

RESEARCH PAPER

2'-Methoxy-6-methylflavone: a novel anxiolytic and sedative with subtype selective activating and modulating actions at GABA_A receptors

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Keywords

GABA_A receptors; *Xenopus*
oocytes; GABA (γ -aminobutyric
acid); dissociated hippocampal
neurons; flavonoids; anxiolytic
agents; sedative effects; α 1- and
 α 2-containing GABA_A receptors

Received

4 April 2011

Revised

12 June 2011

Accepted

28 June 2011

BACKGROUND AND PURPOSE

Flavonoids are known to have anxiolytic and sedative effects mediated via actions on ionotropic GABA receptors. We sought to investigate this further.

EXPERIMENTAL APPROACH

We evaluated the effects of 2'-methoxy-6-methylflavone (2'MeO6MF) on native GABA_A receptors in new-born rat hippocampal neurons and determined specificity from 18 human recombinant GABA_A receptor subtypes expressed in *Xenopus* oocytes. We used ligand binding, two-electrode voltage clamp and patch clamp studies together with behavioural studies.

KEY RESULTS

2'MeO6MF potentiated GABA at α 2 β 1 γ 2L and all α 1-containing GABA_A receptor subtypes. At α 2 β 2/3 γ 2L GABA_A receptors, however, 2'MeO6MF directly activated the receptors without potentiating GABA. This activation was attenuated by bicuculline and gabazine but not flumazenil indicating a novel site. Mutation studies showed position 265 in the β 1/2 subunit was key to whether 2'MeO6MF was an activator or a potentiator. In hippocampal neurons, 2'MeO6MF directly activated single-channel currents that showed the hallmarks of GABA_A Cl⁻ currents. In the continued presence of 2'MeO6MF the single-channel conductance increased and these high conductance channels were disrupted by the γ 2(381–403) MA peptide, indicating that such currents are mediated by α 2/ γ 2-containing GABA_A receptors. In mice, 2'MeO6MF (1–100 mg·kg⁻¹; i.p.) displayed anxiolytic-like effects in two unconditioned models of anxiety: the elevated plus maze and light/dark tests. 2'MeO6MF induced sedative effects at higher doses in the holeboard, actimeter and barbiturate-induced sleep time tests. No myorelaxant effects were observed in the horizontal wire test.

CONCLUSIONS AND IMPLICATIONS

2'MeO6MF will serve as a tool to study the complex nature of the activation and modulation of GABA_A receptor subtypes.

Abbreviations

2'MeO6MF, 2'-methoxy-6-methylflavone; α 5A, 3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-a]phthalazine; DMSO, dimethyl sulphoxide; M1–M4, transmembrane domains 1–4; PTZ, pentylenetetrazole; TES, N-tris(hydroxyl-methyl)methyl-2-amino ethane sulphonic acid; TPA023, 7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine

Introduction

GABA_A receptors not only mediate fast synaptic inhibition in the mammalian brain, but also participate in the regulation of the resting potential of neurons by activating tonic currents in extrasynaptic locations of neurons (Farrant and Nusser, 2005). Therefore, it is not surprising that these receptors are implicated in a variety of neuropsychiatric disorders including anxiety, sleep, cognitive and mood disorders, epilepsies and schizophrenia (Olsen and Sieghart, 2009).

GABA_A receptors are members of the cys-loop family of receptors and form a central chloride ion channel from five homologous subunits. Each subunit contains a large amino-terminal extracellular domain, four transmembrane domains from M1 to M4 and a large intracellular domain between M3 and M4 (Sieghart, 2006). Within the GABA_A receptor class, there exists a great deal of receptor heterogeneity, as different protein subunits (including α 1–6, β 1–3, γ 1–3, δ) can assemble in various combinations. Receptor heterogeneity exists *in vivo*, with α 1-containing and α 2/3-containing isoforms being the most common subunit combinations. These receptors are believed to exist in a 2:2:1 stoichiometry *in vivo* (McKernan and Whiting, 1996). The subunit composition determines the GABA sensitivity, the pharmacological properties of the GABA_A receptor (Olsen and Sieghart, 2009) and the receptor interaction partners mediated by the larger intracellular loop between M3 and M4 (Tierney, 2011).

A number of clinically important drugs including benzodiazepines, neurosteroids, barbiturates, general anaesthetics such as propofol, etomidate and volatile anaesthetics act as positive allosteric modulators of this receptor (Olsen and Sieghart, 2009). Many of these agents possess dual effects, also directly activating GABA_A receptors at high concentrations (Orser *et al.*, 1994).

The α 1-containing GABA_A have been proposed to be responsible for the sedative action of benzodiazepines; the α 2 and/or the α 3 subtypes have been suggested to mediate the anxiolytic activity and the myorelaxation effects, whereas the α 5 subtype has been associated with cognition processes. Given the heterogeneous nature and complex pharmacology of GABA_A receptors, it is therefore important to develop subtype-specific ligands with potentially fewer side effects (Rudolph *et al.*, 1999).

Flavonoids are characterized by a phenylbenzopyran chemical structure. They are secondary plant metabolites that are consumed by humans in significant amounts from beverages, fruits and vegetables (Mattila *et al.*, 2000). Such compounds are reported to have significant health benefits being involved in a variety of biological processes. Recently, flavonoids such as 6-methylflavone (Hall *et al.*, 2004), 6-methylflavanone (Hall *et al.*, 2005), 6-hydroxyflavone (Ren *et al.*, 2010) and 6,2'-dihydroxyflavone (Wang *et al.*, 2007) have also been shown to modulate GABA_A receptors. As flavonoids can vary in activity and function, the development of subtype-selective ligands based on the flavonoid core structure may lead to selective treatment of GABA system-associated pathology (Hanrahan *et al.*, 2011; Jager and Saaby, 2011; Nilsson and Sterner, 2011).

Of particular interest are the synthetic flavonoids, 6-methylflavone and 6-methylflavanone, which acted as flumazenil-insensitive positive modulators of recombinant

GABA_A receptors expressed in oocytes (Hall *et al.*, 2004; 2005). In the case of 6-methylflavanone it was shown that subtype selectivity for α 2- over α 1-containing GABA_A receptors in terms of relative efficacy could be achieved (Hall *et al.*, 2005). Indeed targeting specific GABA_A receptors through subtype-selective efficacy as distinct from potency has been validated for other modulators of GABA_A receptors, including the non-sedating anxiolytic, 7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine (TPA023) and the cognitive enhancer, alpha 5IA [3-(5-methylisoxazol-3-yl)-6-[(1-methyl-1,2,3-triazol-4-yl)methoxy]-1,2,4-triazolo[3,4-a]phthalazine] (Atack, 2010a,b).

In the present study, we examined the actions of an analogue of 6-methylflavone, 2'-methoxy-6-methylflavone (2'MeO6MF) on human recombinant α 1–3, β 1–3 γ 2L and α 1/2 β 2 GABA_A receptors expressed in *Xenopus* oocytes and on native GABA_A receptors in rat newborn hippocampal patches that express multiple receptor subtypes (Seymour *et al.*, 2009). In this study, we report that 2'MeO6MF positively modulates α 1 β 1–3 γ 2L GABA_A receptors, whereas at α 2 β 2/3 γ 2L GABA_A receptors, the flavonoid directly activates these receptors in the absence of GABA. In contrast, 2'MeO6MF positively modulates α 2 β 1 γ 2L GABA_A receptors. The 15' residue located at the extracellular end of the second transmembrane domain of the β -subunit was found to be important in determining activation versus modulatory effects for α 2-containing GABA_A receptors. As 2'MeO6MF showed direct and modulatory effects on α 1/2 containing GABA_A receptors we evaluated its effects in mouse models of sedation and anxiety.

Methods

Drugs

GABA, bicuculline, gabazine, flumazenil and DMSO were purchased from Sigma (St. Louis, MO, USA), whereas diazepam was purchased from Apin Chemicals LTD (Oxon, UK). [³H]-Muscimol and [³H]-flunitrazepam (36.6 Ci·mmol⁻¹) were obtained from Perkin Elmer (Boston, MA, USA). Pentylenetetrazole (PTZ) was purchased from Tocris Biosciences (Bristol, UK). 2'MeO6MF was synthesized according to Supporting Information Scheme 1 and briefly described in the following.

The synthesis of 2'MeO6MF is depicted in Scheme 1 (Supporting Information). The synthesis involves one-step modified Schotten–Baumann reaction, and a Baker–Venkataraman rearrangement followed by acid catalysed cyclization. Substituted 2-hydroxydibenzoyl methane I was prepared in one-step from 2-hydroxy-5-methyl acetophenone and 2-methoxybenzoyl chloride in the presence of lithium diisopropylamide. Intermediate I was further cyclized in the presence of glacial acetic acid and concentrated H₂SO₄ at 120°C to produce the crude product. The crude product was purified by flash chromatography, treated with activated charcoal, filtered through celite and recrystallized twice from acetone to produce 2'MeO6MF (≥98% by NMR and elemental analysis). The assigned structure was in agreement with the ¹H (400 MHz) and ¹³C (100.5 MHz) NMR spectra. Further details concerning synthesis, analytical and spectral characterization data are given in Supporting Information.

Animal use

All animal care and experimental procedures 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.

GABA receptor subunit constructs

Human $\alpha 1$, $\alpha 2$, $\beta 2$ and $\gamma 2L$ DNA in pCDM8 were provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK), $\alpha 3$ and $\beta 3$ in pGEMHE, $\alpha 5$ in pCDNA3 and $\beta 1$ in pCDM8 were a gift from Dr Bjarke Ebert (H. Lundbeck A/S, Valby, Denmark). Human $\rho 1$ DNA in pCDNA1.1 was provided by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA).

Site-specific mutations were introduced into the cDNAs of the GABA_A receptor subunits using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutant clones were submitted to complete sequencing to corroborate the successful incorporation of the point mutation and absence of spurious mutations.

cDNA vectors 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).

Expression of recombinant GABA receptors in *Xenopus oocytes*

The procedures involving the use of female *Xenopus laevis* were carried out according to previously described protocols (Hall *et al.*, 2004) and were approved by the Animal Ethics Committee of the University of Sydney. In brief, female *Xenopus laevis* were anaesthetized by immersion in 0.17% 3-aminobenzoic acid ethyl ester with 0.02% NaCl for 10–15 min, and a lobe of the ovaries was surgically removed. The lobe was rinsed with oocyte releasing buffer 2 (OR2) (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) and treated with collagenase A (2 mg·mL⁻¹ in OR2, Boehringer Mannheim, Germany) for 1–2 h to separate oocytes from connective tissue and follicular cells. Released oocytes were rinsed in ND96 'wash' solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5). Stages V–VI oocytes were collected and kept at 18°C in ND96 'storage' solution (ND96 'wash' solution supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 µg·mL⁻¹ gentamycin).

Oocytes were injected with mRNA in the ratio 1:1:10 (5:5:50 ng) for $\alpha 1$ – $\beta 3$ – $\gamma 2L$ to favour incorporation of the γ subunit (Boileau *et al.*, 2002), 1:1 for $\alpha 1$ / $\beta 2$ and $\rho 1$ (40–60 ng 50 nL⁻¹) before storing at 18°C. Sham-injected oocytes were prepared by injection with nuclease-free water.

Two to five days after injection of RNA, receptor activity was measured by two-electrode voltage clamp recording as previously reported (Hall *et al.*, 2004). In recordings using the flavonoid 2'MeO6MF the DMSO concentration in the perfusate was 0.8% and did not produce any alteration in the recordings.

Oocytes expressing $\alpha 1$ – $\beta 3$ – $\gamma 2L$ GABA_A receptors were screened with 10 µM Zn²⁺ in the presence of two concentra-

tions of GABA (10 and 100 µM) to ensure that the $\gamma 2L$ subunit was incorporated. GABA_A receptors without a $\gamma 2L$ -subunit are sensitive to inhibition by Zn²⁺, whereas those expressing a $\gamma 2L$ subunit are not (Hosie *et al.*, 2003).

Data analysis

The currents elicited directly by 2'MeO6MF were normalized as $I\%_{(comp)} = [I_{comp}/I_{MAX(GABA)}] \times 100$, where I_{comp} is the peak amplitude of the current produced by 2'MeO6MF and $I_{MAX(GABA)}$ is the maximal current produced by GABA measured in each individual cell.

The enhancement effects of 2'MeO6MF were measured in the presence of a GABA concentration eliciting between 7 and 10% of the maximal current amplitude (EC_{7–10}). The EC_{7–10} was determined at the beginning of each experiment. Enhancement of the chloride current was defined as % potentiation = $[(I_{drug+GABA} - I_{GABA})/I_{GABA}]100$ where $I_{drug+GABA}$ is the control GABA current in the presence of a given concentration of drug, and I_{GABA} is the control GABA current. In all cases, a 4–6 min washout period was allowed between drug applications to avoid receptor desensitization.

Standardized responses were plotted against drug concentration on a semi-logarithmic scale using Prism v5 GraphPad software, San Diego, CA, USA. Logarithmically transformed data were tested for significance using a linear regression fit. Provided the slope of the curve significantly deviated from zero, a nonlinear regression fit was performed using a sigmoidal dose–response (variable slope), the equation of which is

$$\%I = \%I_{max} / \{1 + [EC_{50}/[A]]^{n_H}\}$$

where [A] is the agonist concentration, I is the current and I_{max} is the maximum current. EC₅₀ is the concentration of agonist that produces a response that is 50% of the maximum current and n_H is the Hill coefficient. EC₅₀ values are expressed as mean with 95% confidence intervals from a number of different cells over at least two batches.

Hill coefficients (n_H) are expressed as mean $\pm$ SEM. The statistical significance of differences between mean EC₅₀ values was determined by Student's *t*-test and one-way ANOVA.

Neuronal cell culture and electrophysiology

Newborn rat hippocampal neurons were dissociated from Wistar rats and maintained in culture as described previously (Curmi *et al.*, 1993). Single-channel recordings in the inside-out configuration were acquired after 7–15 days. The external solution contained (mM): 135 NaCl, 3 KCl, 2 CaCl₂, 5 MgCl₂ and 10 TES (pH 7.3). Patch electrodes contained (mM): 138 choline chloride, 0.3 KCl, 7 MgCl₂, 10 TES (pH 7.3). Peptides were dissolved in DMSO (final concentration $\leq$ 0.01%), diluted to the required concentration with external solution and applied to the patch by exposing the pipette tip to flow tubes containing the test solutions (Everitt *et al.*, 2009). Currents were recorded with an Axopatch 200A or 200B current-to-voltage converter, filtered at 5–10 kHz and digitized at a frequency of 10–50 kHz using a Data Translation data acquisition card (DT3001-PGL, Data Transition Inc, Marlboro, MA, USA) interfaced with an IBM compatible computer. Current files were collected in Data collect (Mr B. Keys, ANU, Australia).

lia) and segments were analysed using Channel2 (Prof P.W. Gage and Mr M. Smith, ANU, Australia). For the analysis of data, average current amplitudes were measured from all-points probability histograms and subconductance states were measured directly from visual inspection using cursor-measured amplitudes. Single-channel conductance [γ (pS)] was calculated as the current amplitude [I (pA)] divided by the driving force [$\gamma = I/(E_{\text{pipette}} - E_{\text{Cl}})$].

Binding studies

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 previously (Ransom and Stec, 1988). On the day of the assay, the membrane preparation was quickly thawed, suspended in 40 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using an UltraTurax homogenizer (IKA, Wilmington, NC, USA), and centrifuged at 48 000 $\times g$ 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.

Binding assays. Membranes (100 ng of protein/aliquot) in Tris-HCl buffer (50 mM, pH 7.4) were incubated with either [³H]-muscimol (5 nM) and GABA (1 mM) or [³H]-flunitrazepam and diazepam (10 μ M) at 0°C for 60 min in a total volume of 250 μ L. GABA (1 mM) and diazepam (10 μ M) were used to determine non-specific binding. The binding reaction was terminated by rapid filtration through GF/C unilters (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) using a 24 channel cell harvester (Brandel, Gaithersburg, MD, USA) followed by washing with 3 $\times$ 250 μ L of ice-cold binding buffer. ULTIMA-GOLD liquid scintillation cocktail (Perkin Elmer) 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 Life and Analytical Sciences). Data are expressed as percentage of control specific binding. EC₅₀ values are expressed as mean $\pm$ SEM.

In vivo studies

Animals. All behavioural 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 0600 h). Illumination of the experimental room was 80 lux. Drugs were dissolved in the vehicle comprising of DMSO (5%), Tween 80 (1%) and saline (94%), and administered by i.p. injection in a volume of 5 μ L $\cdot$ g⁻¹. Sodium thiopental was dissolved in saline. All behavioural studies were carried out between 0900 and 1400 h.

General behavioural and acute toxicity tests in mice

The overt behavioural effects of 2'MeO6MF on mice after i.p. injection were assessed using the protocol detailed by Irwin (Irwin, 1968) and elsewhere (Chebib *et al.*, 2009). In brief, animal tails were marked with waterproof markers to assign

them to one of four groups each consisting of five animals for random blind allocation to one of four experimental conditions: vehicle; test dose 1; test dose 2; and test dose 3, which were 1, 10 and 100 mg $\cdot$ kg⁻¹.

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 behaviour; prehensile strength; and pupil size, according to the procedure prescribed by Irwin (Irwin, 1968).

Elevated plus maze

The apparatus consisted of two open arms (30 $\times$ 5 cm) and two closed arms (30 $\times$ 5 $\times$ 15 cm) made of black Plexiglas connected by an open central platform (5 $\times$ 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 centre 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 with a digital camera positioned above the maze and at the end of the test behavioural parameters were scored by the experimenter from observing the videos. To assess the involvement of the GABAergic system, animals were either pretreated i.p. with vehicle, flumazenil or PTZ. After individual trials, the apparatus was carefully cleaned with wet paper towel (mixture of ethanol, detergent and water) to remove any residue or odours. The % of open-arm entries and the % of time spent in the open arms were recorded as a measure of anxiety state (File and Pellow, 1985). The absolute number of closed arm entries was also recorded to assess effects on general locomotor activity.

Measurement of locomotor activity

Immediately after completing the plus maze assay, animals were placed in a box made of clear Plexiglas, with a floor of 30 $\times$ 15 cm and 15 cm high walls, which possesses 15 optical beams, which allows the measure of animal activity along a single axis of motion (Fernandez *et al.*, 2008). Spontaneous locomotor activity was measured for 5 min and expressed as total number of light beams crossed.

Holeboard assay

This assay was conducted in an automatic apparatus described previously (Fernandez *et al.*, 2008). Twenty minutes after injection with vehicle or the drug, animals were placed in the centre 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 (File and Pellow, 1985).

Light/dark box test

The light/dark apparatus consists of an acrylic box of dimensions 44 $\times$ 21 $\times$ 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 6×3 cm. The box possesses 16 light beams, 11 in the lit area and five 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 (Bourin and Hascoet, 2003).

Thiopental-induced loss of righting reflex

A sub-threshold dose of sodium thiopental ($40 \text{ mg}\cdot\text{kg}^{-1}$) 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 (Ferrini *et al.*, 1974).

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 (Bonetti *et al.*, 1982).

Statistical analysis

All data are expressed as mean $\pm$ SEM. 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 analysed using Fisher's exact test.

Nomenclature

The nomenclature of all molecular targets (receptors, ion channels, enzymes, etc.) cited in this work conforms to the *British Journal of Pharmacology's* Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

Binding studies

Binding studies were used to evaluate whether 2'MeO6MF could displace [^3H]-flunitrazepam and [^3H]-muscimol binding to rat brain synaptosomal membranes. Specific [^3H]-flunitrazepam binding was defined using diazepam ($10 \mu\text{M}$) and GABA (1 mM) and represented $>80\%$ of the total binding (data not shown). 2'MeO6MF did not displace [^3H]-flunitrazepam in the concentration range of 0.01 – $30 \mu\text{M}$; however, at 100 and $300 \mu\text{M}$, 2'MeO6MF weakly displaced [^3H]-flunitrazepam binding by 5 – 10% (data not shown). In contrast, 2'MeO6MF enhanced [^3H]-muscimol binding to rat brain synaptic membranes in a concentration-dependent manner yielding a mean E_{max} of $219.8 \pm 7.6\%$ and apparent $\text{EC}_{50} = 20.8 \pm 3.7 \text{ nM}$ ($n = 3$ independent experiments). This effect was comparable to diazepam, which also enhanced [^3H]-muscimol binding producing an E_{max} of $276.4 \pm 2.5\%$ and an $\text{EC}_{50} = 20.4 \pm 2.5 \text{ nM}$ ($n = 3$ independent experiments; Supporting Information Figure S1).

Electrophysiological studies

Expression of GABA receptors in Xenopus oocytes. Injection of $\alpha 1/2\beta 2$, $\alpha 1$ – $3,5\beta 1$ – $3\gamma 2\text{L}$ and $\rho 1$ receptor mRNA into *Xenopus* oocytes resulted in GABA sensitive channels. Increasing concentrations of GABA produced a concentration-dependent increase in the inward whole-cell currents until a maximal response was reached (20 – 3000 nA).

2'MeO6MF is a positive allosteric modulator at $\alpha 1$ -containing GABA_A receptors

Like GABA, 2'MeO6MF did not produce a response when tested against sham-injected oocytes ($n = 3$; data not shown). When tested alone 2'MeO6MF ($300 \mu\text{M}$) activated the receptor by $<2\%$ of the maximum effect of GABA. In contrast, 2'MeO6MF ($300 \mu\text{M}$) enhanced the response elicited by a low concentration of GABA (EC_{10}) in a concentration-dependent manner at recombinant $\alpha 1\beta 2\gamma 2\text{L}$ GABA_A receptors expressed in *Xenopus* oocytes (Figure 1A). Interestingly, upon washout, the higher concentrations of 2'MeO6MF produced a rebound current (Figure 1A), and this was observed in 60% of cells. This phenomenon has been observed previously with compounds such as mefenamic acid and etomidate, and may be due to interactions with residues in the second transmembrane region (Hill-Venning *et al.*, 1997; Halliwell *et al.*, 1999). In addition, 2'MeO6MF (1 – $300 \mu\text{M}$) enhanced the response elicited by GABA (EC_{10}) at recombinant $\alpha 1\beta 1,3\gamma 2\text{L}$ and $\alpha 1\beta 2$ GABA_A receptors (Figure 1B) without any direct activation (data not shown).

Flumazenil (1 and $10 \mu\text{M}$) (a competitive benzodiazepine antagonist) did not block the enhancement produced by 2'MeO6MF ($100 \mu\text{M}$) at $\alpha 1\beta 2\gamma 2\text{L}$ GABA_A receptors. At the higher concentration, flumazenil enhanced the effect of GABA by 42% . In contrast, flumazenil (1 and $10 \mu\text{M}$) dose-dependently attenuated the diazepam-induced enhancement of GABA ($10 \mu\text{M}$) at this receptor (Figure 1C).

2'MeO6MF was most efficacious at $\alpha 1\beta 2\gamma 2\text{L}$, enhancing GABA responses by $512.1 \pm 72\%$. The efficacy of 2'MeO6MF at $\alpha 1\beta 1\gamma 2\text{L}$ and $\alpha 1\beta 3\gamma 2\text{L}$ receptors was similar, enhancing the effect of GABA by $395.1 \pm 53\%$ and $371.1 \pm 40.9\%$. 2'MeO6MF also potentiated GABA EC_{10} ($1 \mu\text{M}$) responses on $\alpha 1\beta 2$ receptor by $502.3 \pm 40.9\%$ (Table 1). The apparent order of potency of 2'MeO6MF on $\alpha 1\beta 2$ and $\alpha 1\beta 1$ – $3\gamma 2\text{L}$ was $\alpha 1\beta 2 > \alpha 1\beta 3\gamma 2\text{L} > \alpha 1\beta 2\gamma 2\text{L} > \alpha 1\beta 1\gamma 2\text{L}$; however, the differences in EC_{50} values were not significant (one-way ANOVA, $P > 0.05$; $n = 4$ – 6).

2'MeO6MF directly activates $\alpha 2\beta 2/3\gamma 2\text{L}$ GABA_A receptors without any modulatory effects

Oocytes expressing $\alpha 2\beta 2\gamma 2\text{L}$ GABA_A receptors responded to 2'MeO6MF at concentrations 10 , 30 , 100 and $300 \mu\text{M}$ in the absence of GABA (Figure 2A). 2'MeO6MF appeared to be a partial agonist producing a maximum response of 70% compared with the current generated by the maximum effect of GABA (3 mM) at this receptor (Figure 2D and E). In order to evaluate whether 2'MeO6MF also positively modulates $\alpha 2\beta 2\gamma 2\text{L}$ receptors, we studied various concentrations of 2'MeO6MF alone and in the presence of GABA EC_5 ($3 \mu\text{M}$) and found that the response produced in the presence of GABA was only additive, indicating that 2'MeO6MF does not

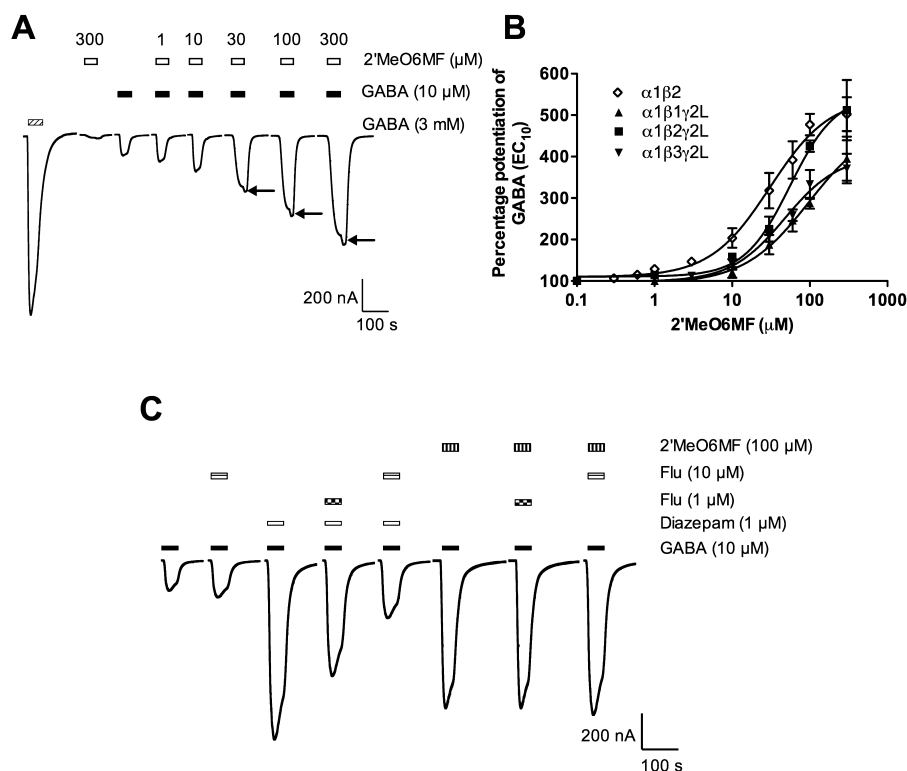


Figure 1

2'MeO6MF is a positive modulator of $\alpha 1$ -containing GABA_A receptors expressed in *Xenopus* oocytes. (A) Representative current trace showing the potentiation of GABA (EC₁₀: 10 μM) by various concentrations of 2'MeO6MF (1, 10, 30, 100 and 300 μM) at human recombinant $\alpha 1\beta 2\gamma 2L$ GABA_A receptors. Horizontal bars show the duration of drug application. In the absence of GABA, 2'MeO6MF (300 μM) produced $\approx 2\%$ of the current produced by the maximum GABA concentration. Note the rebound current following removal of 2'MeO6MF (indicated by the arrows) (B) 2'MeO6MF potentiates the response to GABA (EC₁₀) at recombinant $\alpha 1\beta 2$, $\alpha 1\beta 1\gamma 2$, $\alpha 1\beta 2\gamma 2L$ and $\alpha 1\beta 3\gamma 2L$ receptors expressed in oocytes. Control GABA concentration at each receptor subtype was 10 μM. Data are mean $\pm$ SEM ($n = 3-6$ oocytes). (C) Representative current trace from an oocyte showing potentiation of GABA (10 μM) by 2'MeO6MF (100 μM) at recombinant $\alpha 1\beta 2\gamma 2L$ GABA_A receptors. As shown, the enhancement of GABA (10 μM) by 2'MeO6MF (100 μM) was not inhibited by flumazenil (1 and 10 μM) whereas potentiation of GABA (10 μM) by diazepam (1 μM) was inhibited by flumazenil (1 and 10 μM). Horizontal bars show the duration of drug application.

positively modulate these receptors (Supporting Information Figure S2).

At $\alpha 2\beta 3\gamma 2L$ receptors, 2'MeO6MF directly activated the receptor acting as a full agonist (Figure 2E). Although, 2'MeO6MF is apparently more efficacious at $\alpha 2\beta 3\gamma 2L$ than at $\alpha 2\beta 2\gamma 2L$ receptors, the difference between potencies of 2'MeO6MF at these receptors was not significant (Table 1, $P < 0.05$). 2'MeO6MF also activated $\alpha 2\beta 2/3$ receptors that lacked the $\gamma 2L$ -subunit, producing a maximum effect of $22.8 \pm 1.5\%$ (at $\alpha 2\beta 2$ receptors) and $42.5 \pm 1.3\%$ (at $\alpha 2\beta 3$ receptors) compared with the maximum concentration of GABA (3 mM) (Table 1 and Figure 2E). Again the activities did not significantly differ indicating that the $\gamma 2L$ -subunit is required for maximum efficacy but is not essential for activity.

The direct activation by 2'MeO6MF at $\alpha 2\beta 2/3\gamma 2L$ GABA_A receptors could be attenuated by gabazine, bicuculline and PTZ but not flumazenil

The direct activation of $\alpha 2\beta 2\gamma 2L$ GABA_A receptors by 2'MeO6MF could not be reduced by flumazenil (10 μM);

Figure 2B) despite this concentration inhibiting 60% of the enhancement produced by diazepam (1 μM) (Figure 2B). These data infer that 2'MeO6MF does not bind to the classical flumazenil-sensitive benzodiazepine-binding site supporting the binding data. In contrast, the GABA_A receptor antagonists, bicuculline and gabazine (SR 95531) attenuated the effect of 2'MeO6MF at $\alpha 2\beta 2\gamma 2L$ GABA_A receptors. Pre-incubation with bicuculline (10 μM) for 3 min attenuated the activation produced by 2'MeO6MF (100 μM) by 56%. Similarly, gabazine (1 and 10 μM) attenuated the response produced by 2'MeO6MF (100 μM) on $\alpha 2\beta 2\gamma 2L$ GABA_A receptors (Figure 2C), by 45 and almost 100%, respectively. Concentration-response curves of 2'MeO6MF in the presence of bicuculline (10 μM) and gabazine (1 μM) showed that this inhibition was non-competitive (Figure 2D), despite a four- and fivefold increase in the EC₅₀ for 2'MeO6MF by gabazine and bicuculline, respectively (Figure 2D).

PTZ (1 mM) a GABA_A receptor blocker (Huang *et al.*, 2001) and prototypical anxiogenic drug also attenuated the response produced by 2'MeO6MF (100 μM) by 60% at $\alpha 2\beta 2\gamma 2L$ GABA_A receptors (Supporting Information Figure S3).

Table 1The effects of 2'MeO6MF at the various recombinant ionotropic GABA receptor subtypes expressed in *Xenopus* oocytes

Receptor subtype	2'MeO6MF EC ₅₀ (μM) (95% CI)	Fitted % E _{max} (Mean ± SEM)	% I _{max} (Mean ± SEM)	n _H	n
α1β1γ2L	98.2 (15.3 to 630.5)	395.1 ± 53.0	–	1.5 ± 0.2	6
α1β2γ2L	55.7 (27.9 to 111.2)	512.1 ± 72.0	–	1.0 ± 0.15	8
α1β2	30.7 (18.4 to 51.4)	502.3 ± 40.9	–	1.1 ± 0.4	4
α1β3γ2L	48.1 (29.3 to 77.0)	371.1 ± 25.0	–	0.9 ± 0.2	4
α2β1γ2L	74.3 (36.5 to 151.4)	425.4 ± 18.0	–	1.1 ± 0.4	4
α2β1	28.6 (16.6 to 49.2)	234.3 ± 20.4	–	1.5 ± 0.7	3
α2β2γ2L	54.5 (46.2 to 65.2)	–	70.2 ± 2.1	2.2 ± 0.3	6
α2β2	30.1 (12.1 to 74.7)	–	22.8 ± 1.5	1.1 ± 0.1	3
α2β2N265Sγ2L	61.2 (30.0 to 124.8)	454.6 ± 16.3	–	1.0 ± 0.1	5
α2β1S265Nγ2L	43.3 (29.1 to 64.5)	–	69.4 ± 5.05	1.3 ± 0.7	3
α2β3γ2L	56.3 (38.5 to 71.4)	–	104.6 ± 5.1	1.6 ± 0.5	6
α2β3	21.1 (14.1 to 31.8)	–	42.2 ± 1.3	1.1 ± 0.3	3
α3β1–3γ2L	NE	–	–	–	5
α5β1–3γ2L	NE	–	–	–	5
ρ1	NE	–	–	–	5

95% CI, 95% confidence interval; NE, no effect; E_{max}, Maximum potentiation produced by 2'MeO6MF relative to GABA EC₁₀.

2'MeO6MF is a potentiator of GABA at α2β1 and α2β1γ2L GABA_A receptors

2'MeO6MF had little effect in the absence of GABA when evaluated at α2β1γ2L GABA_A receptors. Instead 2'MeO6MF (1–300 μM) potentiated the effect of a low concentration of GABA (EC₁₀ = 10 μM) at both α2β1 and α2β1γ2L GABA_A receptors. 2'MeO6MF (300 μM) maximally potentiated (Figure 3B) the response to GABA (10 μM) at these receptors. Table 1 summarizes the efficacy and activity of 2'MeO6MF at α2β1 and α2β1γ2L GABA_A receptors.

Mutating β2-N265S converted 2'MeO6MF from an activator to a positive modulator on α2β2γ2L GABA_A receptors

Mutating asparagine to serine at position 265 on the β2 subunit did not inhibit the formation of functional channels when expressed with wild-type α2- and γ2L-subunits in oocytes. The maximal concentration of GABA (3 mM) produced currents ranging from 230 to 550 nA. The EC₅₀ of GABA at α2β2N265Sγ2L was 57.7 μM (34.1 to 97.8; 95% CI, *n* = 4). However, this receptor exhibited a dramatic resistance to the direct activation by 2'MeO6MF. As shown in Figure 3A and B, 2'MeO6MF (300 μM) activated 5–10% of the maximum GABA (3 mM) response at α2β2N265Sγ2L receptors. In contrast, 2'MeO6MF (300 μM) potentiated the current response produced by a low concentration of GABA (10 μM) by 454.6 ± 16.3% at the mutant receptor (Figure 3A and B; Table 1).

The converse mutation in β1-S265N converts 2'MeO6MF from a potentiator to an activator at α2β1S265Nγ2L GABA_A receptors

In order to confirm that the amino acid residue at position 265 participates in the gating mechanism and is responsible

for determining activation versus positive modulation, we generated the converse mutation in the β1-subunit. 2'MeO6MF in the concentration range 1–300 μM directly activated α2β1S265Nγ2L recombinant GABA_A receptors (Figure 3C and D) reaching maximal activation at 300 μM (69.4 ± 5.05%), without potentiating the current response elicited by a low concentration of GABA (10 μM) (Figure 3C). Figure 3C shows that the effect of 2'MeO6MF (100 μM) in the presence of GABA (10 μM) is additive and not synergistic. In addition the difference between the EC₅₀ values of 2'MeO6MF at α2β1S265Nγ2L and α2β2γ2L recombinant GABA_A receptors was not significant (*n* = 4; Student's *t*-test, *P* > 0.05) (Table 1) indicating that this amino acid residue participates only in the gating mechanism and as hypothesized is responsible for determining positive modulation versus activation by 2'MeO6MF of α2-containing receptors.

2'MeO6MF is inactive at α3/5β1–3γ2L and ρ1 GABA receptors

2'MeO6MF (1–300 μM) did not activate or enhance the response to GABA EC₁₀ at recombinant α3/5β1–3γ2L receptors. At α3β2γ2L receptors, 2'MeO6MF (100 μM) slightly shifted the GABA dose-response curve to the left. However, this effect was not significant (*n* = 4, Student's *t*-test: *P* > 0.05; (Supporting Information Figure S4A). At α5β2γ2L receptors, 100 μM 2'MeO6MF inhibited responses to high doses (1000–3000 μM) of GABA and shifted the curve slightly to the right; however, this effect was not significant (*n* = 4, Student's *t*-test: *P* > 0.05) (Supporting Information Figure S4B). 2'MeO6MF (1–100 μM) was found to be inactive at ρ1 receptors; 2'MeO6MF (100 μM) did not significantly affect the dose-response curve of GABA at ρ1 receptors (Supporting Information Figure S4C).

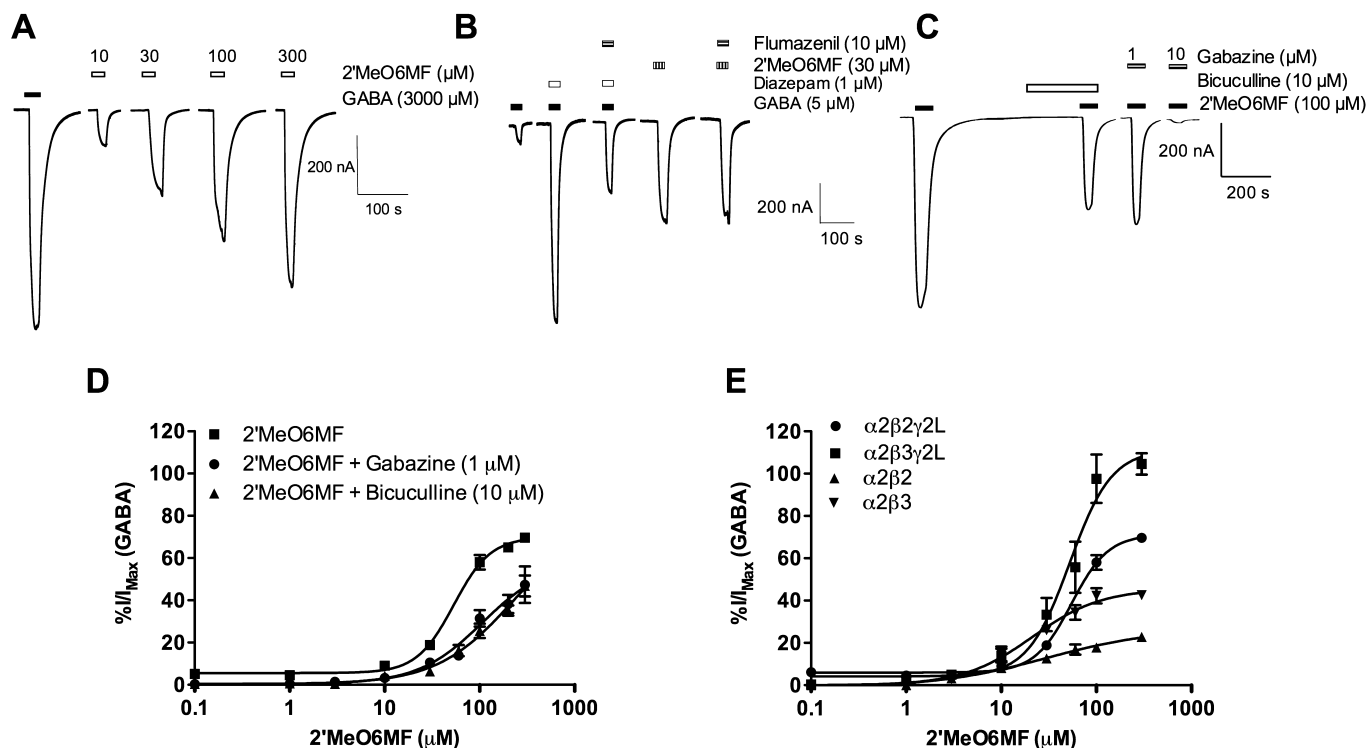


Figure 2

2'MeO6MF directly activates human recombinant $\alpha 2\beta 2/3\gamma 2L$ and $\alpha 2\beta 2/3$ GABA_A receptors expressed in oocytes. (A) Current trace showing the effects of increasing concentrations of 2'MeO6MF on recombinant $\alpha 2\beta 2\gamma 2L$ GABA_A receptors expressed in *Xenopus* oocytes against the maximum effect of GABA (3000 μ M). Horizontal bars indicate duration of drug application. (B) Representative trace showing the potentiation of GABA (EC₁₀: 5 μ M) in the presence of diazepam (1 μ M) and this effect was attenuated by flumazenil (10 μ M) at $\alpha 2\beta 2\gamma 2L$ GABA_A receptors. In contrast, the direct activation of 2'MeO6MF (30 μ M) on the same receptor was not attenuated by flumazenil (10 μ M). (C) Representative trace showing that a 3 min pre-incubation with bicuculline (10 μ M) attenuates the response produced by 2'MeO6MF (100 μ M) by 56 $\pm$ 4% at $\alpha 2\beta 2\gamma 2L$ GABA_A receptors. Gabazine (10 μ M and 100 μ M) also attenuated the response produced by 2'MeO6MF on $\alpha 2\beta 2\gamma 2L$ GABA_A receptors by 45 $\pm$ 4% and 100% respectively. (D) Concentration-response curves for 2'MeO6MF alone and 2'MeO6MF in the presence of bicuculline (10 μ M) and gabazine (1 μ M) at $\alpha 2\beta 2\gamma 2L$ GABA_A receptors expressed in *Xenopus* oocytes. Data are mean $\pm$ SEM (n = 4–6 oocytes). (E) Concentration-response curves for 2'MeO6MF on $\alpha 2\beta 3\gamma 2L$, $\alpha 2\beta 2\gamma 2L$, $\alpha 2\beta 3$ and $\alpha 2\beta 2$ recombinant GABA_A receptors expressed in oocytes. Currents are expressed as a percentage of the peak current elicited by 3 mM GABA. Data are mean $\pm$ SEM (n = 4–6 oocytes).

2'MeO6MF directly activates native γ -containing GABA_A receptors

The ability of 2'MeO6MF to directly activate native GABA_A channels was examined in hippocampal neurons from the newborn rat. Pharmacological (Caraiscos *et al.*, 2004; Mangan *et al.*, 2005; Herd *et al.*, 2008; Seymour *et al.*, 2009) and competitor peptide experiments have shown that these neurons express multiple receptor subtypes including γ - and δ -containing GABA_A channels. Single-channel currents in response to 2'MeO6MF were recorded from inside-out patches pulled from cultured neurons and their characteristics compared with those observed with GABA. All patches were initially held for 5 min to ensure that the patch was electrically silent in the absence of any ligand. A typical example is illustrated in (Supporting Information Figure S5A) showing 2 s of the single-channel trace and an all-points probability histogram measured over 30 s just prior to adding drug. 2'MeO6MF (100 μ M) was subsequently flowed across the patch and within 2 min a current with a small average

amplitude of 0.7 pA was observed ($-V_p$ = +60 mV, Supporting Information Figure S5B). With time, however, the average current amplitude increased (Supporting Information Figure S5C, 2.7 pA), typical of native GABA_A channels, and within approximately 5 min the average amplitude had reached a maximum of 4.1 pA (Supporting Information Figure S5D). The current displayed all the hallmarks of an outwardly rectifying GABA_A current (Curmi *et al.*, 1993); the open probability was noticeably lower and the current amplitude was reduced at $-V_p$ = -60 mV compared with $-V_p$ = +60 mV; compare single-channel traces and all-points probability histograms in Supporting Information Figure S5D, E. With near equimolar Cl⁻ solutions in the bath and pipette and stepping the holding potential to 0 mV the current was negligible, consistent with it being carried by Cl⁻ ions (Supporting Information Figure S5F). In five other patches where 2'MeO6MF (100 μ M) was applied similar Cl⁻ currents were activated, consistent with these being mediated by GABA_A channels – see Supporting Information Figure S5. In 13 additional patches no response to the drug was observed.

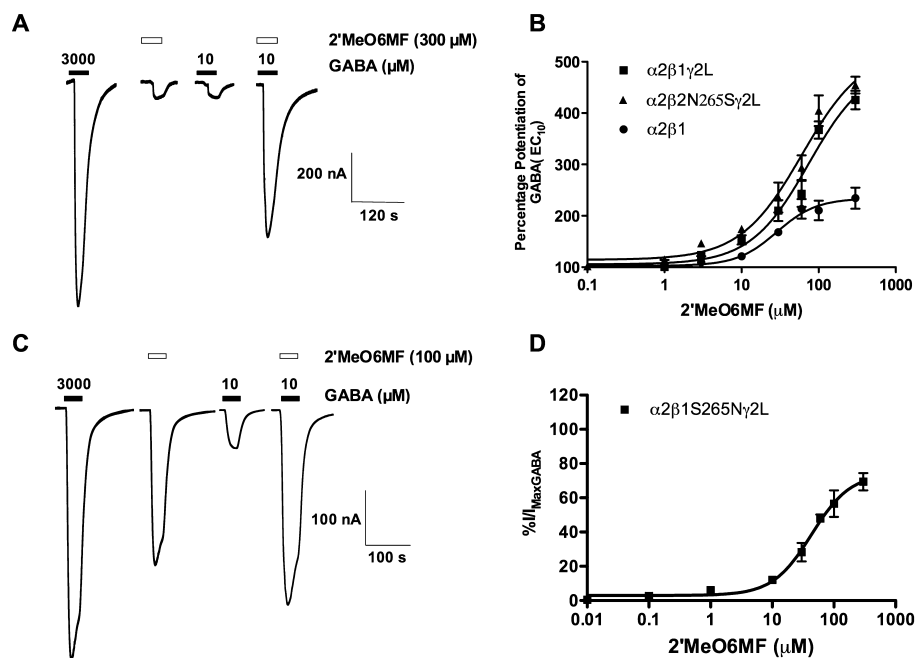


Figure 3

(A) Representative trace showing the activation of 2'MeO6MF (300 μ M) is significantly reduced at the mutant $\alpha 2\beta 2N265S\gamma 2L$ GABA_A receptor. In contrast, 2'MeO6MF (300 μ M) was able to positively modulate GABA (10 μ M) by $454 \pm 16\%$. Horizontal bars show duration of drug application. (B) Concentration–response curves for 2'MeO6MF in the presence of GABA (EC_{10}) at $\alpha 2\beta 1$, $\alpha 2\beta 1\gamma 2L$ and $\alpha 2\beta 2N265S\gamma 2L$. Control GABA dose was 10 μ M. Data are mean $\pm$ SEM ($n = 4$ oocytes). (C) Representative trace showing 2'MeO6MF (100 μ M) directly activating recombinant $\alpha 2\beta 1S265N\gamma 2L$ mutant GABA_A receptors. The current generated by 2'MeO6MF (100 μ M) in the presence of GABA (10 μ M) is the sum of the individual currents produced by GABA (10 μ M) and 2'MeO6MF (100 μ M) alone. Horizontal bars show duration of drug application. (D) Concentration–response curve for 2'MeO6MF expressed relative the maximal GABA concentration (3 mM) at $\alpha 2\beta 1S265N\gamma 2L$ GABA_A receptors showing that the converse mutation converted 2'MeO6MF from a potentiator to a direct activator. Data are mean $\pm$ SEM for three to four oocytes.

Although the absolute composition of native GABA_A channels is still not known, they can be classified into γ - or δ -containing receptors. Both receptor subtypes are able to transition to high conductance states in response to drugs as a consequence of inter-receptor protein interactions (Everitt *et al.*, 2009; Seymour *et al.*, 2009). Competitor peptides, which mimic this receptor-protein binding site and correspond to the unique amino acid sequence for the amphipathic intracellular helix (MA helix) of either the $\gamma 2$ - or δ -subunit, significantly reduce the current mediated by their respective channel subtypes (Everitt *et al.*, 2009; Seymour *et al.*, 2009). On inside-out patches displaying large amplitude currents in response to 2'MeO6MF (100 μ M) a peptide mimicking the $\gamma 2$ MA helix [$\gamma(381-403)$ MA peptide] was subsequently applied in the continued presence of the drug. In the example shown in Figure 4 2'MeO6MF (100 μ M) activated a current with an average amplitude of 2.7 pA ($-V_p = +60$ mV). Upon addition of 1 μ M, $\gamma 2$ MA peptide currents with smaller amplitudes and briefer opening times appeared in the recording, typical of competitor peptide. The 2 s single-channel trace illustrates the variable nature of the current whereas the expanded trace shows the range of multiple current amplitudes observed. The all-points probability histogram along side reveals the current distribution over a longer time period (30 s) with average peaks at 1.2 and 2.8 pA. In all

three patches where the γ MA peptide was applied (1 μ M) a similar effect on current amplitude was observed indicating that 2'MeO6MF (100 μ M) directly activated currents mediated by native γ -containing GABA_A channels.

In vivo studies

Effects on general behaviour and acute toxicity in mice. 2'MeO6MF was tested at doses 1, 10 and 100 mg·kg⁻¹. Overt behavioural 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. An increase in spontaneous activity and escape behaviour was observed at doses of 1 and 10 mg·kg⁻¹ in three out of five animals after 30 and 60 min of injection. Also an increased hypersensitivity (measured as increased irritability and aggressiveness during handling) and increase in escape behaviour was observed in the same animals. At 100 mg·kg⁻¹, four out of five animals appeared slightly drowsy (general observation); however, the righting reflex was not lost. At 24 h to 1 week after injection all animals seemed well with no observable changes in behaviour, spontaneous activity or appearance.

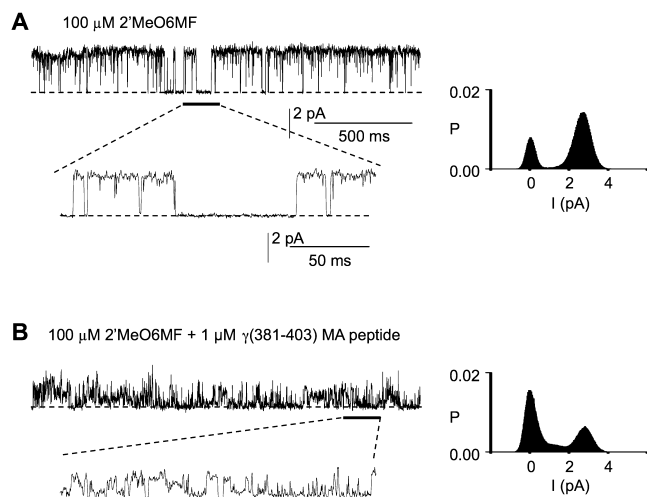


Figure 4

2'MeO6MF directly activates γ -containing GABA_A channels in hippocampal neurons. (A) In an excised inside-out patch 100 μM 2'MeO6MF activated a current with a maximum average amplitude of 2.8 pA ($-V_p = -60$ mV). (B) With the addition of 1 μM γ(381–403) MA peptide, there was a reduction in the current amplitude. Lower amplitudes of around 1.2 pA were now visible in the recording as depicted in (B) and both the reduction in open probability (increase in the zero peak) and current amplitude are reflected in the 30 s all-points probability histogram alongside.

Elevated plus maze test

Results from the ANOVA analysis showed that both the diazepam- and 2'MeO6MF-treated animals displayed significantly different effects from the vehicle controls in both the % number of open-arm entries and % time spent in the open arms [$F(6,49) = 29.1$ $P < 0.001$ and $F(6,49) = 24.7$ $P < 0.001$ respectively]. Dunnett's *post hoc* test revealed that diazepam at doses of 1 and 2 mg·kg⁻¹ caused a significant increase in these two parameters compared with vehicle. 2'MeO6MF at doses of 1, 10, 30 and 100 mg·kg⁻¹ also induced an anxiolytic effect as evidenced in the increase of % open-arm entries and % time spent in the open arms (Figure 5A and B). 2'MeO6MF did not significantly influence the number of entries into the closed arms suggesting that the anxiolytic effects observed at these doses was not due to an increase in the exploratory activity [$F(6,49) = 0.33$ $P > 0.05$]. However, at the higher doses, 2'MeO6MF (30 and 100 mg·kg⁻¹) had a tendency to reduce the number of closed arm entries compared with the vehicle (Supporting Information Figure S6). The reduction in both the open and closed arm entries at high doses may indicate the onset of sedative effects.

PTZ but not flumazenil reversed the anxiolytic effect of 2'MeO6MF in the elevated plus maze test

Co-administration of 2'MeO6MF (10 mg·kg⁻¹) with flumazenil (2.5 mg·kg⁻¹) did not decrease open-arm entries and time spent in open arms confirming the *in vitro* findings that 2'MeO6MF does not bind to the benzodiazepine site. However, flumazenil (2.5 mg·kg⁻¹) significantly decreased

both parameters for the diazepam (2 mg·kg⁻¹)-treated animals (Figure 5C and D). However, as seen in Figure 5E, F, pretreatment with PTZ (20 mg·kg⁻¹) significantly decreased the increase in open-arm entries and time spent in open arms induced by 2'MeO6MF (10 mg·kg⁻¹) in the elevated plus maze, thereby indicating the effect is most likely mediated by a GABAergic system.

Light/dark box test

ANOVA showed a significant difference between groups in the time spent in the lit compartment [$F(6,49) = 27.7$ $P < 0.001$] and in the number of transitions between lit and dark compartments [$F(6,49) = 4.87$ $P < 0.001$, respectively]. Dunnett's *post hoc* analysis revealed that diazepam at doses of 1 and 2 mg·kg⁻¹ significantly ($P < 0.01$) increased the time spent in the lit compartment and the number of transitions between the compartments, indicating anxiolytic activity and validating our paradigm. Similarly, 2'MeO6MF at doses of 1 and 10 mg·kg⁻¹ significantly ($P < 0.01$) increased the % of time spent in the lit area and the number of transitions between areas. Higher doses (30 and 100 mg·kg⁻¹) were ineffective (Figure 6Ai, Aii).

Holeboard test

The influence of treatment on the number of head dips [$F(6,49) = 6.16$ $P < 0.001$] and the total time spend head dipping reached significance [$F(6,49) = 6.16$ $P < 0.001$]. Administration of 2 mg·kg⁻¹ diazepam significantly decreased the number of head dips and both 1 and 2 mg·kg⁻¹ diazepam decreased the total time spent head dipping. In contrast, low doses of 2'MeO6MF (1 and 10 mg·kg⁻¹) did not significantly decrease the number of head dips or time spent head dipping ($P > 0.05$). However, at higher doses of 2'MeO6MF (30 and 100 mg·kg⁻¹), there was a significant decrease in the number of head dips and total time spent in head dipping. These results demonstrate that 2'MeO6MF has sedative effects at higher doses but not at lower doses (Figure 6Bi and ii).

Measurement of locomotor activity

Locomotor activity results are shown in Figure 6C. ANOVA revealed a significant difference between means [$F(6,49) = 22.02$ $P < 0.001$]. Dunnett's *post hoc* comparison test showed a significant decrease ($P < 0.01$) in the number of beam breaks induced by diazepam at doses 1 and 2 mg·kg⁻¹. 2'MeO6MF at doses 30 and 100 mg·kg⁻¹ also significantly ($P < 0.01$) decreased the number of beam breaks; however, doses 1 and 10 mg·kg⁻¹ did not produce any changes in this parameter again demonstrating the lack of sedative effect at these lower doses.

Horizontal wire test

The results from the horizontal wire test are shown in the Table 2. Neither diazepam (1 mg·kg⁻¹) nor 2'MeO6MF (1–100 mg·kg⁻¹) compromised the grasping of wire when compared with vehicle controls pointing to a lack of myorelaxation at these dosages. Diazepam at 3 and 10 mg·kg⁻¹, i.p., in contrast, elicited significant myorelaxation in mice ($P < 0.01$, $P < 0.001$, Fisher's exact tests) (Table 2) as signified by the decrease in percentage of mice grasping the wire.

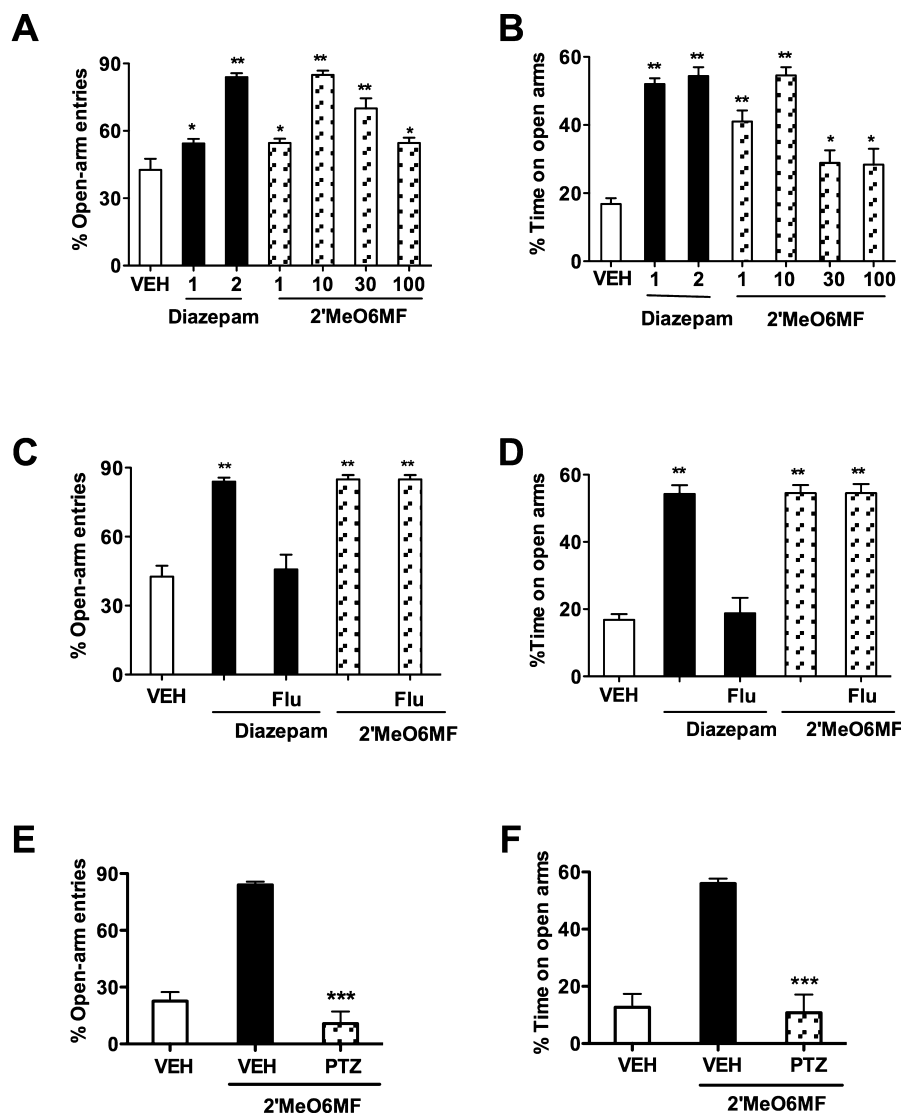


Figure 5

Effect of diazepam and 2'MeO6MF on the behaviour of mice in the elevated plus maze. (A) The % open-arm entries; and (B) % time spent in the open arms recorded over a 5 min session, 20 min after an i.p. injection of diazepam (1 and 2 mg·kg⁻¹), 2'MeO6MF (1–100 mg·kg⁻¹) or vehicle (VEH). Both diazepam and 2'MeO6MF exerted anxiolytic effects in the elevated plus maze. (C) % open-arm entries; and (D) % time spent in open arms in mice given a 5 min test, 30 min after i.p. administration of vehicle (VEH), diazepam (2 mg·kg⁻¹) or 2'MeO6MF (10 mg·kg⁻¹) in the presence of flumazenil (Flu; 2.5 mg·kg⁻¹). $n = 8$ per group. * $P < 0.05$, ** $P < 0.01$ indicate significance from control using one-way ANOVA followed by Dunnett's *post hoc* test. Co-application of flumazenil (Flu; 2.5 mg·kg⁻¹) did not block the anxiolytic-like effect of 2'MeO6MF (10 mg·kg⁻¹), whereas it inhibited the anxiolytic effect of diazepam (2 mg·kg⁻¹) indicating the anxiolytic effect is not mediated via the benzodiazepine site. (E) % open-arm entries; and (F) % time spent in the open arms 20 min after i.p. injection with 2'MeO6MF (10 mg·kg⁻¹) in mice pretreated with PTZ (20 mg·kg⁻¹) or vehicle (VEH). Columns represent mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared with vehicle group using ANOVA followed by Dunnett's multiple comparison test.

Thiopental-induced loss of righting reflex

One-way ANOVA followed *post hoc* Dunnett's multiple comparison test showed that diazepam at doses 1 and 2 mg·kg⁻¹ significantly ($P < 0.01$) increased the duration of loss of righting reflex. Similarly, 2'MeO6MF at doses of 30 and 100 mg·kg⁻¹ significantly ($P < 0.05$ and $P < 0.01$, respectively) increased the duration of loss of righting reflex; however, doses 1 and 10 mg·kg⁻¹ did not significantly affect ($P > 0.05$) this parameter (Table 3).

Discussion

Recent behavioural studies have shown that the anxiolytic and sedative effects of diazepam are mediated by the $\alpha 1/2$ -containing GABA_A receptors, respectively (Atack, 2010a). Thus agents selective for GABA_A receptors containing either $\alpha 1/2$ -subunits would be useful sedative and/or anxiolytic agents. In this study, we investigated the ability of a synthetic flavone, 2'MeO6MF to activate or modulate various α - and

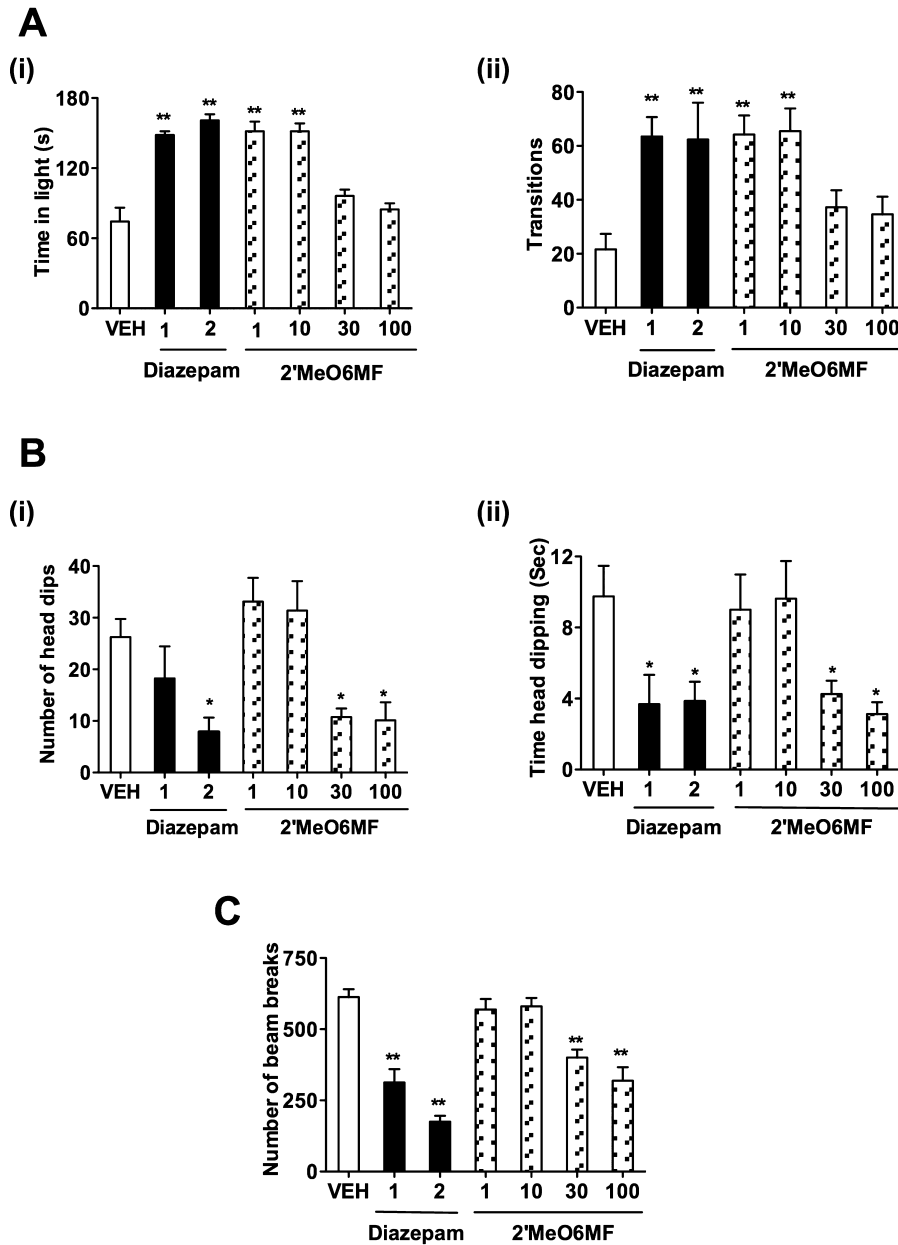


Figure 6

Effect of diazepam (1 and 2 mg·kg⁻¹) and 2'MeO6MF (1–100 mg·kg⁻¹) on the behaviour of mice in the (A) light/dark box test: (i) time spent in the lit compartment; and (ii) number of transitions recorded over a 5 min session, 20 min after i.p. injection of drugs or vehicle (VEH). (B) Holeboard test: (i) number of head dips; and (ii) time head dipping recorded over a 5 min session, 20 min after i.p. injection of drugs or vehicle (VEH). (C) Locomotor test: number of beam breaks recorded over a 5 min session, 20 min after i.p. injection of drugs or vehicle (VEH). Columns represent mean $\pm$ SEM ($n = 8$ /group). * $P < 0.05$, ** $P < 0.01$ compared with vehicle group, one-way ANOVA followed by Dunnett's multiple comparison test.

β -containing GABA_A receptors expressed in *Xenopus* oocytes. The results indicate that 2'MeO6MF differentially affects GABA_A receptor subtypes.

2'MeO6MF was found to directly activate $\alpha 2\beta 2/3$ and $\alpha 2\beta 2/3\gamma 2L$ receptor subtypes without modulating the effect of GABA. Activation by 2'MeO6MF was attenuated by bicuculline and gabazine, but not flumazenil, a result similar to that observed with bicuculline attenuating the direct effect of etomidate (Belelli *et al.*, 1997).

Interestingly, replacing the $\beta 2/3$ with the $\beta 1$ isoform in $\alpha 2$ -containing receptors markedly changed the activation barrier. Instead of activating the receptor, 2'MeO6MF positively modulated $\alpha 2\beta 1$ and $\alpha 2\beta 1\gamma 2L$ receptors indicating that the activation and modulation properties of 2'MeO6MF at $\alpha 2$ -containing receptors are distinct and dependent on the type of β -subunit present. Several drugs are known to display greater efficacy at $\beta 2/3$ - over $\beta 1$ -containing receptors including the general anaesthetic etomidate and sedative anticon-

Table 2

Performance of mice in the horizontal wire test. Percentage of mice grasping wire after i.p. administration of 2'MeO6MF (1, 10, 30 and 100 mg·kg⁻¹), diazepam (1, 3 and 10 mg·kg⁻¹) and vehicle

Treatment	Dose (mg·kg ⁻¹ , i.p.)	Wire test % Of mice grasping wire
Vehicle	–	100
Diazepam	1	100
	3	80**
	10	50***
2'MeO6MF	1	100
	10	100
	30	100
	100	100

P* < 0.01, *P* < 0.001 different from vehicle: Fisher's exact test.

Table 3

Effect of diazepam and 2'MeO6MF on sleep duration in barbiturate induced sleep time

Treatment	Dose (mg·kg ⁻¹ , i.p.)	Sleep duration (mean ± SEM) (min)	% Increase of sleep duration
Vehicle	–	26.1 ± 1.9	–
Diazepam	1	43.5 ± 0.9**	66
	2	56.3 ± 1.1**	84
2'MeO6MF	1	28.9 ± 1.9	–
	10	31.3 ± 1.0	–
	30	34.9 ± 1.8*	35
	100	53.4 ± 3.1**	91

Values represent (mean ± SEM) sleep duration in mice after administration of vehicle, diazepam (1 and 2 mg·kg⁻¹, i.p.) and 2'MeO6MF (1, 10, 30 and 100 mg·kg⁻¹, i.p.).

P* < 0.05, *P* < 0.01 significantly different from vehicle: one-way ANOVA followed by Dunnett *post hoc* multiple comparison test (*n* = 8 per group).

vulsant loreclezole (Hill-Venning *et al.*, 1997). Subsequent studies found that a single asparagine (N) residue located at position 265 on the second transmembrane domain of the $\beta 2$ subunit (and equivalent position at $\beta 3$) conferred this selectivity (Desai *et al.*, 2009). When $\beta 2N265$ is mutated to serine (S), the homologous amino acid in the $\beta 1$ subunit, the ability of etomidate and loreclezole to potentiate GABA currents and the direct activation by etomidate is diminished (Belelli *et al.*, 1997). Accordingly, knock-in mice carrying either $\beta 2N265S$ or $\beta 3N265M$ mutation display impaired sensitivity to the *in vivo* actions of these drugs (Groves *et al.*, 2006).

Interestingly, when N265 of the $\beta 2$ subunit was mutated to serine to mimic the $\beta 1$ residue at the equivalent position, 2'MeO6MF changed from an activator to a modulator at the $\alpha 2$ -containing mutant receptor. At $\alpha 2$ -containing receptors containing the converse mutant subunit $\beta 1S265N$, 2'MeO6MF was converted from a modulator to a direct activator. Interestingly, this same mutation has also been shown to induce direct currents at $\alpha 1\beta 1S265N\gamma 2L$ by mefenamic acid (Halliwell *et al.*, 1999).

As the activation by 2'MeO6MF was significantly diminished on $\alpha 2\beta 2N265S\gamma 2L$ receptors, but had positive modulation at a similar concentration range; this suggests that the modulation and direct activation by 2'MeO6MF are mediated via a single binding site, but that there are two distinct energy barriers. It is proposed that serine can strongly hydrogen-bond via its hydroxyl moiety to stabilize the closed state. Thus 2'MeO6MF requires GABA to help shift the equilibrium from the closed to the open state. In contrast, the hydrogen bond formed by the amide group of asparagine is weaker and the energy required to shift the equilibrium of the channel from the closed to the open state is less. Thus, 2'MeO6MF opens the channel without the aid of GABA. Therefore, the amino acid at position $\beta 265$ appears to contribute to the activation pathway, enabling 2'MeO6MF to go from an activator to a modulator and vice versa at $\alpha 2$ -containing receptors. However, as we did not identify the binding site for 2'MeO6MF, we cannot rule out the possibility that there may be more than one binding site for 2'MeO6MF on these receptors.

In contrast, the effects of 2'MeO6MF on $\alpha 1$ -containing-GABA_A receptors were non-selective. 2'MeO6MF positively modulated all $\alpha 1\beta 2$ and, $\alpha 1\beta 1-3\gamma 2L$ receptor subtypes, but did not directly activate them, indicating the importance of the α -subunit in the gating mechanism.

2'MeO6MF did not activate or potentiate $\alpha 3/5\beta 1-3\gamma 2L$ subtypes. Thus unlike other flavonoids, 2'MeO6MF selectively affects $\alpha 1/2$ -containing GABA_A receptors, and, unlike barbiturates and neurosteroids that non-selectively modulate and directly activate most GABA_A receptor subtypes (Lambert *et al.*, 1996; Thompson *et al.*, 1996; Maitra and Reynolds, 1998), the potentiation and direct activation of 2'MeO6MF are subtype specific.

While recombinant studies revealed that 2'MeO6MF could differentiate between various GABA_A receptor subtypes, *ex vivo* studies in neuronal cells were conducted to confirm that 2'MeO6MF could indeed activate native $\gamma 2$ -containing GABA_A channels. Because native γ -containing GABA_A receptors are involved in receptor-protein interactions that affect their ion permeation (Everitt *et al.*, 2009; Seymour *et al.*, 2009), the disruption of these interactions, using $\gamma 2$ -subunit-specific competitor peptides, enables the underlying receptor's identity to be revealed. 2'MeO6MF directly activated a Cl⁻ current in hippocampal pyramidal neurons that exhibited all the hallmarks of those mediated by GABA_A channels (Eghbali *et al.*, 1997; 2003; Seymour *et al.*, 2009). The reduction in current activity upon application of the $\gamma 2$ MA peptide confirmed that this current was indeed mediated by $\gamma 2$ -containing GABA_A channels. The subtype-specific direct activation of 2'MeO6MF described in the recombinant system together with the activation of native γ -containing GABA_A channels suggest that $\alpha 2\beta 2/3\gamma 2$ receptors may underlie the drug's activation in these neurons.

Recent behavioural studies have shown that the anxiolytic action of benzodiazepines is largely mediated by $\alpha 2/3$ -containing GABA_A receptors, suggesting that these subunits could be useful targets for the development of novel non-sedative anxiolytic agents (Low *et al.*, 2000; Attack, 2010a). In order to correlate the *in vitro* with the *in vivo* effects, 2'MeO6MF was subjected to a battery of behavioural studies in mice. Like diazepam, 2'MeO6MF at doses ranging from 1 to 100 mg·kg⁻¹ induced anxiolytic effects in two unconditional models of anxiety: the elevated plus maze and light/dark box tests. 2'MeO6MF exerted dual effects in these anxiety paradigms. In the elevated plus maze, low doses of 2'MeO6MF (1 and 10 mg·kg⁻¹) showed anxiolytic effects, whereas at higher doses (30 and 100 mg·kg⁻¹), the anxiolytic effects were reduced possibly indicating sedative effects. Like the *in vitro* data, the anxiolytic effects of 2'MeO6MF were not blocked by the benzodiazepine antagonist, flumazenil but were attenuated by the GABA_A channel blocker, PTZ. These data infer that the anxiolytic effects are mediated via the GABAergic system but not through the classical benzodiazepine site despite 2'MeO6MF weakly displacing flunitrazepam binding (Kahnberg *et al.*, 2002). These data indicate that 2'MeO6MF binds to a novel as yet unidentified site on $\alpha 2\beta 2/3\gamma 2L$ GABA_A receptors.

A similar pattern was observed in the light/dark box test, 1 and 10 mg·kg⁻¹ of 2'MeO6MF produced an increased anxiolytic effect whereas 30 and 100 mg·kg⁻¹ were ineffective, suggesting that at high doses, $\alpha 1$ -mediated sedation might have led to a decrease in the anxiolytic activity (Rudolph *et al.*, 1999). Similarly, 2'MeO6MF at doses 30 and 100 mg·kg⁻¹ decreased exploratory parameters in the hole-board and spontaneous locomotor activity. Furthermore, at 100 mg·kg⁻¹, 2'MeO6MF significantly potentiated the central depressant action of the barbiturate sodium thiopental, confirming that 2'MeO6MF acts as an anxiolytic at low doses and a sedative at high doses. In addition, 2'MeO6MF showed no effects in the horizontal wire test at all doses tested indicating no myorelaxant effects.

In contrast, diazepam at doses 1 and 2 mg·kg⁻¹ decreased time spent in head dipping but only the higher dose of diazepam decreased the number of head dips in the hole-board test. Diazepam at the doses tested also decreased spontaneous locomotor activity, increased the duration of loss of righting reflex in the thiopental-induced sleep time and impaired the ability of mice to grasp the horizontal wire demonstrating sedative and myorelaxant effects at the doses tested. Classical benzodiazepines indiscriminately modulate GABA_A receptors containing the $\alpha 1$ –3/5-subunits and are regarded as relatively non-selective modulators (Attack *et al.*, 2005; Whiting, 2006). Our results and those of others (Rudolph *et al.*, 1999) showed that the administration of diazepam to animals leads to a series of pharmacological actions within a narrow dose range.

Interestingly, 2'MeO6MF at doses as low as 1 mg·kg⁻¹ as effective as diazepam (1 mg·kg⁻¹) as an anxiolytic agent *in vivo*. Given that 2'MeO6MF is approximately 100- to 1000-fold weaker at activating GABA_A receptors than diazepam, the enhanced *in vivo* effect of 2'MeO6MF may be that other as yet untested receptors or enzymes are involved in its activity. Indeed it has been shown that the anxiolytic effects of neurosteroids are eliminated in δ -knock-out mice (Mihalek

et al., 1999) implicating the δ -subunit in anxiety. In addition it has been shown that PKC ϵ regulates anxiety effects mediated via the GABA_A receptor (Hodge *et al.*, 2002). Given that retrochalcones (a class of molecules related to flavonoids) and flavonoids themselves can modulate δ -containing GABA_A receptors (Jiang *et al.*, 2011) and PKC enzymes (Gamet-Payraastre *et al.*, 1999), respectively, it may be that 2'MeO6MF acts at δ -containing GABA_A receptors and or modulates PKC ϵ activity. However, the involvement of these and other possible targets is yet to be determined. Furthermore, unlike diazepam, 2'MeO6MF does not require GABA to mediate its effects, acting preferentially as an allosteric activator at the $\alpha 2\beta 2/3\gamma 2L$ receptors rather than as a positive modulator and this may also be contributing to its enhanced *in vivo* activity.

2'MeO6MF, despite modulating the $\alpha 1$ -containing receptors, showed clear segregation of sedative and anxiolytic doses, suggesting that its direct actions on $\alpha 2$ -containing receptors are more effective than its modulatory actions on $\alpha 1$ -containing receptors. It is also important to note that not all compounds conform to the hypothesis that the $\alpha 1$ -subunit mediates sedation and that high efficacy at this subtype is accompanied by sedation (Lippa *et al.*, 2005; Popik *et al.*, 2006). While the reason for the lack of sedation in these compounds is unclear, at the very least, the data suggest that efficacy as measured in recombinant $\alpha 1$ -containing GABA_A receptors may not necessarily be predictive of sedative liability (Popik *et al.*, 2006).

The myorelaxant effects mediated by diazepam have been shown to be mediated by $\alpha 2$ -containing receptors although $\alpha 3$ - and $\alpha 5$ -containing receptors are also involved (Crestani *et al.*, 2001; 2002). In this study, 2'MeO6MF, but not diazepam, failed to exert myorelaxant effects. A number of agents lack myorelaxant effects despite selective effects for $\alpha 2$ -containing GABA_A receptors (Fernandez *et al.*, 2008). The reasons why 2'MeO6MF and other $\alpha 2$ -selective agents do not exert myorelaxant effects is not yet understood. It is possible that the myorelaxant effects are mediated by $\alpha 3/5$ -containing receptors and 2'MeO6MF lacks activity at these receptors. It may be that 2'MeO6MF is acting at other subtypes such as $\alpha 4/6\beta 1$ –3 $\gamma 2L$, δ -containing GABA_A receptors and or non-GABAergic targets to counteract the effect. Interestingly, a recent study showed that limiting activity at $\beta 1$ -containing GABA_A receptors reduces ataxia (Gee *et al.*, 2010). As 2'MeO6MF has limited effects on $\beta 1$ -containing $\alpha 1/\alpha 2$ GABA_A receptors, the reduced activity at these receptors may also be a contributing factor to the lack of myorelaxant effects.

In summary, 2'MeO6MF positively modulated all $\alpha 1$ -containing and $\alpha 2\beta 1\gamma 2L$ while it directly activated $\alpha 2\beta 2/3$ and $\alpha 2\beta 2/3\gamma 2L$ GABA_A receptors. Behavioural studies revealed that 2'MeO6MF exerts anxiolytic effects at low doses and sedative effects at high doses without myorelaxant effects. Due to its differential effects on GABA_A receptor subtypes, 2'MeO6MF can serve as a tool to study the complex mechanisms of activation and modulation of these receptors, and highlights another potential site on GABA_A receptors. It is envisaged that the mechanism of direct activation by flavonoids such as 2'MeO6MF on both recombinant and native systems will emerge as an area of interest and may help in understanding how such activation is produced by barbiturates and steroids.

Acknowledgments

We are very grateful to the Department of Pharmacology, the University of Sydney, for managing and maintaining the *Xenopus laevis* colony. We are also grateful to Dr Paul Whiting, Prof Bjarke Ebert and Dr George Uhl for GABA receptor subunits cDNAs. The authors are thankful to A/Prof Petrine Wellendorph, Dr Sebastian Fernandez, and Hye-Lim Kim for initial technical assistance. NK acknowledges funding from The University of Malakand, Pakistan (Faculty Development Programme Scholarship) and a John Lamberton Scholarship and NG acknowledges support from an Endeavour International Postgraduate Research Scholarship (EIPRS) and a John Lamberton Scholarship.

Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Enhancement of [^3H] muscimol binding by diazepam and 2'MeO6MF. Each data point is the mean of triplicate determinations, which varied by <15%.

Figure S2 Example of a trace from an oocyte expressing human recombinant $\alpha 2\beta 2\gamma 2\text{L}$ receptors. 2'MeO6MF (30 μM) and a submaximal concentration of GABA (EC_{10} 10 μM) activated the receptor (duration indicated by open and black bars respectively). When 2'MeO6MF (30 μM) and GABA (10 μM) are applied together, the resultant peak current (735 nA) is approximately the sum of the individual currents 550 nA (2'MeO6MF 30 μM) and 165 nA (GABA 10 μM).

Figure S3 Recording from $\alpha 2\beta 2\gamma 2\text{L}$ GABA $_A$ receptors expressed in *Xenopus* oocytes showing PTZ (1 mM) blocking the effect of 2'MeO6MF (100 μM). Horizontal bars indicate duration of drug application.

Figure S4 Concentration–response curves for GABA alone (■) and GABA in the presence of 100 μM 2'MeO6MF (▲) on (A) $\alpha 3\beta 2\gamma 2\text{L}$; (B) $\alpha 5\beta 2\gamma 2\text{L}$; and (C) $\rho 1$ GABA receptors. Data are expressed as mean $\pm$ SEM ($n = 4\text{--}5$ oocytes).

Figure S5 2'MeO6MF activates an outwardly rectifying single-channel Cl^- current in neurons, typical of those mediated by GABA. (A) In the absence of ligand an excised inside-out patch from a cultured hippocampal neuron is electrically silent as depicted in the 2 s trace and 30 s all-points probability histogram taken at the end of 5 min. (B) Within 2 min of perfusing 100 μM 2'MeO6MF onto the patch a current was

observed with an average amplitude of 0.7 pA ($-V_p = -60$ mV, ~ 12 pS). The current amplitude increased with time showing transitions to intermediate, substates as shown in panel C (1.5 and 2.7 pA). A maximum average amplitude was reached measuring 4.1 pA with three channels open in the patch. All traces are 2 s recordings with an expanded trace of 200 ms together with a 30 s all-points histogram. When the membrane potential was switched to $-V_p = +60$ mV the single-channel current rectified, showing a reduced average amplitude of 3.3 pA and lower open probability as depicted in the all-points probability histogram in panel E. At 0 mV and in near equimolar chloride solutions there was current was negligible consistent with it being carried by chloride ions (F).

Figure S6 Effect of diazepam (1 and 2 $\text{mg}\cdot\text{kg}^{-1}$) and 2'MeO6MF (1, 10, 30 and 100 $\text{mg}\cdot\text{kg}^{-1}$) on the number of closed arm entries of the elevated plus maze over a 5 min test period in the mice. Each bar represents mean $\pm$ SEM ($F = 0.33$ $P > 0.05$ ANOVA followed by Dunnet's *post hoc* test).

Scheme 1 Synthesis of 2'-methoxy-6-methylflavone (2'MeO6MF).

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