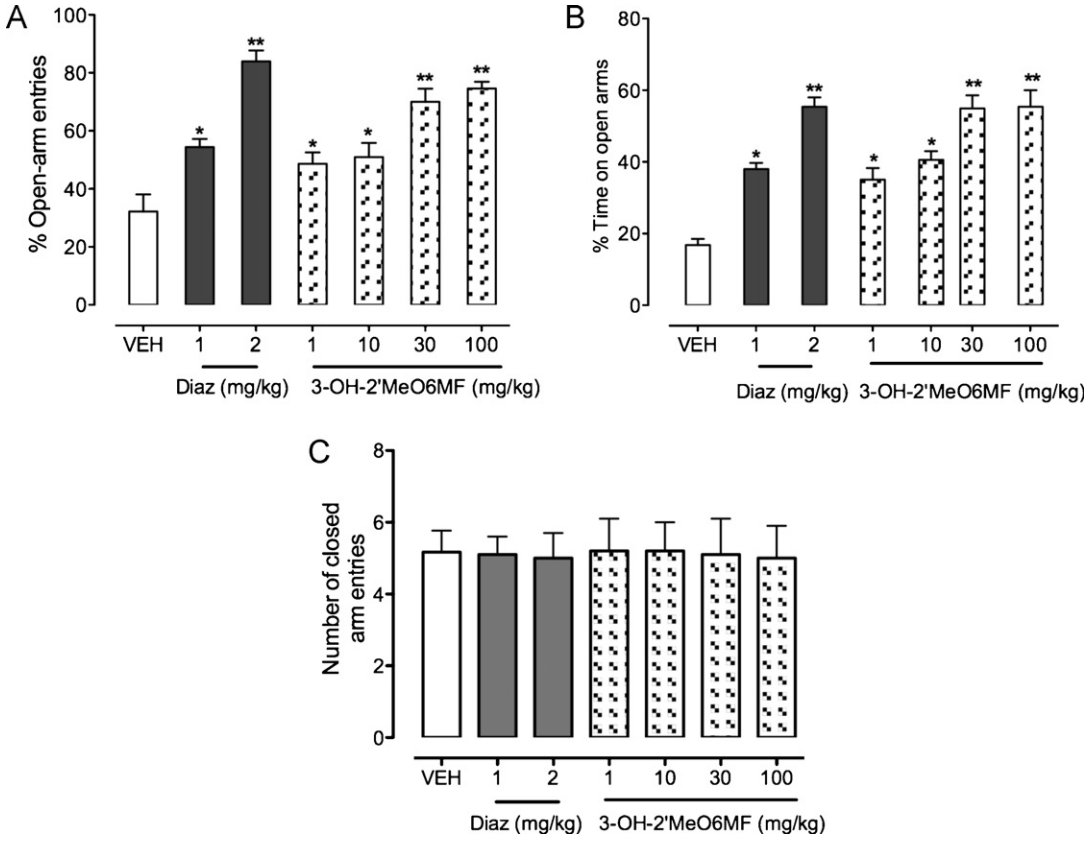

Fig. 8. Effect of diazepam (Diaz) and 3-OH-2′MeO6MF on the behavior of mice in the elevated plus maze. (A) % Open arm entries; (B) % time spent on the open arms; and (C) number of closed arm entries recorded over a 5 min session, 20 min after an *i.p.* injection of diazepam (1 and 2 mg/kg), 3-OH-2′MeO6MF (1–100 mg/kg) or vehicle; both diazepam and 3-OH-2′MeO6MF exerted anxiolytic effects in the elevated plus maze ($n = 8$ mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle group using ANOVA followed by Dunnett's multiple comparison test.

increased irritability, reactivity and aggressiveness during handling and towards cage mates), increased escape behavior (measured by general observation and finger escape behavior), and decreased tolerance to handling, corneal and pinna reflexes. An increased spontaneous activity and escape behavior was observed at doses of 30 mg/kg in the same two of eight animals after 30 and 60 min of injection. At 100 mg/kg, one of eight animals was found to be slightly ataxic after 30 min of injection, however this effect did not persist when checked 60 min post injection. At 24 h to 1 week after injection all animals seemed well with no observable changes in behavior, spontaneous activity or appearance.

3.4. 3-OH-2′MeO6MF exerts anxiolytic effects in the elevated plus maze

Both diazepam and 3-OH-2′MeO6MF showed a significant difference from vehicle in both % open arm entries (ANOVA – $F_{6,49} = 18.3$, $p < 0.001$) and % time spent in open arms of the

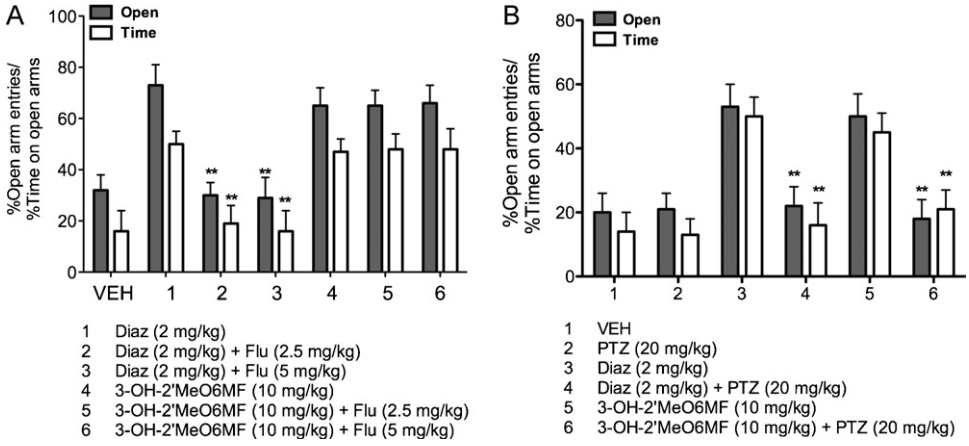

Fig. 9. (A) Effect of the % open arm entries and % time spent on open arms in mice given a 5 min test, 30 min after *i.p.* administration of vehicle, diazepam (2 mg/kg) or 3-OH-2′MeO6MF (10 mg/kg; $n = 16$ mice per group) in the presence of flumazenil (2.5 and 5 mg/kg) ($n = 8$ mice per group). (B) Effect of % open arm entries and % time spent on the open arms 20 min after *i.p.* treatment with diazepam (2 mg/kg), 3-OH-2′MeO6MF (10 mg/kg) in mice pretreated with PTZ (20 mg/kg), PTZ (20 mg/kg) alone, or vehicle ($n = 8$ mice per group). Bars represent mean ± S.E.M. Results were analyzed by performing separate one-way ANOVA among the groups. $^{**}p < 0.01$ compared to diazepam or 3-OH-2′MeO6MF alone using ANOVA followed by Dunnett's multiple comparison test.

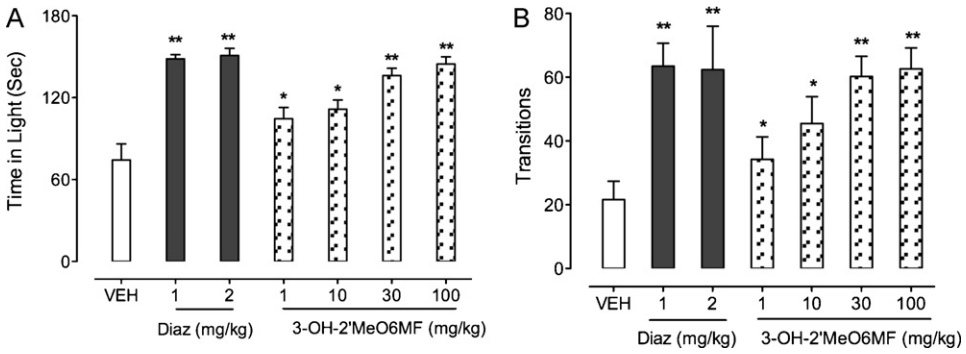

Fig. 10. Effect of diazepam (Diaz; 1 and 2 mg/kg) and the 3-OH-2′MeO6MF (1–100 mg/kg) on the behavior of mice in the light/dark box test. Bars represent mean ± S.E.M. (n = 8 mice per group) of (A). The time spent in the lit compartment; and (B) the number of transitions recorded over a 5 min session, 20 min after an *i.p.* injection of drugs or vehicle. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test.

elevated plus maze (Fig. 8A and B). Like diazepam at 1 mg/kg, 3-OH-2′MeO6MF significantly increased the % open arm entries and % time spent in open arms at 1 and 10 mg/kg ($p < 0.01$; ANOVA with Dunnett's post-hoc test). The increase in % open arm entries and % time spent in the open arms by 3-OH-2′MeO6MF at doses of 30 mg/kg and 100 mg/kg was comparable to that of diazepam at 2 mg/kg ($p < 0.001$). To investigate general locomotor activity, the number of closed arm entries was assessed and it was found that 3-OH-2′MeO6MF displayed no effect on general locomotor activity, as the number of closed arm entries was unaffected (Fig. 8C).

Co-administration of flumazenil (2.5 mg/kg or 5 mg/kg) did not reverse the increase in % open arm entries and % time spent in open arms exerted by 3-OH-2′MeO6MF (10 mg/kg) while both 2.5 and 5 mg/kg concentrations completely abolished both parameters in diazepam (2 mg/kg) treated animals (Fig. 9A).

Mice treated with PTZ (20 mg/kg) alone had no significant effect on the % open arm entries and % time spent in open arms (Fig. 9B). Pretreatment with PTZ (20 mg/kg) significantly decreased open arm entries (ANOVA – $F_{5,50} = 5.8$, $p < 0.01$) and time spent on open arms (ANOVA – $F_{5,50} = 6.4$, $p < 0.01$) exerted by exerted by 3-OH-2′MeO6MF (10 mg/kg; Fig. 9B) and diazepam (2 mg/kg) in the elevated plus maze (Fig. 9B). The data implicate that the effect of both diazepam and 3-OH-2′MeO6MF are most likely mediated by the GABAergic system.

3.5. 3-OH-2′MeO6MF exerts anxiolytic effects in the light–dark test

3-OH-2′MeO6MF significantly increased the time spent in light ($F_{6,49} = 16.42$, $p < 0.001$) and number of transitions between compartments ($F_{6,49} = 4.11$, $p < 0.001$) at doses 1, 10 mg/kg ($p < 0.01$), 30 and 100 mg/kg ($p < 0.001$). Diazepam also significantly increased both parameters at doses 1 and 2 mg/kg ($p < 0.001$). The results of light dark test are shown in Fig. 10A and B.

3.6. 3-OH-2′MeO6MF lacked sedative effects in the holeboard test

Administration of 3 mg/kg diazepam significantly decreased the number of head dips ($F_{6,49} = 2.8$ $p < 0.001$) and time spent head dipping ($F_{6,49} = 3.5$ $p < 0.001$) (Fig. 11A and B). In contrast, 3-OH-2′MeO6MF (1–100 mg/kg) did not significantly alter the number of head dips and time spent in head dipping ($p > 0.05$) demonstrating that 3-OH-2′MeO6MF according to this test, it lacked sedative effects at the anxiolytic dose range.

3.7. 3-OH-2′MeO6MF lacked locomotor activity in the actimeter

The motor effects of treatments are shown in Fig. 11C. ANOVA followed by Dunnett's post-hoc comparison test showed that

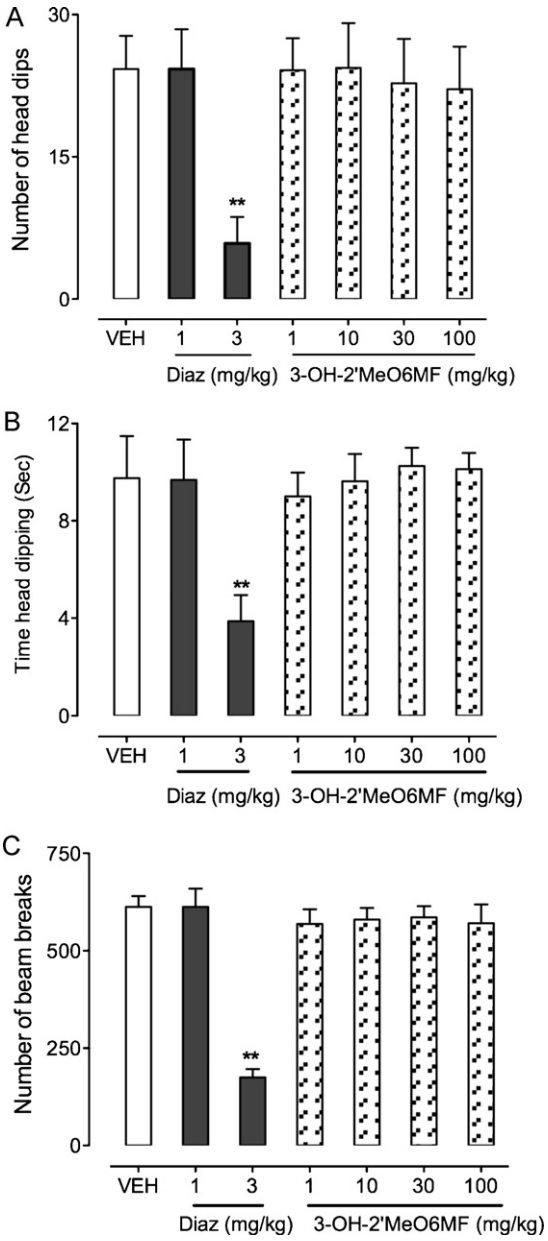

Fig. 11. Effect of diazepam (Diaz; 1 and 3 mg/kg) and the 3-OH-2′MeO6MF (1–100 mg/kg) on the behavior of mice in the holeboard and actimeter tests. Bars represent mean ± S.E.M. (n = 8 mice per group) of (A). Number of head dips; (B) time head dipping; and (C) number of beam breaks recorded over a 5 min session, 20 min after an *i.p.* injection of drugs or vehicle. $^{**}p < 0.01$ compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test.

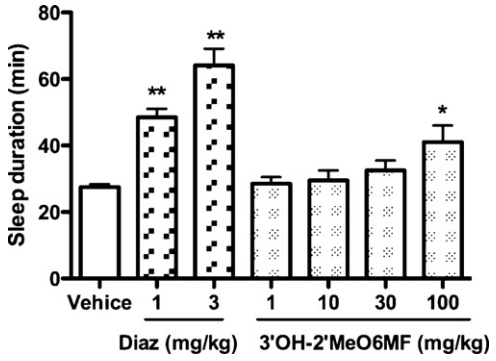

Fig. 12. Effect of diazepam (1 and 3 mg/kg) and 3′OH-2′MeO6MF (1–100 mg/kg) on thiopental-induced sleep time in mice. Values represent mean ± S.E.M. from 8 animals per group. $^{*}p < 0.05$ or $^{**}p < 0.01$ compared to vehicle group, ANOVA followed by Dunnett's multiple comparison test; $F_{6,49} = 16.02$.

diazepam at 3 mg/kg significantly ($F_{6,49} = 19.60$ $p < 0.001$) decreased the number of beam breaks and reduced locomotor activity while 3-OH-2′MeO6MF did not affect the locomotor activity at the doses tested.

3.8. High doses of 3-OH-2′MeO6MF increased thiopental-induced sleep time

Like diazepam (1 and 3 mg/kg; $F_{6,49} = 16.2$ $p < 0.01$; One-way ANOVA followed by Dunnett's post-hoc multiple comparison test), 3-OH-2′MeO6MF significantly increased the duration of thiopental-induced sleep time at the highest dose of 100 mg/kg ($F_{6,49} = 16.2$ $p < 0.05$; Fig. 12).

3.9. 3-OH-2′MeO6MF did not induce myorelaxant effects in the horizontal wire test

Mice treated with 3 mg/kg diazepam revealed deficits in their performance in the horizontal wire test in comparison to vehicle. Compared to diazepam, 3-OH-2′MeO6MF at doses 1, 10 and 30 did not result in a significant change in the performance of mice in the horizontal wire test, however at 100 mg/kg there was a tendency of reduction in the ability of mice grasping the wire but this effect was not significant ($p = 0.497$: Fisher's exact test; Table 3).

4. Discussion

In the present study, we investigated the ability of a synthetic flavonoid, 3-OH-2′MeO6MF to activate or modulate 29 different $GABA_A$ receptor subtypes recombinantly expressed in *Xenopus* oocytes, including the extrasynaptic α4/6βδ receptors. The results indicate that 3-OH-2′MeO6MF differentially affect $GABA_A$ receptor subtypes.

3-OH-2′MeO6MF modulated all γ2L-containing receptors that contained α1/2/4/6- but not α3/5-subunits. The modulatory effects of 3-OH-2′MeO6MF at αβγ2L receptors were not attenuated by flumazenil nor were the effects dependent on the γ2L-subunit, as 3-OH-2′MeO6MF also potentiated GABA-evoked currents at α–β combinations. Given that 3-OH-2′MeO6MF did not displace [^{3}H]flunitrazepam binding implicates that 3-OH-2′MeO6MF does not bind to the benzodiazepine binding site. The affinities of 3-OH-2′MeO6MF for these receptor combinations were not significantly different but the efficacy of 3-OH-2′MeO6MF at α2β2/3γ2L $GABA_A$ receptor subtypes was 4–11-fold greater compared to all other γ2L-containing receptors.

Preference for α2β2γ2L $GABA_A$ receptors has been reported for certain flavonoids including 6-hydroxyflavone [39], 6-methylflavanone [29], 3-acetoxy-4′-methoxyflavan (Fa131) [22] with the selectivity based on efficacy as distinct from potency. However there are many differences in their pharmacology and chemical properties. For example Fa131 is a flavan-3-ol possessing two chiral centers. It exerts both direct activating and modulating actions at α2β2γ2L $GABA_A$ receptors, while 3-OH-2′MeO6MF is a flavonoid with no chiral center and is devoid of direct actions at this receptor subtype.

Of the flavonoids reported thus far, only 3-OH-2′MeO6MF and its analogue 2′-methoxy-6-methylflavone (2′MeO6MF) are known to be devoid of activity at α3- and α5-containing receptors: 2′MeO6MF was recently shown to have a similar selectivity profile for α1-, α3- and α5- but not for α2-containing $GABA_A$ receptors [40]. At α2β2/3γ2L $GABA_A$ receptors, 2′MeO6MF acted as an allosteric agonist devoid of modulatory effects. The sole structural difference between 2′MeO6MF and 3-OH-2′MeO6MF is the hydroxyl in the 3-position of the phenylbenzopyran ring system; this hydroxyl appears to be the chemical trigger converting an allosteric activator to a positive modulator at these receptors, possibly by altering the population of rotamers around the bond joining rings B and C.

Furthermore, 3-OH-2′MeO6MF but not 2′MeO6MF showed significant selectivity for β2/3- over β1-containing $GABA_A$ receptors; a selectivity profile similar to the pharmacology observed for lorecrezole, etomidate and certain nonsteroidal anti-inflammatory agents [41]. In the case of 3-OH-2′MeO6MF, the efficacy difference for β2/3- over β1- at α2-containing receptors is as high as 10-fold, whereas the efficacy difference for lorecrezole, etomidate and the nonsteroidal anti-inflammatory agents at α1-containing receptors ranged between 1.5 and 4-fold [41].

3-OH-2′MeO6MF also acted as an agonist, directly activating extrasynaptic α4β1–3δ receptors with a minimum 10-fold higher affinity for α4β2/3δ over α4β1δ or γ2L-containing receptors. The direct effects were devoid of any potentiating effects at these combinations. Interestingly bicuculline, a GABA-site competitive antagonist, blocked the actions of 3-OH-2′MeO6MF in a non-competitive manner at α4β3δ receptors, strongly inferring that 3-OH-2′MeO6MF binds to an allosteric site.

The efficacy of 3-OH-2′MeO6MF at α4β2/3δ extrasynaptic receptors was similar to GABA, while acted as a partial agonist at α4β1δ $GABA_A$ receptors indicating 3-OH-2′MeO6MF has a preference for β2/3- over β1-subunits. Given that 3-OH-2′MeO6MF was at least 10-fold more potent for the δ-containing receptors than for the γ2L-containing receptors may indicate that the binding site(s) differ between δ- and γ-containing receptors.

3-OH-2′MeO6MF exerted both direct and modulatory effects at α6β2/3δ $GABA_A$ receptors, similar to what is observed with, for example, neurosteroids [42] and propofol [43]. At α6β1δ $GABA_A$ receptors, 3-OH-2′MeO6MF directly activated the receptor with

Table 3
Performance of mice in the horizontal wire test. Percentage of mice grasping wire after *i.p.* administration of 3-OH-2′MeO6MF (1, 10, 30 and 100 mg/kg) and diazepam (3 mg/kg) and vehicle.

Treatment	Dose (mg/kg, *i.p.*)	Wire test (% of mice grasping wire)
Vehicle	–	100
Diazepam	3	75**
3-OH-2′MeO6MF	1	100
	10	100
	30	100
	100	98

$^{**}p < 0.01$; different from vehicle: Fisher's exact test.

significantly reduced efficacy. The pharmacological profile of 3-OH-2′MeO6MF at δ-containing $GABA_A$ receptors also differed to retrochalchones (a class of molecules related to flavonoids) [26]. The retrochalchones acted in a manner similar to known $GABA_A$ modulators such as barbiturates, etomidate, propofol, and neurosteroids in that they had a tri-modal action: directly activating the receptor; enhancing GABA effects at low concentrations; and inhibiting GABA at higher concentrations.

Numerous studies have shown that flavonoids readily permeate the blood–brain barrier [19,44,45] and exert anxiolytic effects [18,21,22,39,46]. As 3-OH-2′MeO6MF shows clear preference for α2β2/3γ2L and α4β2/3δ extrasynaptic receptors, we subsequently evaluated this compound using *in vivo* mouse models of anxiety: the elevated plus maze and light dark tests [47,48]. The anxiolytic-like effects induced by diazepam and not 3-OH-2′MeO6MF could be attenuated by flumazenil. The flumazenil-insensitive effects exerted *in vivo* by 3-OH-2′MeO6MF support the electrophysiological and binding studies, indicating that 3-OH-2′MeO6MF does not mediate its effects via the classical BDZ binding site. Instead the $GABA_A$ receptor antagonist, PTZ was able to attenuate the anxiolytic effects exerted by both diazepam and 3-OH-2′MeO6MF implicating that the GABAergic system is involved.

3-OH-2′MeO6MF did not exert any sedative effects as there was an increase in the exploratory parameters in the holeboard test, nor did it reduce spontaneous locomotor activity at any of the doses tested. In contrast, sedative and locomotor effects were observed with diazepam in these two tests.

3-OH-2′MeO6MF weakly potentiated the central depressant action of thiopental indicating some hypnotic effects at these doses, possibly via activity at α1- and or β2-containing $GABA_A$ receptors as this subunit has been implicated in the hypnotic effects of ethanol and gaboxadol [49]. In the horizontal wire test, no significant myorelaxant effects were observed at any of the doses tested for 3-OH-2′MeO6MF although at 100 mg/kg, there was a slight tendency for the mice to have reduced ability in grasping the wire. In contrast diazepam potentiated the central depressant action of thiopental indicating hypnotic effects and induced a clear-cut myorelaxant effect.

Interestingly there are dramatic differences in the *in vivo* profile between 3-OH-2′MeO6MF and its analogue 2′MeO6MF. 2′MeO6MF showed anxiolytic effects at low doses while at high doses sedative effects were observed with this compound. The sedative effects may be attributed to the non-selective action of 2′MeO6MF at α1-containing receptors.

The subtype selectivity profile and *in vivo* effects of 3-OH-2′MeO6MF support the hypothesis that α2-containing $GABA_A$ receptors may be involved in its anxiolytic effects. However given the wide dose range used in this study and that a dose as low as 1 mg/kg of 3-OH-2′MeO6MF can exert anxiolytic effects indicates that the α2-containing receptors may not be the only contributors to this effect. Indeed, the anxiolytic effects of neurosteroids [50–52], gaboxadol [53], low doses of alcohol [54] and AA29504 [55] have been attributed to their effects on extrasynaptic receptors. Thus, the direct activation of α4β2/3δ extrasynaptic $GABA_A$ receptors, by 3-OH-2′MeO6MF, may also be contributing to the anxiolytic effects of 3-OH-2′MeO6MF considering the higher potency of 3-OH-2′MeO6MF for δ- over γ-containing receptors. As we have not ruled out activity of 3-OH-2′MeO6MF for non-GABAergic sites, other targets may also contribute to the anxiolytic effects of this compound.

The myorelaxant effects of diazepam are thought to be mediated by α2-containing receptors, although α3- and α5-containing receptors are also involved [56,57]. However, a number of agents selective for α2-containing $GABA_A$ receptors are devoid of myorelaxant effects [22,58]. In this study, 3-OH-2′MeO6MF failed to exert myorelaxant effects despite its selective effects at α2-containing $GABA_A$ receptors, although at 100 mg/kg there was a tendency for 3-OH-2′MeO6MF to exert weak myorelaxant effects. In contrast, diazepam showed clear myorelaxant effects at the doses tested. The reasons why 3-OH-2′MeO6MF and other α2-selective agents [22,40] or α2/3-selective agents [39] have no or reduced myorelaxant effects is far from understood. It may be speculated that the myorelaxant effects are mediated in part by other subunits such as β1- and α6-subunits. Indeed reducing activity at β1- and not β2/3-containing $GABA_A$ receptors leads to a reduction in ataxia [59], while activation of α6-containing receptors located exclusively on cerebellar granule cells affects motor coordination [60]. Thus the limited activity of 3-OH-2′MeO6MF at these subtypes may serve to reduce myorelaxant effects.

In conclusion, classical BDZ's such as diazepam non-discriminate between $GABA_A$ receptors containing α1–3,5-containing $GABA_A$ receptors, and are regarded as non-selective modulators [61,62]. Functional studies have demonstrated that diazepam exhibits little variability in modulating these receptor subtypes [63,64]. We, and others [12,14] have shown that the administration of diazepam to animals leads to a series of pharmacological actions within a narrow dose range. The findings that 3-OH-2′MeO6MF is inactive at α3/5-containing receptors, has greater efficacy in modulating α2β2/3γ2L and higher affinity for the extrasynaptic α4β2/3δ receptors, implicates 3-OH-2′MeO6MF as an important tool with a unique pharmacological profile to study $GABA_A$ receptor subtypes, exhibiting anxiolytic effects without significant sedative, myorelaxant and motor coordination effects.

Conflicts of interest statement

The authors have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.09.002.

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