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6-Methylflavanone, a more efficacious positive allosteric modulator of γ-aminobutyric acid (GABA) action at human recombinant $\alpha_2\beta_2\gamma_{2L}$ than at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes

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Abstract

6-Methylflavanone acted as a positive allosteric modulator of γ-aminobutyric acid (GABA) responses at human recombinant $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. It was essentially inactive at ρ_1 $GABA_C$ receptors.

The EC_{50} values for 6-methylflavanone for the positive modulation of the $EC_{10–20}$ GABA responses were 22 μM, 10 μM and 6 μM and the maximum potentiations were 120%, 417% and 130% at $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors respectively.

Thus 6-methylflavanone was much more efficacious as a positive modulator at $\alpha_2\beta_2\gamma_{2L}$ than at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors. This may be significant since diazepam-induced anxiolysis is considered to be mediated via α_2-containing $GABA_A$ receptors, while sedation is thought to be mediated via α_1-containing $GABA_A$ receptors.

We have previously reported that 6-methylflavone (1–100 μM) produced positive allosteric modulation at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors with no significant difference between the enhancement seen at either receptor subtype. In the present study, 6-methylflavone was tested at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors and found to maximally potentiate the $EC_{10–20}$ GABA response by 183 ± 39% which is similar to that previously observed for 6-methylflavone at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Thus, 6-methylflavone did not show a preference for $\alpha_2\beta_2\gamma_{2L}$ over $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors in terms of efficacy.

Compared to 6-methylflavone, 6-methylflavanone is more efficacious as a positive allosteric modulator at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors, and less efficacious at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. This may represent a relatively unique type of selectivity for positive modulators of GABA-A receptor subtypes based on efficacy as distinct from potency.

As was previously shown for 6-methylflavone at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, the positive modulation of GABA responses at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors by 6-methylflavanone was insensitive to antagonism by flumazenil, indicating that this action is not mediated via "high-affinity" benzodiazepine sites.

Keywords: GABA (γ-aminobutyric acid); 6-methylflavanone; 6-methylflavone; Benzodiazepine; Flavonoid; $GABA_A$ receptor

1. Introduction

$GABA_A$ receptors belong to the superfamily of ligand-gated ion channels that include $GABA_C$, nicotinic acetylcholine, strychnine-sensitive glycine and $5\text{-}HT_3$ receptors. $GABA_A$ receptors are hetero-oligomeric receptors forming a pentameric structure. At least 16 human $GABA_A$ receptor subunits have been described and are classified under 6 subfamilies of protein subunits: α, β, γ, δ, ε, θ (Chebib and Johnston, 2000).

Classically, benzodiazepine agonists act as positive modulators at a subpopulation of $GABA_A$ receptors by increasing the frequency of chloride channel openings (Chebib and Johnston, 2000; Rogers et al., 1994). The

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benzodiazepine binding sites for this action, which can be blocked by flumazenil, are considered to lie between the α and γ subunits (Klausberger et al., 2001). Flumazenil-insensitive positive modulation of $GABA_A$ receptors has been described in receptors lacking a γ subunit by benzodiazepines at micromolar concentrations. For example, Malherbe et al. (1990) found that recombinant $\alpha_1\beta_1$ $GABA_A$ receptors from rat brain were sensitive to potentiation by benzodiazepine receptor ligands, with both diazepam and flumazenil acting as positive modulators. Walters et al. (2000) found that classical benzodiazepines produce biphasic potentiation at rat recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors via two distinct mechanisms. This biphasic potentiation is believed to be mediated via two sites referred to as high-affinity and low-affinity benzodiazepine sites. Both sites are present at receptors composed of $\alpha_1\beta_2\gamma_2$ $GABA_A$ subunits and low-affinity potentiation can be selectively observed at receptor combinations lacking a γ subunit such as $\alpha_1\beta_2$ $GABA_A$ receptors. Furthermore, low-affinity potentiation at both receptor combinations is insensitive to flumazenil.

Flavonoids are a group of structurally related phenylbenzopyrones subdivided into the following main classes: flavones, flavanones, flavonols, isoflavones, flavans and flavan-3-ols. They are found in all plants and therefore form part of our diet. Our estimated average daily intake of flavonoids of 1–2 g is derived from fruits, vegetables, chocolate and beverages such as tea, coffee and red wine as well as herbal preparations.

Flavonoids are known to exert both peripheral and central nervous system effects. Much of the research into flavonoids has focussed on their peripheral actions, however more recently, they have been the subject of many studies using rat or bovine brain membrane binding assays which suggest that they possess a selective affinity for the benzodiazepine binding site at $GABA_A$ receptors (Dekermendjian et al., 1999; Hong and Hopfinger, 2003; Huang et al., 2001; Hui et al., 2000; Kahnberg et al., 2002; Marder et al., 1997, 1998; Medina et al., 1998; Nielsen et al., 1988). Many behavioural studies have also been carried out, often in conjunction with binding studies, which indicate that flavonoids exert anxiolytic effects in rodents without many of the unwanted side effects of benzodiazepines (Griebel et al., 1999; Marder et al., 1995, 1996; Salgueiro et al., 1997; Viola et al., 1995, 1997, 2000a; Wolfman et al., 1994, 1996, 1998). More recently, this has been extended to include functional studies of flavonoid activity at recombinant $GABA_A$ receptors (Ai et al., 1997; Hall et al., 2004; Hanrahan et al., 2003; Huen et al., 2003; Kavvadias et al., 2004; Viola et al., 2000b).

Flavanones have not been studied as GABA receptor modulators as extensively as the flavones. Flavanones differ from flavones by the absence of the 2–3 double bond (Fig. 1), resulting in a less planar structure and a chiral centre at the 2 position of the pyrone ring, giving rise to two or four possible isomers depending on the substituents at the 3 position.

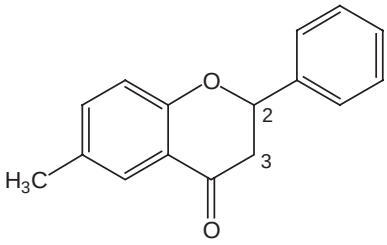

Fig. 1. 6-Methylflavanone.

Some examples of flavanones that have been investigated for an ability to displace [^{3}H]diazepam are eriodictyol (3′,4′,5,7-tetrahydroxyflavanone), hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone), 7-hydroxyflavanone, kaempferol (3,4′,5,7-tetrahydroxyflavanone), 4′-methoxyflavanone and naringenin (4′,5,7-trihydroxyflavanone). All were found to be inactive or only weakly active as determined by having an IC_{50} value greater than 25 μM (Ai et al., 1997).

Flavanone itself had no effect on [^{3}H]flunitrazepam binding up to a concentration of 40 μM while flavone inhibited [^{3}H]flunitrazepam binding with a K_i of 1 μM (Marder et al., 1996). Also, the flavone apigenin inhibited benzodiazepine binding with a K_i of 4 μM (Viola et al., 1995) while naringenin, the flavanone analogue of apigenin, had no effect up to a concentration of 25 μM (Ai et al., 1997). Thus, it would appear that flavones are more active than flavanones at binding to classical benzodiazepine sites. According to structure–activity analysis, in order to bind to the classical benzodiazepine site at $GABA_A$ receptors, flavonoids should be planar in structure (Dekermendjian et al., 1999). Thus, the less planar structure of flavanones would be expected to reduce their affinity for classical benzodiazepine binding sites. However, it has also been found that a planar conformation is not an absolute requirement for flavonoid activity at the benzodiazepine site of $GABA_A$ receptors (Hong and Hopfinger, 2003). Hence, there remain discrepancies regarding the structure–activity requirements for the flavonoid pharmacophore at the benzodiazepine site.

Previously, we described the flumazenil-insensitive positive modulatory action of 6-methylflavone at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors (Hall et al., 2004). To date, no studies have investigated the action of 6-methylflavanone (Fig. 1) at $GABA_A$ receptors. Based on previous findings, 6-methylflavanone would be predicted to be less active than 6-methylflavone on benzodiazepine binding. However, functional studies in our laboratory have already shown that 6-methylflavone is a positive modulator of GABA-induced currents through a mechanism other than an action at classical benzodiazepine sites. Hence, testing 6-methylflavanone in a functional study at $GABA_A$ receptors is also likely to yield different results to those expected to be obtained in a benzodiazepine binding study.

The present study set out to characterise the effects of 6-methylflavanone at human recombinant $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, $\alpha_1\beta_2$ and ρ_1 GABA receptors expressed in *Xenopus laevis* oocytes.

2. Materials and methods

2.1. Drugs

Diazepam and flumazenil were gifts from Hoffman-La Roche (Nutley, NJ, USA). GABA and dimethyl sulfoxide (DMSO) were obtained from Sigma (St Louis, MO, USA).

2.2. Synthesis of 6-methylflavanone

2.2.1. p-Cresyl benzoate

Acetic anhydride (9.2 g; 90 mmol) was added slowly with stirring to a solution of *p*-cresol (8.00 g; 74 mmol) in pyridine (100 ml). The reaction mixture was heated to reflux, stirred overnight, then cooled to room temperature and the solvent removed under vacuum. The crude product was extracted into dichloromethane and the solvent removed to give a yellow oil (10.03 g; 91%). δ_H (300 MHz; $CDCl_3$) 2.28 (3 H, s, $ArCH_3$), 2.34 (3 H, s, $COCH_3$), 6.96 (2 H, dd, *J* 6.6, 2.4, ArH), 7.17 (2 H, dd, *J* 6.6, 2.4, ArH).

2.2.2. 2-Acetyl-4-methylphenol

Aluminium chloride (8.90 g, 67 mmol) was added slowly (over approximately 1 h) to *p*-cresyl benzoate (10.00 g, 67 mmol) at 130 °C. The temperature was raised to 160 °C and kept there for 45 min. The reaction mixture was cooled to room temperature and 1 M hydrochloric acid (20 ml) was added. The crude product was extracted into dichloromethane and the solvent removed to give dark brown crystals (8.51 g; 85%). δ_H (300 MHz; $CDCl_3$) 2.32 (3 H, s, $ArCH_3$), 2.63 (3 H, s, $COCH_3$), 6.92 (1 H, d, *J* 8.1, C(6)–H), 7.33 (1 H, dd, *J* 8.4, 2.1, C(5)–H), 7.52 (1 H, d, *J* 1.2, C(3)–H), 12.10 (1 H, s, OH).

2.2.3. 2′-Hydroxy-5′-methylchalcone

Chilled aqueous sodium hydroxide (27 ml of 10% w/v) was added to a cooled stirred solution of benzaldehyde (5.86 g; 55 mmol) and 2-acetyl-4-methylphenol (8.29 g; 55 mmol) in ethanol (~30 ml). A yellow precipitate formed immediately and became a dark orange oil after a few hours. The reaction mixture was stirred for 48 h then poured into ice/water (100 ml) to become a tacky yellow solid. The crude product was extracted into dichloromethane and the organic layer was washed with brine (2 × 100 ml) and dried over anhydrous magnesium sulphate to give a dark orange oil (12.77 g). Thin layer chromatography (TLC) analysis showed that the reaction only went to 25% completion. Crude 2′-hydroxy-5′-methylchalcone along with starting material was separated from other impurities by vacuum column chromatography (eluent: 3% ethyl acetate in hexane). Recrystallisation from ethanol afforded the title compound as orange crystals (1.27 g; 10%). δ_H (300 MHz; $CDCl_3$) 2.36 (3 H, s, $ArCH_3$), 6.95 (1 H, d, *J* 8.1, C(6)–H), 7.33 (1 H, dd, *J* 8.4, 2.1, C(5)–H), 7.44–7.46 (2 H, m, C(2′)–H), 7.65–7.70 (5 H, m, HC=*C*H, C(3)–H, C(3′)–H, C(4′)–H), 7.92 (1 H, d, *J* 15.6, H*C*=CH), 12.65 (1 H, s, OH).

2.2.4. 6-Methylflavanone

2′-Hydroxy-5′-methylchalcone (1.27 g, 5.3 mmol) was mixed with glacial acetic acid (~50 ml) and phosphoric acid (0.3 ml, 5.3 mmol) then heated under reflux overnight. The acetic acid was removed under reduced pressure and the product extracted into dichloromethane (2 × 50 ml) to give a yellow oil (1.14 g). The crude product was recrystallised twice from ethanol to afford the title compound as white crystals (0.14 g; 10.7%), m.p. 107–109°C. δ_H (300 MHz; $CDCl_3$) 2.34 (3 H, s, $ArCH_3$), 2.88 (1 H, dd, *J* 16.8, 3, C(3)–H′), 3.08 (1 H, dd, *J* 16.8, 13.2, C(3)–H), 5.46 (1 H, dd, *J* 13.5, 3, C(2)–H), 6.97 (1 H, d, *J* 8.4, C(8)–H), 7.33 (1 H, dd, *J* 8.4, 2.1, C(7)–H), 7.39–7.50 (5 H, m, C(2′)–H, C(3′)–H, C(4′)–H), 7.73 (1 H, d, *J* 2.1, C(5)–H).

2.3. Expression of recombinant GABA receptors in X. laevis oocytes

The procedures involving the use of *X. laevis* were carried out according to those described by Huang et al. (2003) and were approved by the Animal Ethics Committee of the University of Sydney. Female *X. laevis* were anaesthetised 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 $MgCl_2$.6H2O, 5 mM HEPES, pH 7.5) and treated with Collagenase A (2 mg/ml in OR2, Bohringer Manheim, Germany) for 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 $MgCl_2$.6H2O, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Stage V–VI oocytes were collected and stored on an oscillator at 16 °C in ND96 'storage' solution (ND96 'wash' solution supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 μg/ml gentamycin).

Human α_1, α_2, β_2 and γ_{2L} DNA in pcDM8 were provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). Human ρ_1 DNA in pcDNA1.1 (Invitrogen, San Diego, CA, USA) was provided by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA). Plasmids containing α_1, α_2, β_2 or γ_{2L} DNA and ρ_1 DNA were linearised using the restriction enzymes *NOT*1 and *Xba*1, respectively. RNA was synthesised from linearised DNA using the 'mMessage mMachine' kit from Ambion (Austin, TX, USA). A 'Nanoject' (Drummond Scientific Co., Broomali, PA, USA) was used

to inject either $\alpha_1\beta_2\gamma_{2L}$ (20 ng/50 nl), $\alpha_2\beta_2\gamma_{2L}$ (20 ng/50 nl), $\alpha_1\beta_2$ (20 ng/50 nl) or ρ_1 (10 ng/50 nl) RNA dissolved in nuclease-free water into the cytoplasm of the oocytes, which were then stored at 16 °C in ND96 storage solution. For $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors the RNA for each subunit was injected in the ratio 1:1:2 respectively to favour incorporation of the γ subunit. For $\alpha_1\beta_2$ $GABA_A$ receptors the RNA for each subunit was injected in a 1:1 ratio. Sham-injected oocytes were prepared by injection with nuclease-free water.

2.4. Recording from oocytes

Two to seven days after injection of RNA, receptor activity was measured by two-electrode voltage clamp recording. Glass micropipettes for the electrodes were made using a micropipette puller (Narishige Scientific Instrument Lab, Tokyo, Japan) and filled with 3 M potassium chloride. Oocytes were transferred into a cell bath, impaled by two micropipettes, and voltage clamped using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA). The membrane potential was clamped at −60 mV and the electrodes had resistance values between 0.5 and 2 MΩ. In the cell bath, oocytes were continuously superfused with ND96 solution and drugs were applied in the perfusate. Current traces were recorded using a Mac Lab 2e recorder (ADInstruments, Sydney, NSW, Australia) and Chart v.3.5.2.

Oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors were screened with 10 μM Zn^{2+} in the presence of two concentrations of GABA (10 μM and 100 μM) to ensure that the γ subunit was incorporated. $GABA_A$ receptors without a γ subunit are sensitive to inhibition by zinc, whereas those expressing a γ subunit are not (Hosie et al., 2003). To test for positive modulation, low GABA doses at each subtype were chosen for the control doses since benzodiazepines produce greatest enhancement of the GABA response at lower GABA doses. 5 μM GABA, 3 μM GABA and 1 μM GABA were used as control GABA doses at $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors respectively as these doses corresponded to approximately an EC_{10} response at each subtype. Stock solutions in DMSO of 100 mM (6-methylflavone, diazepam and flumazenil) concentrations were prepared. GABA stock solutions (100 mM) were prepared in milli-Q water. For experiments where any drugs were dissolved in DMSO, all drug solutions were standardised to contain 0.8% DMSO, which had no significant effect on the oocytes.

2.5. Data analysis

Standardised responses were plotted against drug concentration on a semi-logarithmic scale using GraphPad Prism version 2.0. 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. EC_{50} is the concentration of agonist that produces a response that is 50% of the maximum current and n_H is the Hill coefficient. EC_{50} values are expressed as mean with 95% confidence intervals. Hill coefficients (n_H) are expressed as mean ± S.E.M. Significant differences were determined using two-way analysis of variances (ANOVAs) producing F and P values which have been quoted where appropriate.

3. Results

3.1. Direct activity at GABA receptors

6-Methylflavanone (100 μM) did not have any effect at sham-injected oocytes ($n=3$). Also, 6-methylflavanone (100 μM) had no activity at $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, $\alpha_1\beta_2$ and ρ_1 GABA receptors when administered alone.

3.2. 6-Methylflavanone as a positive allosteric modulator with a higher efficacy at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors compared to $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors

6-Methylflavanone enhanced the response to 3 μM GABA at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors ($n=4$–5; deviation from zero: significant, $F=55.45$, $P<0.0001$). The EC_{50} for this effect was 9.9 μM (95% CI: 7.01 to 14.02) with a Hill coefficient of 1.36 ± 0.28. 6-Methylflavanone maximally enhanced the response to 3 μM GABA by 417 ± 30% at a concentration of 60 μM ($n=3$; Fig. 2A) and shifted the GABA dose–response curve at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors to the left ($n=3$; two-way ANOVA: $F=105.41$, $P<0.0001$), decreasing the mean GABA EC_{50} from 18.2 μM (95% CI: 14.46 to 22.77) to 2.60 μM (95% CI: 1.935 to 3.481; Fig. 3A).

6-Methylflavanone also enhanced the response to a low dose of GABA at $\alpha_1\beta_2\gamma_{2L}$ ($n=4$–5; deviation from zero: significant, $F=60.56$, $P<0.0001$; Fig. 2B) and $\alpha_1\beta_2$ ($n=3$–6; deviation from zero: significant, $F=26.45$, $P<0.0001$; Fig. 2C) $GABA_A$ receptors, however the level of enhancement was lower than that observed at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors. At $\alpha_1\beta_2$ $GABA_A$ receptors, the EC_{50} value for 6-methylflavanone-induced enhancement was 5.72 μM (95% CI: 3.09 to 10.6). At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, an accurate EC_{50} value could not be determined as the dose–response curve was incomplete due to the solubility limit for 6-methylflavanone. At a concentration of 100 μM, 6-methylflavanone enhanced the response to 5 μM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors and 1 μM GABA at $\alpha_1\beta_2$ $GABA_A$ receptors by 120 ± 11% ($n=5$) and 116 ± 36% ($n=6$) respectively. 6-Methylflavanone shifted the GABA

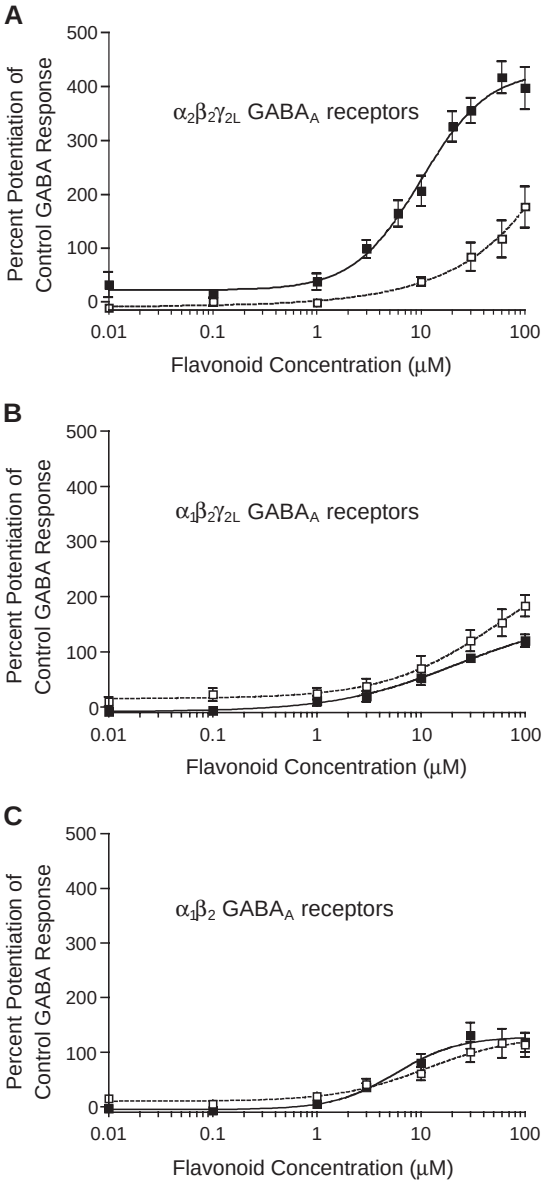

Fig. 2. Enhancement of the response to a low dose of GABA by 6-methylflavanone (■; solid line) and 6-methylflavone (□; dotted line) at (A) $\alpha_2\beta_2\gamma_{2L}$, (B) $\alpha_1\beta_2\gamma_{2L}$ and (C) $\alpha_1\beta_2$ GABA$_A$ receptors. Control GABA doses used at each receptor subtype were 3 μM, 5 μM and 1 μM respectively. Data for 6-methylflavone in B, C are from Hall et al. (2004). Data are expressed as mean ± S.E.M.

dose–response curve at $\alpha_1\beta_2\gamma_{2L}$ ($n=4$; two-way ANOVA: $F=19.04$, $P<0.0001$) GABA$_A$ receptors to the left, decreasing the mean GABA EC$_{50}$ from 21.47 μM (95% CI: 17.18 to 26.84) to 13.39 μM (95% CI: 8.87 to 20.22; Fig. 3B).

6-Methylflavanone (0.01–100 μM) did not enhance the response to 0.3 μM GABA at ρ_1 GABA$_C$ receptors (data not shown). Linear regression analysis produced a line with a slope that did not significantly deviate from zero ($n=3$; $F=1.668$, $P=0.2129$). 6-Methylflavanone also had no significant effect on the GABA dose–response curve at ρ_1 GABA$_C$ receptors ($n=5$; two-way ANOVA: $F=0.13$, $P=0.7214$; data not shown).

3.3. 6-Methylflavone as a positive allosteric modulator at $\alpha_2\beta_2\gamma_{2L}$ GABA$_A$ receptors

6-Methylflavone also enhanced the response to 3 μM GABA at $\alpha_2\beta_2\gamma_{2L}$ GABA$_A$ receptors ($n=3$–5, deviation from zero: significant, $F=35.35$, $P<0.0001$), however this was to a lesser extent than that by 6-methylflavanone. Solubility constraints prevented the determination of an accurate EC$_{50}$ for this effect of 6-methylflavone at $\alpha_2\beta_2\gamma_{2L}$ GABA$_A$ receptors; however, 6-methylflavone (100 μM) maximally potentiated the control response to GABA by 183 ± 39% ($n=5$; Fig. 2A). 6-Methylflavone (60 μM)

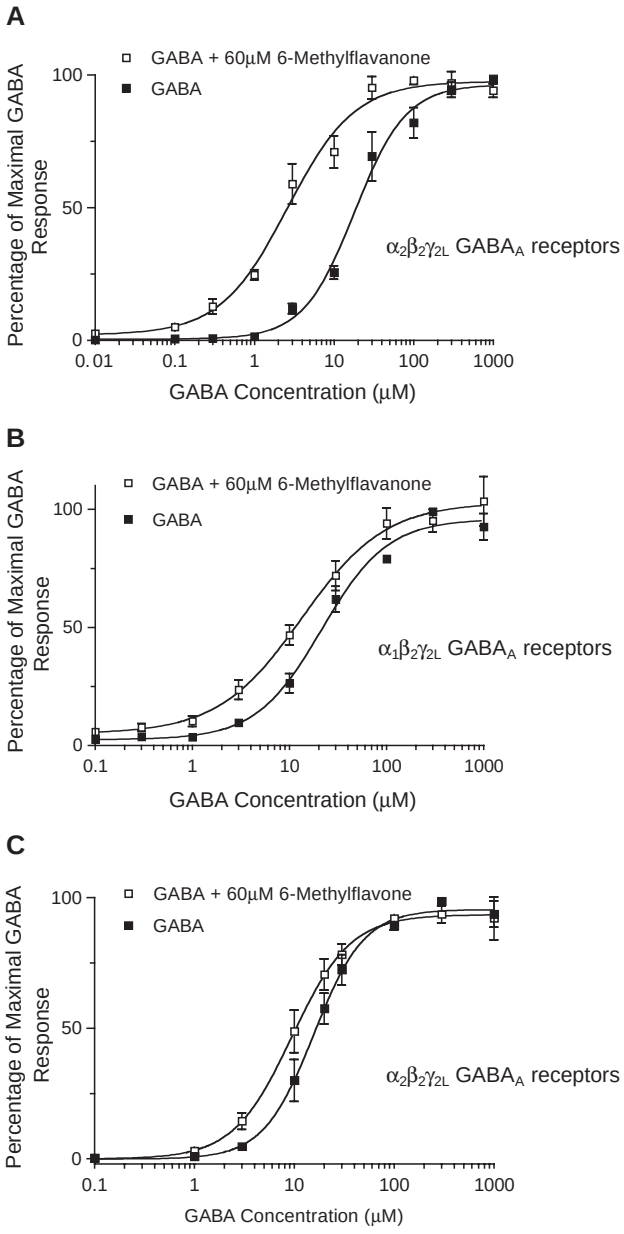

Fig. 3. Enhancement of the GABA dose–response curves at (A) $\alpha_2\beta_2\gamma_{2L}$, (B) $\alpha_1\beta_2\gamma_{2L}$ GABA$_A$ receptors by 60 μM 6-methylflavanone and (C) $\alpha_2\beta_2\gamma_{2L}$ GABA$_A$ receptors by 60 μM 6-methylflavone. Data are expressed as mean ± S.E.M.

shifted the GABA dose–response curve $\alpha_2\beta_2\gamma_{2L}$ ($n=4$, two-way ANOVA: $F=5.57$, $P=0.0219$) $GABA_A$ receptors to the left, decreasing the mean GABA EC_{50} from 15.7 μM (95% CI: 13.13 to 18.74) to 9.46 μM (95% CI: 7.26 to 12.3; Fig 3C).

3.4. Flumazenil insensitive action of 6-methylflavanone

Application of increasing concentrations of flumazenil (0.01–10 μM) in the presence of GABA (3 μM) and 6-methylflavanone (3 μM) did not block the 6-methylflavanone-induced enhancement of the GABA response at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 4A). Similar results were observed at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors using 5 μM GABA in the presence of 10 μM 6-methylflavanone. These 6-methylflavanone concentrations were chosen to test for flumazenil sensitivity as they represent approximately the lowest dose producing observable enhancement at each receptor subtype. In contrast, the enhancement of the GABA (3 μM) response produced by diazepam (1 μM) was completely abolished by 0.1 μM flumazenil at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 4B).

4. Discussion

These results show that 6-methylflavanone, like 6-methylflavone, is an allosteric positive modulator at human recombinant $\alpha_1\beta_2\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ expressed in *X. laevis* oocytes, as it produced no response when administered alone but enhanced the response to a low dose of GABA. This enhancement by 6-methylflavanone does not appear to be mediated via classical benzodiazepine sites, since it was insensitive to antagonism by flumazenil (Fig. 4A). Furthermore, the action of 6-methylflavanone was not dependent on the presence of a γ subunit, which is required for the formation of classical benzodiazepine sites. Although flumazenil (10 μM) slightly enhanced the response to 3 μM GABA in the presence of 3 μM 6-methylflavanone at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 4A), it is unlikely that this was responsible for the apparent flumazenil insensitivity of 6-methylflavanone-induced positive modulation, since under the same conditions, diazepam-induced positive modulation was completely abolished by the presence of flumazenil (0.1–10 μM; Fig. 4B).

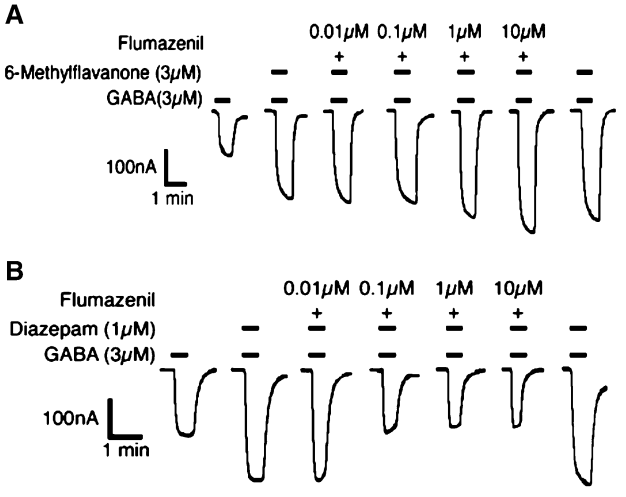

Fig. 4. Representative current traces from individual oocytes showing that potentiation of the GABA (3 μM) response at human recombinant $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors by (A) 6-methylflavanone (3 μM) is not inhibited by flumazenil (0.01–10 μM) whereas under the same conditions, (B) potentiation by 1 μM diazepam is inhibited by flumazenil.

6-Methylflavanone enhanced the response to GABA at $\alpha_2\beta_2\gamma_{2L}$, $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors (Fig. 2A–C), but not at ρ_1 $GABA_C$ receptors. A complete dose–response curve for 6-methylflavanone action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was not obtained, as the limit of solubility for 6-methylflavanone in 0.8% DMSO is 100 μM. This makes a comparison between 6-methylflavanone action at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors difficult, however two-way ANOVA showed that there was no significant difference between the dose–response curves for 6-methylflavanone enhancement at each receptor subtype ($F=3.01$, $P=0.088$). It seems that 6-methylflavanone was more potent at $\alpha_1\beta_2$ $GABA_A$ receptors, since a maximal response was reached and an EC_{50} value obtained. The EC_{50} value of 5.75 μM also appears to be lower than any possible estimate for an EC_{50} value at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, since, at these receptors, 6-methylflavanone at a concentration of 5 μM produced ~40% enhancement of the GABA response which was only about a third of the maximal observed enhancement of $120\pm11\%$ at 100 μM. At $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors, 6-methylflavanone enhanced the control response to GABA with an EC_{50} of 9.9 μM which is comparable to, but slightly higher than, the EC_{50} at $\alpha_1\beta_2$ $GABA_A$ receptors.

More significant however, was the difference in 6-methylflavanone efficacy at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors compared with the α_1-containing $GABA_A$ receptor subtypes tested. 6-Methylflavanone had a similar efficacy at both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors, producing a maximal enhancement at each receptor subtype of $120\pm11\%$ and $130\pm24\%$ respectively (*t*-test showed no significant difference: $P=0.727$). In contrast, at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors, 6-methylflavanone maximally enhanced the response to GABA by $417\pm30\%$ (*t*-tests showed significant differences between $\alpha_2\beta_2\gamma_{2L}$ and each α_1-containing subtype: $P<0.0001$ for $\alpha_1\beta_2\gamma_{2L}$ and $P=0.0002$ for $\alpha_1\beta_2$). Fig. 2A, B and C are all displayed with a *y*-axis spanning from 0 to 500% to illustrate this difference more clearly. Two-way ANOVA showed that there was a significant difference between the dose–response curves for 6-methylflavanone enhancement at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors ($F=160.25$, $P<0.0001$).

6-Methylflavanone (60 μM) shifted the GABA dose–response curve to the left at both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 3A–B), but not at ρ_1 $GABA_C$ receptors (data not shown). The greatest shift was seen at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors, where 6-methylflavanone

decreased the mean GABA EC_{50} to 2.60 μM (Fig. 3A), which is only 14% of the original mean GABA EC_{50} value of 18.2 μM. At $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, 6-methylflavanone decreased the mean GABA EC_{50} to 62% of the original value (Fig. 3B).

Previously, we investigated the effect of 6-methylflavone at recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes and found that it is a flumazenil-insensitive, positive modulator at recombinant $GABA_A$ receptors of $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ subtypes (Hall et al., 2004). Comparing our previous results with those of the current study, it appears that 6-methylflavone had a slightly higher efficacy at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, since the maximal observed enhancement at 100 μM 6-methylflavone (183 ± 20%; Hall et al., 2004) was higher than that produced by 6-methylflavanone in the current study (120 ± 11%). In contrast to 6-methylflavone, 6-methylflavanone is a racemic mixture of two enantiomers, thus the concentration of the active component may be only half the stated concentration. At $\alpha_1\beta_2$ $GABA_A$ receptors, 6-methylflavone and 6-methylflavanone appear to have similar potencies, with EC_{50} values of 8.7 μM (Hall et al., 2004) and 5.7 μM respectively. 6-Methylflavanone also had a similar efficacy to 6-methylflavone at $\alpha_1\beta_2$ $GABA_A$ receptors, the former maximally enhancing the response to 1 μM GABA in the current study by 130 ± 24% compared with the maximal enhancement of 116 ± 27% produced by 6-methylflavone in the previous study (Hall et al., 2004). The effect of each compound on the GABA dose–response curve at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was similar, as 6-methylflavanone and 6-methylflavone decreased the mean GABA EC_{50} values to 62% and 67% (Hall et al., 2004) of their original values, respectively.

In the current study, 6-methylflavone was tested at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors for comparison with 6-methylflavanone since this subtype was not investigated in our previous study on 6-methylflavone (Hall et al., 2004). Fig. 2A shows that 6-methylflavanone had both a greater efficacy and potency than 6-methylflavone. While no EC_{50} value was obtained for the enhancing effect of 6-methylflavone at these receptors, the EC_{50} value of 9.9 μM for 6-methylflavanone was well below any EC_{50} value that could be estimated for 6-methylflavone, hence 6-methylflavanone appears to be more potent. For instance, at a concentration of 10 μM, 6-methylflavone produced an average enhancement which was less than a quarter of the average enhancement observed at the maximal test concentration of 100 μM. The efficacy of 6-methylflavanone at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors was much higher than that of 6-methylflavone since the former produced a maximal enhancement of 417 ± 30% compared with 183 ± 39% for 6-methylflavone. Furthermore, 60 μM 6-methylflavanone shifted the GABA dose–response curve at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors to the left to a greater extent than 60 μM 6-methylflavone. 6-Methylflavanone decreased the mean GABA EC_{50} to a mere 14% of its original value (Fig. 3A), while 6-methylflavone only decreased the mean GABA EC_{50} to 60% of its original value (Fig. 3C).

In conclusion, 6-methylflavanone is a flumazenil-insensitive, positive modulator at $GABA_A$ receptors. This action appears to be selective for $GABA_A$ receptors, since 6-methylflavanone was inactive at ρ_1 $GABA_C$ receptors. At $GABA_A$ receptors, 6-methylflavanone, unlike 6-methylflavone (Hall et al., 2004), displayed some subtype selectivity, as it was much more efficacious at $GABA_A$ receptors of the $\alpha_2\beta_2\gamma_{2L}$ subtype compared with the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ subtypes. The greater efficacy of 6-methylflavanone at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors compared to the α_1-containing $GABA_A$ receptors may be important behaviourally, as it is known that the actions of benzodiazepines depend on the $GABA_A$ receptor subtype, with the type of α subunit present being particularly important.

Rudolph et al. (1999) showed that α_1-containing receptors are responsible for mediating diazepam-induced sedation, anterograde amnesia and impairment of locomotor activity, while α_2-containing $GABA_A$ receptors have been shown to be responsible for mediating diazepam-induced anxiolysis (Löw et al., 2000). Therefore, it is possible that a compound like 6-methylflavanone that has a greater efficacy at $\alpha_2\beta_2\gamma_{2L}$ receptors may be useful for treating anxiety with a reduction in some of the other effects of classical benzodiazepines, particularly sedation. Clearly 6-methylflavanone as a racemic mixture is not as potent with an EC_{50} of 9.9 μM at $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors, but resolution of the active isomer may afford a compound with a likely EC_{50} of ~5 μM. 6-Methylflavanone provides a lead for the discovery of more potent analogues as positive GABA modulators with an even greater selectivity for α_2-containing $GABA_A$ receptors.

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References

Ai, J., Dekermendjian, K., Wang, X., Nielsen, M., Witt, M., 1997. 6-Methylflavone, a benzodiazepine receptor ligand with antagonistic properties on rat brain and human recombinant $GABA_A$ receptors in vitro. Drug Dev. Res. 41, 99–106.

Chebib, M., Johnston, G.A.R., 2000. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. J. Med. Chem. 43, 1–21.

Dekermendjian, K., Kahnberg, P., Witt, M.R., Sterner, O., Nielsen, M., Liljefors, T., 1999. Structure–activity relationships and molecular modeling analysis of flavonoids binding to the benzodiazepine site of the rat brain $GABA_A$ receptor complex. J. Med. Chem. 42, 4343–4350.

Griebel, G., Perrault, G., Tan, S., Schoemaker, H., Sanger, D.J., 1999. Pharmacological studies on synthetic flavonoids: comparison with diazepam. Neuropharmacology 38, 965–977.

Hall, B.J., Chebib, M., Hanrahan, J.R., Johnston, G.A.R., 2004. Flumazenil-independent positive modulation of γ-aminobutyric acid action by 6-methylflavone at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors. Eur. J. Pharmacol. 491, 1–8.

Hanrahan, J.R., Chebib, M., Davucheron, N.L.M., Hall, B.J., Johnston, G.A.R., 2003. Semisynthetic preparation of amentoflavone: a negative modulator at $GABA_A$ receptors. Bioorg. Med. Chem. Lett. 13, 2281–2284.

Hong, X., Hopfinger, A.J., 2003. 3D-pharmacophores of flavonoid binding at the benzodiazepine $GABA_A$ receptor site using 4D-QSAR analysis. J. Chem. Inf. Comput. Sci. 43, 324–336.

Hosie, A.M., Dunne, E.L., Harvey, R.J., Smart, T.G., 2003. Zinc-mediated inhibition of $GABA_A$ receptors: discrete binding sites underlie subtype specificity. Nat. Neurosci. 6, 362–369.

Huang, X.Q., Liu, T., Gu, J.D., Luo, X.M., Ji, R.Y., Cao, Y., Xue, H., Wong, J.T.F., Wong, B.L., Pei, G., Jiang, H.L., Chen, K.X., 2001. 3D-QSAR model of flavonoids binding at benzodiazepine site in $GABA_A$ receptors. J. Med. Chem. 44, 1883–1891.

Huang, S.H., Duke, R.K., Chebib, M., Sasaki, K., Wada, K., Johnston, G.A.R., 2003. Bilobalide, a sesquiterpene trilactone from *Gingko biloba*, is an antagonist at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Eur. J. Pharmacol. 464, 1–8.

Huen, M.S.Y., Hui, K., Leung, J.W.C., Sigel, E., Baur, R., Wong, T., Xue, H., 2003. Naturally occurring 2′-hydroxyl-substituted flavonoids as high-affinity benzodiazepine site ligands. Biochem. Pharmacol. 66, 2397–2407.

Hui, K.M., Wang, X.H., Xue, H., 2000. Interaction of flavones from the roots of *Scutellaria baicalensis* with the benzodiazepine site. Planta Med. 66, 91–93.

Kahnberg, P., Lager, E., Rosenberg, C., Schougaard, J., Camet, L., Sterner, O., Nielsen, E.O., Nielsen, M., Liljefors, T., 2002. Refinement and evaluation of a pharmacophore model for flavone derivatives binding to the benzodiazepine site of the $GABA_A$ receptor. J. Med. Chem. 45, 4188–4201.

Kavvadias, D., Sand, P., Youdim, K.A., Qaiser, M.Z., Rice-Evans, C., Baur, R., Sigel, E., Rausch, W., Riederer, P., Schreier, P., 2004. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood–brain barrier and exhibits anticonvulsive effects. Br. J. Pharmacol. 142, 811–820.

Klausberger, T., Sarto, I., Ehya, N., Fuchs, K., Furtmuller, R., Mayer, B., Huck, S., Sieghart, W., 2001. Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. J. Neurosci. 21, 9124–9133.

Löw, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J.A., Fritschy, J., Rulicke, T., Bluethmann, H., Mohler, H., Rudolph, U., 2000. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290, 131–134.

Malherbe, P., Draguhn, A., Multhaup, G., Beyreuther, K., Mohler, H., 1990. $GABA_A$-receptor expressed from rat brain α- and β-subunit cDNAs displays potentiation by benzodiazepine receptor ligands. Mol. Brain Res. 8, 199–208.

Marder, M., Viola, H., Wasowski, C., Wolfman, C., Waterman, P.G., Medina, J.H., Paladini, A., 1995. 6,3′-Dinitroflavone, a novel high affinity ligand for the benzodiazepine receptor with potent anxiolytic properties. Bioorg. Med. Chem. Lett. 5, 2717–2720.

Marder, M., Viola, H., Wasowki, C., Wolfman, C., Waterman, P.G., Cassels, B.K., Medina, J.H., Paladini, A., 1996. 6-Bromoflavone, a high affinity ligand for the central benzodiazepine receptors is a member of a family of active flavonoids. Biochem. Biophys. Res. Commun. 223, 384–389.

Marder, M., Zinczuk, J., Colombo, M.I., Wasowki, C., Viola, H., Wolfman, C., Medina, J.H., Ruveda, E.A., Paladini, A., 1997. Synthesis of halogenated/nitrated flavone derivatives and evaluation of their affinity for the central benzodiazepine receptor. Bioorg. Med. Chem. Lett. 7, 2003–2008.

Marder, M., Viola, H., Bacigaluppo, J.A., Colombo, M.I., Wasowki, C., Wolfman, C., Medina, J.H., Ruveda, E., Paladini, A., 1998. Detection of benzodiazepine receptor ligands in small libraries of flavone derivatives synthesized by solution phase combinatorial chemistry. Biochem. Biophys. Res. Commun. 249, 481–485.

Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1998. Neuroactive flavonoids: new ligands for the benzodiazepine receptors. Phytomedicine 5, 235–243.

Nielsen, M., Frokjaer, S., Braestrup, C., 1988. High affinity of the naturally-occurring biflavonoid, amentoflavon, to brain benzodiazepine receptors in vitro. Biochem. Pharmacol. 37, 3285–3287.

Rogers, C.J., Twyman, R.E., Macdonald, R.L., 1994. Benzodiazepine and β-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurons in culture. J. Physiol. 475, 69–82.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J.A., Fritschy, J., Martin, J.R., Bluethmann, H., Mohler, H., 1999. Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. Nature 401, 796–800.

Salgueiro, J.B., Ardenghi, P., Dias, M., Ferreira, M.B.C., Izquierdo, I., Medina, J.H., 1997. Anxiolytic and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory tasks in rats. Pharmacol. Biochem. Behav. 58, 887–891.

Viola, H., Wasowki, C., Levi de Stein, M., Wolfman, C., Silveira, R., Dajas, F., Medina, J.H., Paladini, A., 1995. Apigenin, a component of *Matricaria recutita* flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. Planta Med. 61, 213–216.

Viola, H., Marder, M., Wolfman, C., Wasowki, C., Medina, J.H., Paladini, A., 1997. 6-Bromo-3′-nitroflavone, a new high affinity benzodiazepine receptor agonist recognizes two populations of cerebral cortical binding sites. Bioorg. Med. Chem. Lett. 7, 373–378.

Viola, H., Marder, M., Wasowski, C., Giorgi, O., Paladini, A.C., Medina, J.H., 2000a. 6,3′-Dibromoflavone and 6-nitro-3′-bromoflavone: new additions to the 6,3′-disubstituted flavone family of high-affinity ligands of the brain benzodiazepine binding site with agonistic properties. Biochem. Biophys. Res. Commun. 273, 694–698.

Viola, H., Wolfman, C., Marder, M., Goutman, J.D., Bianchin, M., Wasowski, C., Calvo, D.J., Izquierdo, I., Paladini, A.C., Medina, J.H., 2000b. 6-Chloro-3′-nitroflavone is a potent ligand for the benzodiazepine binding site of the $GABA_A$ receptor devoid of intrinsic activity. Pharmacol. Biochem. Behav. 65, 313–320.

Walters, R.J., Hadley, S.H., Morris, D.W., Amin, J., 2000. Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms. Nat. Neurosci. 3, 1274–1281.

Wolfman, C., Viola, H., Paladini, A., Dajas, F., Medina, J.H., 1994. Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from *Passiflora Coerulea*. Pharmacol. Biochem. Behav. 47, 1–4.

Wolfman, C., Viola, H., Marder, M., Wasowski, C., Ardenghi, P., Izquierdo, I., Paladini, A.C., Medina, J.H., 1996. Anxioselective properties of 6,3′-dinitroflavone, a high-affinity benzodiazepine receptor ligand. Eur. J. Pharmacol. 318, 23–30.

Wolfman, C., Viola, H., Marder, M., Ardenghi, P., Wasowki, C., Schroder, N., Izquierdo, I., Ruveda, E., Paladini, A., Medina, J.H., 1998. Pharmacological characterisation of 6-bromo-3′-nitroflavone, a synthetic flavonoid with high affinity for the benzodiazepine receptors. Pharmacol. Biochem. Behav. 61, 239–246.