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$GABA_A$ receptor subtype selectivity underlying anxiolytic effect of 6-hydroxyflavone

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ABSTRACT

6-Hydroxyflavone (6HF), a naturally occurring flavonoid, was previously reported to bind to type A γ-aminobutyric acid ($GABA_A$) receptors benzodiazepine (BZ) site with moderate binding affinity. In the present study, we showed that 6HF partially potentiated GABA-induced currents in native $GABA_A$ receptors expressed in cortical neurons *via* BZ site, as the enhancement was blocked by the antagonist flumazenil. Furthermore, in patch clamp studies, 6HF displayed significant preference for α_2- and α_3-containing subtypes, which were thought to mediate anxiolytic effect, compared to α_1- and α_5-containing subtypes expressed in HEK 293T cells. In mice, 6HF exhibited anxiolytic-like effect in the elevated plus-maze test, unaccompanied at anxiolytic doses by the sedative, cognitive impairing, myorelaxant, motor incoordination and anticonvulsant effects commonly associated with classical BZs when tested in the hole-board, step-through passive avoidance, horizontal wire, rotarod, and pentylenetetrazol (PTZ)-induced seizure tests, respectively. The findings therefore identified 6HF as a promising drug candidate for the treatment of anxiety-like disorders.

1. Introduction

Type A γ-aminobutyric acid ($GABA_A$) receptors, belonging to the fast acting ligand gated ion channel superfamily, are the major inhibitory neurotransmitter receptors in the central nervous system (CNS) [1]. The ionotropic $GABA_A$ receptors are transmembrane proteins consisting of five subunits to form a heteromeric pentamer. Seven classes of subunits with multiple isoforms have been identified by sequence homology (α_1–α_6, β_1–β_3, γ_1–γ_3, δ, ε, π, θ) [2], and the majority of $GABA_A$ receptors in the brain contain two α, two β and one single γ subunit [3].

The BZ site on $GABA_A$ receptors modulates the inhibitory effects of GABA [4]. BZ site agonists such as diazepam and flunitrazepam increase the GABA-induced chloride channel opening frequency [5], exerting anxiolytic, anticonvulsant, muscle relaxant, sedative-hypnotic and cognition-impairing effects [6], rendering them the most important $GABA_A$ receptor-modulating drugs in clinical use. Despite the usefulness of conventional BZs as potent anxiolytics, unwanted side effects such as sedation, myorelaxation, amnesia, dependence and tolerance [7] have spearheaded a search for alternatives without side effects.

Experiments with site-directed mutagenesis have located the BZ site between α and γ subunits [5]. Since the majority of γ subunit in brain is the γ_2 subunit [8], different pharmacological effects of BZ are likely mediated by different α subunits. $GABA_A$ receptors are termed "diazepam sensitive" when they contain α_1, α_2, α_3 or α_5 subunits, or "diazepam-insensitive" when they contain α_4 or α_6 subunits which do not recognize the classical benzodiazepines (BZs) such as diazepam [9]. The sedation, anterograde amnesia, muscle relaxant effects and in part the anticonvulsant action are thought to be mediated by α_1 subunit-containing receptors [10–12], anxiolytic effect by α_2- or α_3-containing receptors [13–16], whereas α_5-containing receptors may play an important role in learning and memory [17]. On this basis, the search for new anxiolytics without side effects has been mainly focused on the development of agonists for the BZ site selective toward α_2 or α_3 subunit-containing receptors. Since the discovery of high-affinity binding of amentoflavone to the BZ site [18], flavonoids have drawn much interest as candidate drugs for the BZ site. A range of naturally occurring or synthetic flavonoids are found to be BZ site agonists [19,20], with only a small number of them displaying subunit-selective agonist activity [21,22].

The compound 6-hydroxyflavone (6HF) is a naturally occurring flavone found in the leaves of *Barleria prionitis*, a species of plants in the *Acanthaceae* family native to India that is widely used against neurological disorders such as paraplegia, sciatica, etc. [23]. Previous studies in our lab showed that 6HF binds to $GABA_A$ receptors with moderate affinity [24]. In the present study, the efficacies of 6HF for native $GABA_A$ receptors expressed in cortical neurons, and different recombinant $GABA_A$ receptor subtypes

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expressed in HEK 293T cells, were investigated. The results characterized 6HF as a novel selective agonist, exerting anxiolytic-like effect in the elevated plus-maze test but devoid of sedative, cognitive impairing, myorelaxant, motor incoordination and anticonvulsant effects at effective anxiolytic dosages in the hole-board, step-through passive avoidance, horizontal wire, rotarod, and PTZ-induced seizure tests, respectively.

2. Materials and methods

2.1. Chemicals

Radioactive [^{3}H]-flunitrazepam (N-methyl-[^{3}H], 88.0 Ci/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). 6HF (Indofine Chemical Company, NJ, USA; purity 97%) was dissolved in deionized-distilled water (dd water). Diazepam, flumazenil, PTZ and GABA were obtained from Sigma Chemical (St. Louis, MO, USA). Diazepam was dissolved in dd water in the presence of 1% DMSO for animal tests. These drugs were administered to mice at 10 ml/kg.

2.2. $GABA_A$ receptor subunit constructs

The human $GABA_A$ receptor α_1, α_2, α_3, α_5, β_2, β_3 and γ_{2L} subunits were amplified from ResGen™ human brain cDNA library (Invitrogen) by PCR. Full-length cDNA clones of α_2, α_3, α_5, β_2 and β_3 were constructed with the pcDNA3.1 vector (Invitrogen) [25], while full-length cDNAs of other subunits were inserted into the EcoRI–XbaI (for α_1) or EcoRI–BamHI (for γ_{2L}) sites of this vector. All constructs were verified by DNA sequencing.

2.3. Cell culture and transfection

2.3.1. Cortical neurons

Cortical tissue was dissected from E18 Sprague–Dawley rat embryos, incubated for 15 min in 0.125% trypsin–EDTA in Hank's buffered salt solution (HBSS) without calcium and magnesium, before transferring to Dulbecco's modified Eagle's medium (DMEM)/10% horse serum/0.5 mM glutamine. The dissociated cells were plated at a density of $8 \times 10^4/cm^2$ in poly-L-lysine-coated 30 mm dishes. After 4 h, the culture medium was changed to neurobasal medium/2% B27 supplement/2 mM glutamine. The cultures were maintained under an atmosphere of water saturated 5% CO_2, 95% air at 37 °C. Half of the culture medium was exchanged once a week, and 2–3-week-old cultures were used for electrophysiological recording.

2.3.2. Human embryonic kidney 293T cells

Human embryonic kidney (HEK) 293T cells were cultured in DMEM containing 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 95% air/5% CO_2. HEK 293T cells were transiently co-transfected with 1 μg of each plasmid DNA encoding $GABA_A$ receptor $\alpha_1\beta_3\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, $\alpha_3\beta_2\gamma_{2L}$, or $\alpha_5\beta_2\gamma_{2L}$ using the Lipofectamine reagent (Invitrogen) following manufacturer's instruction, and employed for radioligand binding analysis and whole-cell patch clamp studies 48 h after transfection.

2.4. Radioligand binding assays

The [^{3}H]-flunitrazepam binding assay was performed with synaptosomal membranes isolated from male Sprague–Dawley rat cerebral cortex [26] and membranes from HEK 293T cells [27] transfected with $\alpha_1\beta_3\gamma_{2L}$, $\alpha_2\beta_2\gamma_{2L}$, $\alpha_3\beta_2\gamma_{2L}$, or $\alpha_5\beta_2\gamma_{2L}$ subunits by means of filtration methods [4]. Briefly, 1 nM [^{3}H]-flunitrazepam was incubated with aliquots of membranes at 4 °C for 90 min in the presence of different concentrations of 6HF. Non-specific binding was determined by the addition of 10 μM diazepam. After incubation, the mixtures were filtered onto Whatman GF/B filters on a Brandel 24-well harvester. Each filter was incubated for at least 1 h with 4 ml scintillation cocktail before measurement of radioactivity in a Bechman-Coulter LS 6500 scintillation counter. The concentration at which 6HF elicited half maximum inhibition (IC_{50}) on the competitive inhibition curve was determined by non-linear regression analysis (Prism 3.0, Graphad Software). K_i value was calculated from $K_i = IC_{50}/[1 + ([^3H]/K_d)]$, where [^{3}H] was the concentration of [^{3}H]-flunitrazepam, and K_d the dissociation constant of [^{3}H]-flunitrazepam.

2.5. Whole-cell patch clamp

Whole-cell patch clamp current was recorded at room temperature (20–22 °C) on cortical neurons or HEK 293T cells using the EPC9 amplifier and Pulse/PulseFit Software (HEKA, Germany) [25]. The membrane potential was voltage clamped at −40 mV. Patch electrodes (3.0–6.0 MΩ) were filled with an internal solution containing 145 mM CsCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA and 4 mM Mg^{2+}-ATP, adjusted to pH 7.4 with CsOH. The cells were constantly perfused with external solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with 1 mM NaOH at 5 ml/min. Drugs were applied via gravity perfusion for 10 s with 3–5 min wash-out between applications to ensure complete recovery from desensitization. Diazepam and 6HF were prepared daily from stock (10 mM in DMSO); GABA was dissolved directly in the external solution. Data was fitted with a non-linear fitting program to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$ where x was drug concentration, EC_{50} the drug concentration eliciting half maximum response, and n the Hill coefficient.

2.6. Animals

Male ICR mice (Animal Care Center, HKUST) weighing 20–36 g were housed in groups of four to five with food and water *ad libitum* and kept on a 08:00 to 20:00 h light cycle. All animal experiments were pre-approved by the HKUST Animal Ethics Committee and conducted in accordance with the Code of Practice for Care and Use of Animals for Experimental Purposes, which was approved by the Hong Kong Department of Agriculture, Fisheries and Conservation, and Department of Health. All the animals were test-naive and used only once in each test.

2.7. Elevated plus-maze test

The maze consisted of two opposing open arms (25 cm × 5 cm), and two opposing closed arms of the same dimension but enclosed by 20 cm high walls. The four arms extended from a central platform (5 cm × 5 cm), and the maze was elevated 40 cm above ground and kept in a dimly lit room. Male ICR mice were randomized into five groups (n = 12–18/group), and received vehicle (dd water, pH 9.0, p.o.), 6, 12 or 25 mg/kg 6HF (p.o.), or 1 mg/kg diazepam (p.o.). Forty-five min after treatment, each mouse was placed individually at the center of the maze facing a closed arm. The number of entries into and time spent in the open arms and closed arms were recorded over a 5-min period. An arm entry was defined as having all four paws inside the arm. At the end of the test, the number of entries into and time spent in open arms were expressed as a percentage of total number of arm entries and total time spent in the arms.

To test whether the effects of 6HF in the elevated plus-maze experiment could be blocked by the BZ site antagonist flumazenil, male ICR mice were randomly divided into three groups (n = 12–

21/group). The control group received dd water 45 min (p.o.) and 15 min (i.p.) before the elevated plus-maze test. The second group was pretreated with 25 mg/kg 6HF (p.o.) 45 min and dd water (i.p.) 15 min prior to the test. The third group received 25 mg/kg 6HF (p.o.) 45 min and 1.25 mg/kg flumazenil (i.p.) 15 min before the test.

2.8. Hole-board test

This test was conducted in a walled black wood arena with 60 cm × 60 cm floor and 30 cm high walls, and four centered and equally spaced 3-cm diameter holes in the floor. Male ICR mice were randomized into the following groups (n = 12–22/group): vehicle control (dd water, pH 9.0, p.o.), diazepam (1 or 3 mg/kg, p.o.), 6HF (6, 12, 25, 50 or 100 mg/kg, p.o.). Forty-five min after treatment, the mice were placed and released singly in the center of the board, facing away from the observer. The number of head-dips and rearings were recorded in a given 5 min interval. After each trial the apparatus was wiped clean with 70% ethanol and dried thoroughly with tissue to remove traces of the previous assay. A decrease in the number of head-dips and rearings revealed a sedative behavior [28].

2.9. Step-through passive avoidance test

The apparatus was a two-compartment box with shock grid that consisted of one illuminated and one dark compartments separated by a guillotine door (Chinese Academic of Chinese Medical Science, Beijing, China). Male ICR mice were randomized into seven groups (n = 12–21/group) that received vehicle (dd water, p.o.), 6, 12, 25, 50 or 100 mg/kg 6HF (p.o.), or 1 mg/kg diazepam (p.o.) 45 min prior to training. During training, each mouse was placed into the lighted chamber. After 10 s, the door leading to the dark chamber was opened. Mice that did not enter the dark chamber within 15 s were excluded from the experiment. Once the mouse entered the dark compartment, the door was closed immediately and an inescapable electric foot-shock (0.4 mA, 1 s) was delivered from the grid floor. The mouse was then removed from the dark chamber and placed in the home cage 10 s later. Twenty-four hours later, the mouse was put back into the lighted chamber and the door was opened 10 s later. The time for the mouse to re-enter the dark chamber was recorded up to a 300 s cut off as the step-through latency.

2.10. Horizontal wire test

Male ICR mice were randomized into seven groups (n = 12–19/group). Vehicle (dd water, p.o.), 6, 12, 25, 50 or 100 mg/kg 6HF (p.o.), or 1 mg/kg diazepam (p.o.) were administrated 45 min prior to training. Mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm long, and placed 20 cm above the table) with their forepaws, and then released. Testing took place after two trials performed at 5-min intervals. The number of mice from each treatment group that did not grasp the wire with the forepaws or actively grasped the wire with at least one hind paw within 3 s was recorded. A myorelaxant drug would impair the ability of the mice to grasp the wire.

2.11. Rotarod test

The rotarod test utilized a custom-built apparatus consisting of an elevated cylinder (2.5 cm diameter) placed 0.5 m above the ground with a textured surface. Male ICR mice were randomly allocated into the following groups (n = 12–18/group): vehicle control (dd water, p.o.), 6HF (6, 12, 25, 50 or 100 mg/kg, p.o.), and diazepam (1 or 3 mg/kg, p.o.). Before drug administration, mice were trained to stay on the rotarod revolving at 16 rpm for two consecutive 120 s trials. Vehicle or drugs were orally administered 45 min before test. The duration, up to 120 s, that a mouse could remain on the rotarod was recorded.

2.12. PTZ-induced seizure test

Clonic convulsions were induced by the injection of PTZ (72 mg/kg, i.p.). ICR mice were randomly assigned into seven groups (n = 16–22/group). Vehicle (dd water, p.o.), 6HF (6, 12, 25, 50 or 100 mg/kg, p.o.) or diazepam (1 mg/kg, p.o.) were administrated 45 min prior to PTZ injection. The mice were placed individually in Perspex cages and observed for 30 min. The latency to onset of seizure and incidence of death following seizure after PTZ injection were recorded. If no clonic seizure was observed within 30 min, a latency of 1800 s was recorded.

2.13. Data analysis

Data were presented as mean ± standard error of the mean (SEM). Behavioral data were subjected to one-way ANOVA, and multiple group comparisons were made by Newman–Keuls' test for those responses that were significant in the ANOVA test. Fisher's exact test was used for comparing death rate in the convulsion test and percentage of mice grasping the wire in horizontal wire test. The accepted level of significance for the tests was $P < 0.05$.

3. Results

3.1. In vitro binding affinity

The addition of 6HF inhibited [^{3}H]-flunitrazepam binding to rat cerebral cortex membranes with a half-inhibitory K_i of 1.53 ± 0.09 μM (Fig. 1). Compared to the full agonist diazepam (K_i = 0.0064 ± 0.00021 μM) [24], 6HF was approximately 200-fold less potent.

3.2. Effects of 6HF on GABA-induced currents in cortical neurons

The effect of 6HF on GABA-induced currents was determined by whole-cell patch clamp recordings of rat cortical neurons. EC_{20} for the dose response of GABA in cortical neurons was determined to be 2.1 ± 0.4 μM, and the effect of 6HF was analyzed through co-application with GABA at this EC_{20} concentration. The current elicited with 2.1 μM GABA was increased to 192.5 ± 15.0% of control by 10 μM 6HF, which was smaller than the increase to 267.4 ± 19.0% by 1 μM diazepam (Fig. 2A). Half-maximal stimulation was observed at 1.2 ± 0.3 μM 6HF, or 0.11 ± 0.04 μM diazepam (Fig. 2A). The current

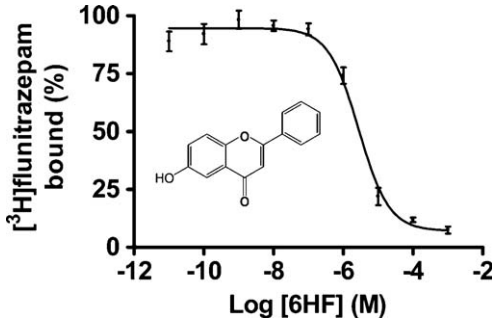

Fig. 1. Inhibition of [^{3}H]-flunitrazepam binding to rat cortical membrane by 6HF (structure shown on graph). Data represent mean ± SEM of four independent experiments each performed in duplicates.

Table 1
Binding affinity of 6HF to recombinant human $\alpha_1\beta_2\gamma_2$, $\alpha_x\beta_3\gamma_2$ (x = 2, 3, 5) $GABA_A$ receptors expressed in HEK 293T cells.

Subunit combination	Binding affinity, K_i (μM)
$\alpha_1\beta_2\gamma_2$	1.34 ± 0.44
$\alpha_2\beta_3\gamma_2$	4.46 ± 2.01
$\alpha_3\beta_3\gamma_2$	4.96 ± 3.21
$\alpha_5\beta_3\gamma_2$	4.76 ± 4.15

Data represent mean ± SEM of 4–5 independent experiments.

increase induced by 10 μM 6HF was blocked by simultaneous co-application of 1 μM flumazenil, a $GABA_A$ receptor BZ site antagonist (Fig. 2B and C).

3.3. Subtype selectivity of 6HF

The binding affinities and efficacies of 6HF on different recombinant $GABA_A$ receptor subtypes were investigated in HEK 293T cells. Although 6HF about 3-fold exhibited higher binding affinity for $\alpha_1\beta_2\gamma_2$- than for $\alpha_2\beta_3\gamma_2$-, $\alpha_3\beta_3\gamma_2$- and $\alpha_5\beta_3\gamma_2$-containing receptors (Table 1), it was not statistically significant ($P = 0.71$). Moreover, in whole-cell patch clamp analysis, 6HF differentially potentiated GABA-induced currents at $GABA_A$ receptors with different subunit compositions. From the GABA dose response curves, the EC_{20} for GABA in current induction was found to be 0.91 ± 0.37 μM, 0.29 ± 0.04 μM, 0.12 ± 0.04 μM and 0.69 ± 0.23 μM for the $\alpha_1\beta_2\gamma_2$-, $\alpha_2\beta_3\gamma_2$-, $\alpha_3\beta_3\gamma_2$-, and $\alpha_5\beta_3\gamma_2$-containing receptor subtypes, respectively. At a GABA concentration corresponding to the receptor EC_{20}, the effects of diazepam or 6HF on each receptor subtype were analyzed. Under these conditions, addition of diazepam yielded maximal currents of 167.3 ± 7.5%, 200.4 ± 12.2%, 171.3 ± 6.5% and 153.4 ± 6.5% relative to controls, and EC_{50} of 6.0 ± 2.1 nM, 7.4 ± 2.7 nM, 22.3 ± 10.1 nM and 9.9 ± 5.1 nM for the $\alpha_1\beta_2\gamma_2$-, $\alpha_2\beta_3\gamma_2$-, $\alpha_3\beta_3\gamma_2$-, and $\alpha_5\beta_3\gamma_2$-containing subtypes, respectively (Fig. 3). Addition of 6HF, on the other hand, yielded maximal currents of 127.4 ± 3.2%, 165.4 ± 7.3%, 161.8 ± 5.5% and 126.1 ± 4.7%, and EC_{50} of 301.5 ± 127.9 nM, 29.1 ± 14.8 nM, 87.3 ± 45.3 nM and 452.5 ± 383.9 nM for the same four subtypes (Fig. 3). Thus, in contrast to the full agonist diazepam which elicited largely similar responses from the different receptor subtypes in terms of maximal currents or EC_{50}, 6HF elicited a range of different maximal currents and EC_{50} from the different subtypes.

3.4. Anxiolytic-like effect in elevated plus-maze test

Both diazepam and 6HF showed a significant difference from vehicle in both % open-arm entries ($P < 0.01$) (Fig. 4A) and % time spent in open arms ($P < 0.01$) (Fig. 4B) based on ANOVA analysis. The post hoc Newman–Keuls' test revealed that, like diazepam at dose of 1 mg/kg, 6HF significantly increased the % open-arm entries at 25 mg/kg ($P < 0.01$) (Fig. 4A) and the % time spent in the open arms at 12 and 25 mg/kg (both $P < 0.01$) (Fig. 4B). When 6HF (25 mg/kg) was co-administrated with the BZ site antagonist flumazenil (1.25 mg/kg), the 6HF-induced decreasing effects on open-arm entries and time spent in open arms were essentially abolished (Fig. 5). The influence of diazepam or 6HF treatment on activity-related parameters such as the number of entries into closed arms (Fig. 4C) and general locomotor activity did not reach statistical significance (Fig. 4D).

3.5. Lack of sedative effect in the hole-board test

Administration of 3 mg/kg diazepam significantly decreased the number of head-dips ($P < 0.01$) (Fig. 6A) and rears ($P < 0.01$) (Fig. 6B). In contrast, 6HF (6–100 mg/kg) treatment did not significantly alter the number of head-dips ($P = 0.93$) (Fig. 6A) or rears ($P = 0.99$) (Fig. 6B), demonstrating that 6HF was lacking in sedative effect at its anxiolytic dose range.

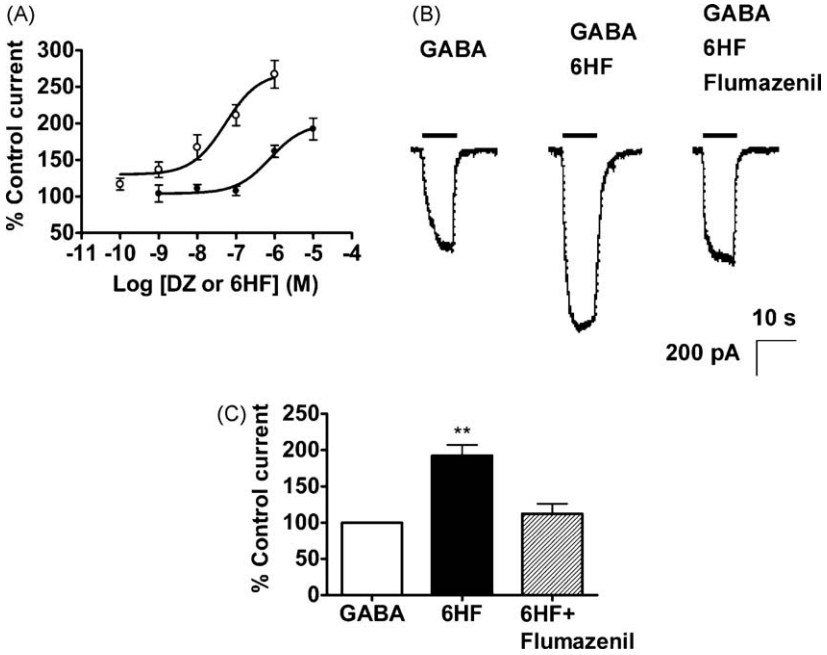

Fig. 2. Modulatory effects of 6HF on GABA-induced currents in rat cortical neurons. (A) GABA currents elicited by 2.1 μM GABA and modulated by different concentrations of diazepam (DZ, ○) or 6HF (●). Data expressed as mean ± SEM of 7–8 independent experiments. (B) Current response to GABA at EC_{20} with or without co-application of 6HF (10 μM) in the absence or presence of flumazenil (1 μM). Bar above current tracing shows duration of stimulation pulse. Scaling is indicated by representation of 200 pA and 10 s. (C) GABA currents in the presence of 6HF (10 μM) with or without flumazenil (1 μM) relative to control. **Indicates $P < 0.01$ significant difference between GABA and GABA plus 6HF groups based on unpaired *t*-test. Data represent mean ± SEM of 7–8 independent experiments.

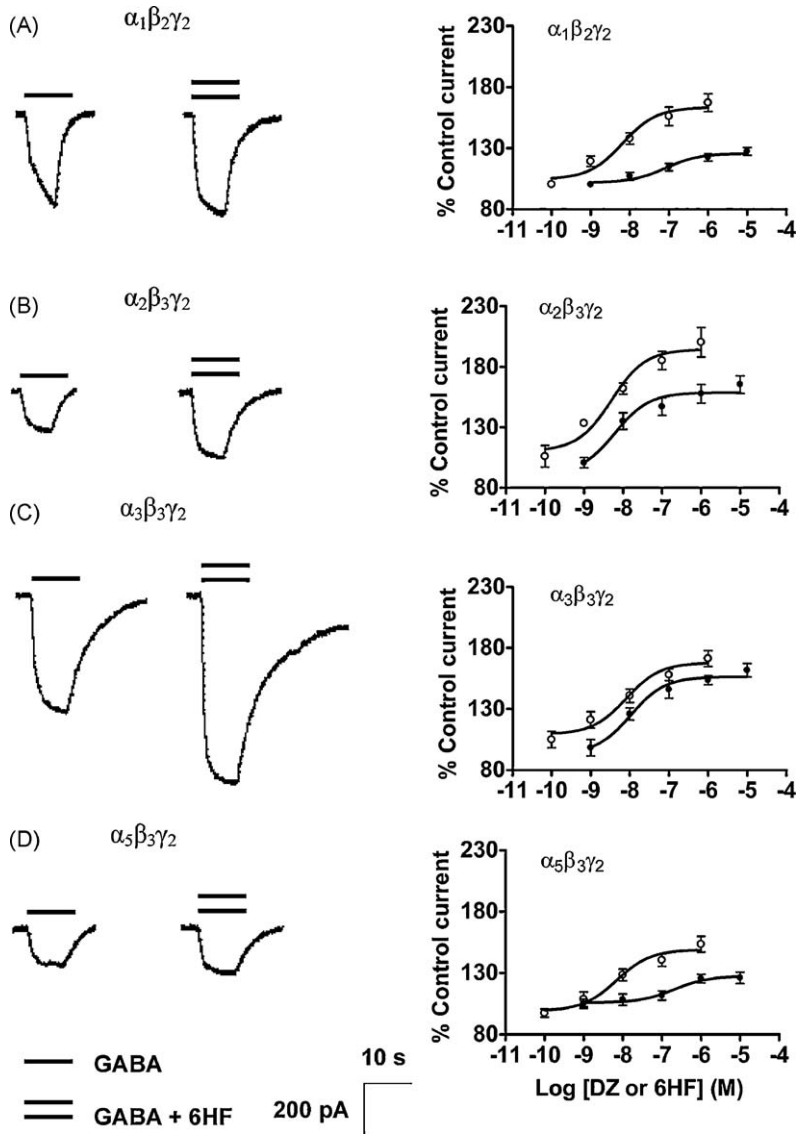

Fig. 3. Efficacies of 6HF on recombinant $GABA_A$ receptor subtypes expressed in HEK 293T cells under whole-cell patch clamp. GABA currents, elicited by GABA at EC_{20} concentration and modulated by different concentrations of 6HF, were recorded with HEK 293T cells expressing $\alpha_1\beta_2\gamma_2$ (A), $\alpha_2\beta_3\gamma_2$ (B), $\alpha_3\beta_3\gamma_2$ (C), or $\alpha_5\beta_3\gamma_2$ (D) receptors. Data in the dose response curves for diazepam (DZ, ○) and 6HF (●) represent mean ± SEM (n = 6–8) for each subtype.

3.6. Lack of cognition impairment in step-through passive avoidance test

In the training trials, 2/14 vehicle-treated mice, 0/18 diazepam-treated mice, 0/12, 0/12, 3/20, 0/12, and 0/12 6HF-treated (at 6, 12, 25, 50 and 100 mg/kg, respectively) mice were excluded from the test for failure to enter the dark chamber within 15 s. The difference between these numbers was not statistically significant ($P > 0.05$). Administration of 1 mg/kg diazepam significantly decreased the step-through latency in the passive avoidance test ($P < 0.01$) (Fig. 7). However, 6HF-treated mice (at 6, 12, 25, 50 and 100 mg/kg) did not significantly differ from controls ($P = 0.97$) (Fig. 7).

3.7. Lack of myorelaxant effect in horizontal wire test

Treatment with 1 mg/kg diazepam decreased the number of mice successfully grasping the wire relative to controls ($P < 0.01$) (Fig. 8). Compared to diazepam, 6HF treatment did not result in a significant change in the performance of mice in the horizontal wire test at any of the dosages tested (Fig. 8).

3.8. Rotarod test and PTZ-induced seizure test

Although diazepam at 1 mg/kg did not affect the ability of mice to remain on the rotarod, it brought about significant motor incoordination at 3 mg/kg compared to controls ($P < 0.01$) (Fig. 9). In contrast, the performance of 6HF-treated mice on the rotarod was unaffected at doses up to 100 mg/kg ($P = 0.07$) (Fig. 9).

When mice were subjected to 1 mg/kg diazepam, the latency of the first seizure in PTZ-treated mice was significantly increased ($P < 0.01$), and the percentage of death was decreased to 0% compared with 27.3% for vehicle-treated mice (Fig. 10). In contrast, 6HF at 6–100 mg/kg did not significantly alter either the latency of the first seizure ($P = 0.67$) or the percentage of death in PTZ-treated mice (Fig. 10).

4. Discussion

Ligands of the BZ site of $GABA_A$ receptors, of which BZs represent a major class, are known to exert pharmacologically important actions including anxiolysis, sedation, anticonvulsion, and muscle-relaxation. Despite being one of the most widely

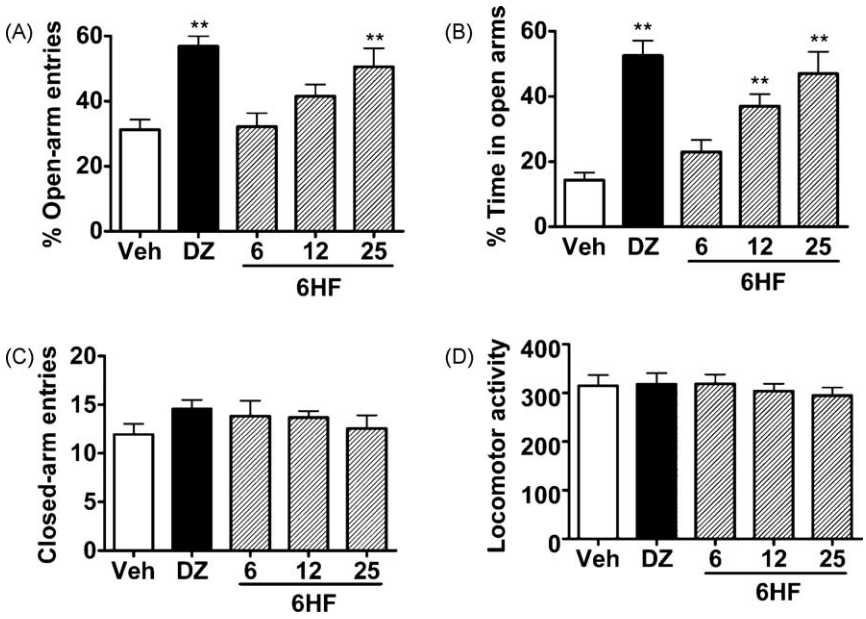

Fig. 4. Anxiolytic-like effects of 6HF in elevated plus-maze test. Data expressed as mean ± SEM for open-arm entries (A), time spent in open arms (B), total number of closed-arm entries (C) and locomotor activity counts (D) in 5-min period, monitored 45 min after the administration of 6HF (6–25 mg/kg, p.o.), diazepam (DZ, 1 mg/kg, p.o.) or vehicle (dd water, p.o.). $N = 12–18$ mice per group. $^{**}P < 0.01$ significant difference from controls based on one-way ANOVA.

employed classes of anxiolytics, the usefulness of classical BZs is seriously limited by their unwanted side effects such as sedation, cognition impairment and dependence [7]. To develop alternative therapeutics with minimal side effects, there is accordingly a need for new BZ site ligands with a more selective induction of anxiolytic effect without accompanying sedation or muscle relaxation. The search for new $GABA_A$ receptor ligands has led to the identification of a variety of high-affinity compounds structurally different from the BZ nucleus, such as β-carbolines [29], trizolopyridazines, quinolines [30,31], and flavonoids [19,20]. To date only a small number of flavonoids have demonstrated selective anxiolytic action [21,22].

4.1. Partial agonist activity of 6HF

In the present study, 6HF acted as a partial positive allosteric modulator of the $GABA_A$ receptor at the BZ site. In the cortical neurons, maximal stimulation by 6HF yielded only about 70% of the electrophysiological response induced by diazepam with an EC_{50} 10-fold higher than that for diazepam. The fact that the potentiation of GABA-induced current by 10 μM 6HF was blocked by the BZ site antagonist flumazenil indicated that 6HF acted through the BZ site.

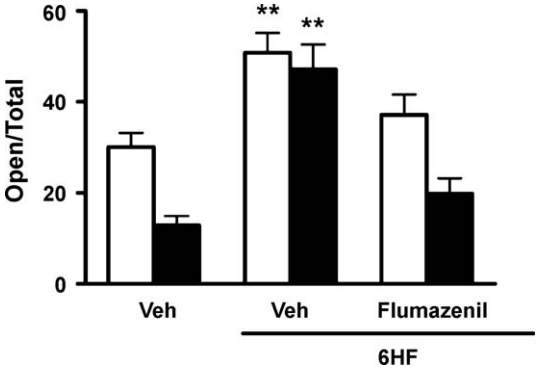

Fig. 5. Co-application of flumazenil blocks the anxiolytic-like effects of 6HF. Data represent mean ± SEM of % open-arm entries (empty column) or % time spent in open arms (solid column) in mice given a 5 min test, 45 min after oral administration of the vehicle (dd water), 6HF (25 mg/kg) or 6HF (25 mg/kg) in the presence of flumazenil (1.25 mg/kg, i.p., 15 min prior testing). $N = 12–21$ mice per group. $^{**}P < 0.01$ significantly different from control using the unpaired *t*-test.

4.2. Preference for α_2 and α_3 subunit-containing $GABA_A$ receptors

Recent genetic and pharmacological studies have demonstrated that α_2 and α_3 subunit-containing $GABA_A$ receptors mediate the anxiolytic effects of BZs, suggesting that these subunits could be useful targets in developing novel, non-sedative anxiolytic agents. In order to investigate the subunit specificity of 6HF, subunit selectivities were examined for both binding affinity and efficacy.

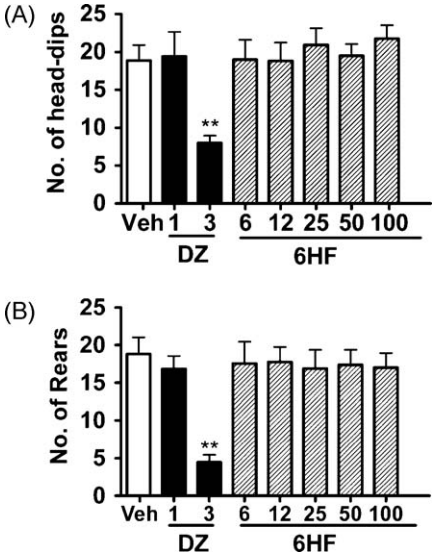

Fig. 6. Lack of sedative effects of 6HF in hole-board test. Data represent mean ± SEM of number of head-dips (A) and rears (B) made during a 5-min period, monitored 45 min after the administration of 6HF (6–100 mg/kg, p.o.), diazepam (DZ, 1–3 mg/kg, p.o.) or vehicle (dd water, p.o.). $N = 12–22$ mice per group. $^{**}P < 0.01$ significant difference from controls based on one-way ANOVA.

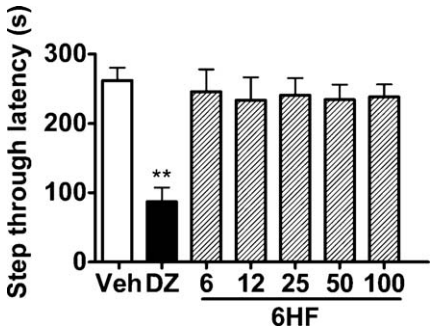

Fig. 7. Lack of memory-impairing effects of 6HF in the step-through passive avoidance test. Mice were trained 45 min after administration of 6HF (6–100 mg/kg, p.o.), diazepam (DZ, 1 mg/kg, p.o.) or vehicle (dd water, p.o.). Data represent mean ± SEM of step-through latency in seconds at 24 h after training. N = 12–21 mice per group. $^{**}P < 0.01$ significant difference from controls based on one-way ANOVA.

The test compound 6HF did not exhibit significant difference in binding affinity for the α1, α2, α3 or α5 subunit-containing $GABA_A$ receptors (Table 1). Moreover, whole-cell patch clamp experiments were performed to measure 6HF efficacies toward different α-containing subtypes of recombinant $GABA_A$ receptors expressed on HEK 293T cells. The results showed that the full agonist diazepam unselectively potentiated GABA-induced currents with similar maximal currents and EC_{50} in α_1, α_2, α_3 and α_5 subunit-containing $GABA_A$ receptors. In contrast, while 6HF also significantly increased GABA-induced currents in the α_2 and α_3 subunit-containing $GABA_A$ receptors, it only potentiated GABA-induced currents weakly with low maximal stimulation and high EC_{50} in the α_1 and α_5 subunit-containing $GABA_A$ receptors. These findings characterized 6HF as a subtype-selective partial agonist of $GABA_A$ receptors with preference for the α_2 and α_3 subunits over the α_1 and α_5 subunits.

4.3. Selective anxiolytic action without side effects at effective anxiolytic dosages

In mice, 6HF significantly increased the percentage of open-arm entries and time spent in the open arms with dose dependence in the elevated plus-maze test (Fig. 4). This anxiolytic-like effect was essentially abolished by the BZ site antagonist flumazenil (Fig. 5), suggesting that 6HF exerted its anxiolytic-like effect via the BZ site. In the hole-board test, 3 mg/kg diazepam significantly decreased the number of head-dips and rears, whereas 6HF exerted no sedative effect at anxiolytic dosages (Fig. 6). In the step-through passive avoidance test, 1 mg/kg diazepam produced a clear-cut cognitive impairment effect, but 6–100 mg/kg 6HF did not significantly change the step-through latency (Fig. 7). Furthermore, 1 mg/kg diazepam significantly impaired the ability of mice

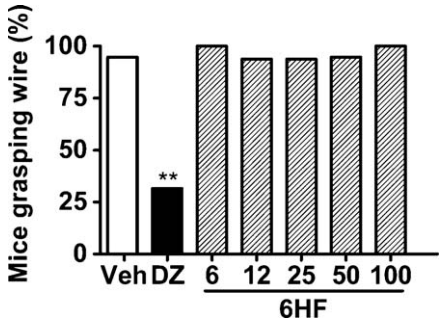

Fig. 8. Lack of myorelaxant effects of 6HF in horizontal wire test. Data expressed as percentage of mice grasping the wire. $^{**}P < 0.01$ significant difference from control, using Fisher's exact test, N = 16–19 per group.

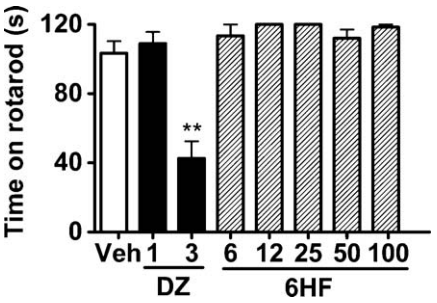

Fig. 9. Lack of motor incoordination effects of 6HF in rotarod test. Data represent mean ± SEM of time on the revolving rod at 45 min after administration of 6HF (6–100 mg/kg, p.o.), diazepam (DZ, 1–3 mg/kg, p.o.) or vehicle (dd water, p.o.). N = 12–18 mice per group. $^{**}P < 0.01$ significant difference from controls using one-way ANOVA.

to grasp the wire in the horizontal wire test, but 6HF exhibited no significant myorelaxant effect (Fig. 8). In the rotarod test, no significant motor incoordination effect was observed after administration of 6HF (6–100 mg/kg). In contrast, diazepam at 3 mg/kg significantly impaired the ability of mice to remain on the revolving rod (Fig. 9). Treatment of 1 mg/kg diazepam significantly increased the latency to first seizure and totally prevented the occurrence of death in the PTZ-treated mice, indicating thereby an anticonvulsant effect, whereas no anticonvulsant effect was observed at 6–100 mg/kg 6HF (Fig. 10). Taken together, unlike diazepam, 6HF brought about anxiolysis without the sedative, cognitive impairment, myorelaxant, motor incoordination, or anticonvulsant activity at effective anxiolytic dosages.

4.4. Relationship between subtype selectivity and pharmacological effect

An early study with α_2- and α_3-containing subtype-selective agonists indicated that α_2- and α_3-containing subtypes mediated the anxiolytic effect of BZ site ligands [32,33]. Furthermore, the study with α_2 (H101R) and α_3 (H126R) mice demonstrated that the anxiolytic effect of BZs was primarily mediated by α_2-containing

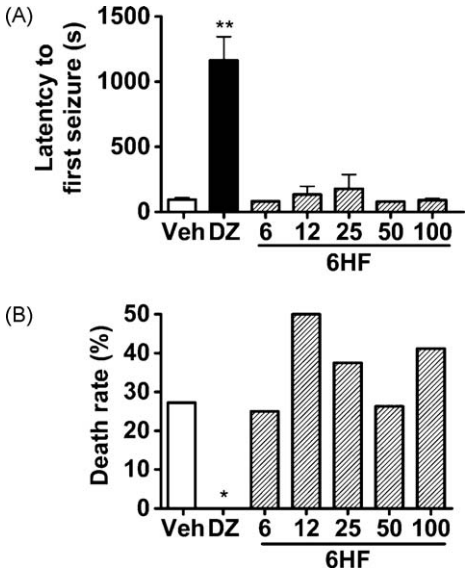

Fig. 10. Lack of anticonvulsant effects of 6HF in PTZ-induced seizure test. Vehicle (dd water, p.o.), diazepam (DZ, 1 mg/kg, p.o.) or 6HF (6–100 mg/kg, p.o.) was administered 45 min prior to PTZ injection. The latency to first clonic seizure (A) and % death within 30 min (B) were recorded after PTZ injection. $^{**}P < 0.01$ or $^{*}P < 0.05$ significant difference from controls, using one-way ANOVA or Fisher's exact test, N = 16–22 per group.

subtypes [13], and under conditions of high receptor occupancy also by α_3-containing subtypes [34,35]. The subtype selectivity of 6HF characterized in the present study supported the notion that α_2- and/or α_3-containing $GABA_A$ receptors participate in the anxiolytic effect of BZ site ligands.

In keeping with studies on α_1 (H101R) mice demonstrating that α_1-containing subtypes mediated the sedative, amnesic, and in part the anticonvulsant and motor incoordination effects of BZs [10–12], and the low activity of 6HF on α_1-containing subtypes, 6HF exhibited no sedative, amnesic, anticonvulsant, or motor incoordination effects in mice.

$GABA_A$ receptors containing α_5 subunit are thought to play a role in learning and memory [17], and we have observed that the partial inverse agonist 6,2′-dihydroxyflavone, which inhibited GABA-induced current at α_5-containing subtypes, exerted memory-enhancing effect [22]. In accord with the limited activity of 6HF on α_5-containing subtypes, 6HF exhibited no cognitive impairment (Fig. 6).

In conclusion, in the present study 6HF was found to be a subtype-selective partial agonist of $GABA_A$ receptors, exhibiting anxiolytic effects without sedative, amnesic, myorelaxant, motor incoordination, or anticonvulsant effects. Therefore, this flavonoid represents a promising drug candidate for the treatment of anxiety-like disorders. The preferential actions of 6HF on α_2- and α_3-containing subtypes also confirmed that α_2- and α_3-containing subtypes constitute useful drug targets for flavonoid anxiolytics. In conclusion, 6HF represents an important addition to the growing number of flavone derivatives equipped with different side chains and endowed with different affinities and efficacies toward $GABA_A$ receptors. Its investigation advances the delineation of the flavonoid structure–activity relationships toward the $GABA_A$ receptors.

Acknowledgements

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