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Coronaridine congeners induce sedative and anxiolytic-like activity in naïve and stressed/anxious mice by allosteric mechanisms involving increased $GABA_A$ receptor affinity for GABA

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Running title: Sedative and anxiolytic activity of coronaridine congeners

Abbreviations: LORR, loss of righting reflex; NSFT, novelty-suppressed feeding test; GABA, γ-aminobutyric acid; $GABA_A$ receptor, GABA type A receptor; (-)-18-MC, (-)-18-methoxycoronaridine; (+)-catharanthine, (+)-3,4-didehydrocoronaridine; (-)-ibogaine, 12-methoxyibogamine; (-)-voacangine, (-)-10-methoxyibogamine-18-carboxylic acid methyl ester; PTZ, pentylenetetrazole; RT, room temperature; IC_{50}, ligand concentration that produces 50% inhibitory response (binding or function); potentiating EC_{50}, ligand concentration that produces half-maximal potentiation response; K_i, inhibitory constant.

Keywords: Coronaridine congeners; Sedative activity; Anxiolytic activity; $GABA_A$ receptors; Fluorescence; Radioligand

Abstract

The sedative and anxiolytic-like activity of two coronaridine congeners, (+)-catharanthine and (-)-18-methoxycoronaridine (18-MC), was studied in male and female mice. The underlying molecular mechanism was subsequently determined by fluorescence imaging and radioligand binding experiments. The loss of righting reflex and locomotor activity results showed that both (+)-catharanthine and (-)-18-MC induce sedative effects at doses of 63 and 72 mg/kg in a sex-independent manner. At a lower dose (40 mg/kg), only (-)-18-MC induced anxiolytic-like activity in naïve mice (elevated O-maze test), whereas both congeners were effective in mice under stressful/anxiogenic conditions (light/dark transition test) and in stressed/anxious mice (novelty-suppressed feeding test), where the latter effect lasted for 24 h. Coronaridine congeners did not block pentylenetetrazole-induced anxiogenic-like activity in mice. Considering that pentylenetetrazole inhibits $GABA_A$ receptors, this result supports a role for this receptor in the activity mediated by coronaridine congeners. Functional and radioligand binding results showed that coronaridine congeners interact with a site different from that for benzodiazepines, increasing $GABA_A$ receptor affinity for GABA. Our study showed that coronaridine congeners induce sedative and anxiolytic-like activity in naïve and stressed/anxious mice in a sex-independent fashion, likely by a benzodiazepine-independent allosteric mechanism that increases $GABA_A$ receptor affinity for GABA.

Introduction

γ-Aminobutyric acid type A receptors ($GABA_A$ receptors) mediate most of the fast inhibitory neurotransmission in the brain and spinal cord and are involved in many physiological functions, including healthy anxiety, fear, and stress responses, whereas their dysfunctional activity may progress in pathological conditions such as addiction, chronic pain, anxiety and major depressive disorders, epilepsy, schizophrenia, and autism spectrum disorder (Deidda et al., 2014; Möhler, 2006), $GABA_A$ receptors are positively modulated by a large variety of compounds, including benzodiazepines, general anesthetics, anticonvulsants, and neuroactive steroids (Greenfield, 2013; Sieghart and Savić, 2018; Siegwart et al., 2002; Weir et al., 2017; Wingrove et al., 1994; Ziemba et al., 2018).

Coronaridine congeners such as (-)-ibogaine (and its metabolite noribogaine), (+)-catharanthine, (-)-voacangine, and 18-methoxycoronaridine (18-MC) (Fig. 1) show a very interesting set of behavioral effects, each one determined by specific molecular mechanisms. The current notion is that the anti-addictive activity of these compounds is mediated by the inhibition of habenular α3β4-containing nicotinic acetylcholine receptors (Ach receptors) (Arias et al., 2017; Arias et al., 2015; Glick et al., 2011; Maisonneuve and Glick, 2003), the antinociceptive and anti-inflammatory activity through combined inhibitory effects on $Ca_V2.2$ channels and α9α10 Ach receptors (Arias et al., 2020a), and the antidepressant-like activity through differential inhibition of norepinephrine and serotonin transporters (Arias et al., 2023b; Rodrıguez et al., 2020).

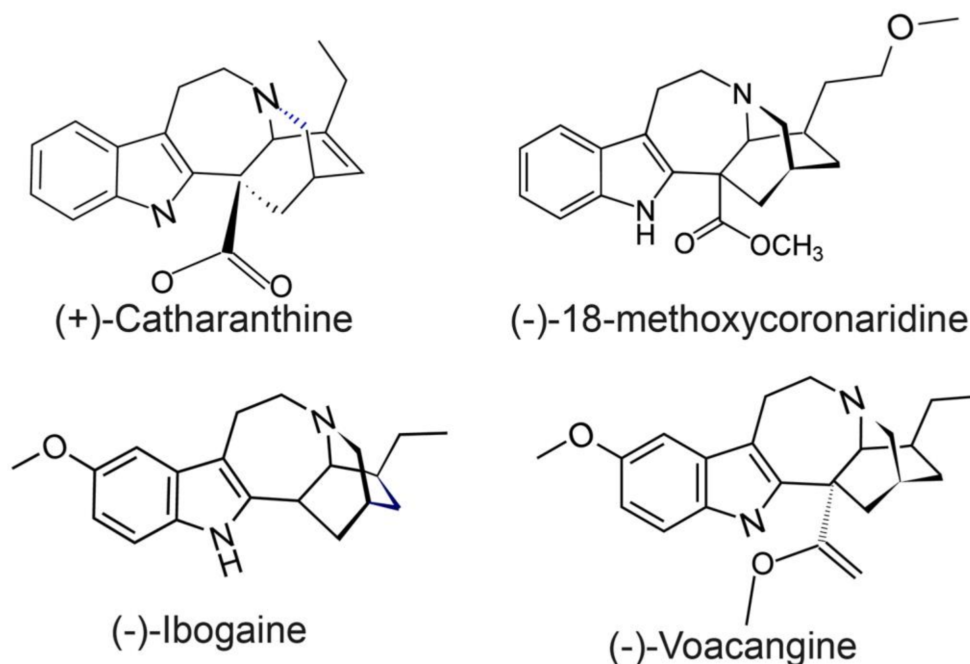

Figure 1. Molecular structures of coronaridine congeners, including (-)-18-methoxycoronaridine [(-)-18-MC], (+)-catharanthine [(+)-3,4-didehydrocoronaridine], (-)-ibogaine [12-methoxyibogamine], (-)-voacangine [(-)-10-methoxyibogamine-18-carboxylic acid methyl ester], and (-)-voacangarine [20S-hydroxy-12-methoxyibogamine-18-carboxylic acid methyl ester].

The observed sedative activity of coronaridine congeners was assigned to a mechanism involving $GABA_A$ receptor potentiation (Arias et al., 2020b). Considering that $GABA_A$ receptors are involved in anxiety and sedation (Deidda et al., 2014; Möhler, 2006), it is likely that coronaridine congeners may induce anxiolytic effects, at least partially, through $GABA_A$ receptor potentiation.

Thus, we want to compare the anxiolytic-like activity of (+)-catharanthine and (-)-18-MC between male and female mice, and after acute and repeated treatments, and determine the sedative activity of (-)-18-MC to complement our initial results with (+)-catharanthine (Arias et al., 2020). In this regard, different animal paradigms (Pennanen et al., 2006) (Blasco-Serra et al., 2017; Bourin and Hascoët, 2003; Shepherd et al., 1994) will be used to determine the anxiolytic-like and sedative activity in naïve and

stressed/anxious mice. To determine the potential involvement of $GABA_A$ receptors, additional radioligand and fluorescence imaging experiments were devised.

2. Methods

2.1. Material

[^{3}H]Flunitrazepam (83 Ci/mmol) was purchased from Perkin Elmer New England Nuclear (Waltham, Massachusetts, USA). Diazepam was obtained from Nycomed (Opfikon, Switzerland). GABA, fetal bovine serum (FBS), and pentylenetetrazole (PTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA; and Saint Quentin Fallavier, France). Dulbecco's modified Eagle medium (DMEM), GlutaMAX®, NEM (non-essential amino acids), penicillin, and streptomycin were obtained from ThermoFisher (Waltham, Massachusetts, USA). 18-Methoxycoronaridine hydrochloride [(-)-18-MC and (±)-18-MC] was purchased from Obiter Research, LLC (Champaign, IL, USA). (+)-Catharanthine (free base) was obtained from Henan Tianfu Chemical Co. (Zhengzhou, China). (-)-Voacangine (free base) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Salts, solvents, and reagents were purchased from commercial suppliers and used as received.

2.2. Animals

All experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, approved by the Regional Ethical Committee for Animal Experimentation and performed according to the European Communities Council Directive (86/609/EEC + 2010/63/UE), and by the Institutional Animal Care Committee from each institution.

Adult male and female Swiss albino CD1 mice (30-35 g), purchased from Janvier Labs (Le Genest Saint Isle, France), were used in the behavioral studies. Animals were housed in Makrolon cages (L: 37 cm, W: 21 cm, H: 14 cm), with free access to a standard semisynthetic laboratory diet (SERLAB,

Montataire, France). All animals were kept in a ventilated room, at a temperature of 22 ± 1 °C, under a 12-h light/12-h dark cycle (light between 7:00 a.m. and 7:00 p.m.).

For tissue binding experiments, xx female rats (4 weeks old) were purchased Hugo, please complete

2.3. Behavioral Experiments

2.3.1. Loss of righting reflex (LORR) test

To compare the sedative-hypnotic effects of (+)-catharanthine and (-)-18-MC between male and female mice, LORR tests were performed as previously described (Arias et al., 2020a). Male and female mice (n = 10/condition) were habituated to the experimental room 24 h before the experiments. On the next day, mice were injected (i.p.) (+)-catharanthine or (-)-18-MC (63 and 72 mg/kg) [freshly dissolved in the vehicle: DMSO (1%), Tween 80 (1%), NaCl (0.9%)], or vehicle. Thirty minutes after the drug's injection, when mice become motionless, they were placed individually on their back in a position of dorsal recumbence in a plexiglass V-shaped trough until they were able to right themselves within 10 min. The experimenter then observed the mice until they turned over onto all four paws, righting themselves. Immobility time was defined as the time from being placed in the supine position until the animal regained their righting reflexes.

2.3.2. Open field test

To compare the effect of different doses of (+)-catharanthine or (-)-18-MC (63 and 72 mg/kg) on locomotor activity of male and female mice, open field experiments were performed as previously described (Arias et al., 2020a). Male and female mice (n = 10/condition) were habituated to the experimental room 24 h before the experiments. On the next day, mice were injected (i.p.) (+)-catharanthine or (-)-18-MC, or vehicle, and after 10 min, placed individually in 20 x 20 x 30 cm compartments, in a dimly illuminated and quiet room. Mice locomotor activity was automatically

monitored by a computerized actimeter (Versamax, AccuScan Instruments, Inc., OH, USA). Horizontal movements determined by the number of crossed beams were tracked every 10 min for a total time of 60 min.

2.3.3. Elevated O-maze test

The anxiolytic-like activity of (+)-catharanthine and (-)-18-MC was determined on naïve and PTZ-treated (Jung et al., 2002) mice using the elevated O-maze test (Shepherd et al., 1994). The elevated O-maze test exploits the predisposition of rodents to avoid open and/or elevated areas, which is compensated by their natural tendency to explore areas unfamiliar to them. Anxiolytic drugs are reported to increase open-arms exploration, whereas anxiogenic drugs such as PTZ operate in the opposite way (Aragão et al., 2006; Jung et al., 2002).

Male mice (n = 10/condition) were habituated to the experimental room 24 h before the experiments. On the next day, mice were pretreated (i.p.) with 40 mg/kg (-)-18-MC or (+)-catharanthine, or vehicle, and the acute activity was determined at 1 h and 24 h using the elevated O-maze test. To determine the activity after repeated treatment, mice were pretreated for 14 days, and the activity was determined at day 7 and day 14, respectively. One hour after the last injection, mice were treated (i.p.) with 20 mg/kg PTZ (Jung et al., 2002), and the behavioral activity was determined after 15 min using the elevated O-maze test.

The elevated O-maze session began by gently placing the mouse with the nose facing one of the closed arms of the maze and allowing it to freely explore it. The session was recorded with a videotape for 5 min. The relative degree of anxiety or fear can be assessed by comparing the time spent in closed arms and the time spent in open arms, and the number of entries in enclosed arms. The maze was cleaned with 20% ethanol solution and dried with a cloth between sessions.

2.3.4 Novelty-suppressed feeding test

To assess the anxiolytic-like activity of coronaridine congeners in mice under stressful conditions, the novelty-suppressed feeding test (NSFT) was used (Blasco-Serra et al., 2017). This test examines anxiety-related behaviors and feeding behavior when the animal is exposed to a novel stressful environment without previous training of the animals. The animal experiences a conflicting situation between the anxiety-inducing environment and the hunger-induced behavior. An anxiolytic agent will decrease the latency associated with feeding in animals subjected to this anxiogenic environment.

Male mice (n = 10/condition) were deprived of food for 24 h, and subsequently treated (i.p.) with 40 mg/kg (-)-18-MC or (+)-catharanthine, or vehicle. One, 24, and 48 h after the injection, the following parameters were measured (Fig. 6A): (a) latency to approach the center of the field (sec). Anxious mice prefer to stay at the periphery of the arena, taking longer time to go to the center; (b) latency to eat (sec). Anxious mice take longer time to eat because anxiety overrides the desire to eat. Each animal was then returned to its cage and provided with the habitual food, and (c) food consumption (mg) and (d) in-cage latency to eat (sec) respectively were measured. These two parameters serve to assess whether fasting induces "unusual" feeding behavior that might interfere with the anxiety behavior and to assure that mice recover from fasting's effects.

The apparatus features a highly illuminated open field (40 cm x 60 cm). A small pellet of the usual food was positioned on a platform consisting of a Petri dish with a white circle cut out from a Whatman paper using the sidewalls of the Petri dish as a base to steady the location in the bedding (Fig. 6A). On the day of testing, each mouse was delicately and randomly positioned in a corner of the open field and the timer started immediately. Animals initially approached the food pellet and sniffed it without biting, then grabbed the food pellet with their front paws and bit it. Latency to approach the center and/or eat the food was recorded for 5 min.

2.4. Fluorescence imaging assays using $GABA_A$ receptors-expressing HEK293T cells

To determine whether coronaridine congeners modify the affinity of GABA for $GABA_A$ receptors, fluorescence imaging experiments were carried out in HEK293T-α1β3γ2L cells using gabazine-oregon green (Gzn-OG), a $GABA_A$ receptor antagonist that becomes fluorescent upon binding to the orthosteric binding site (Sakamoto et al., 2019). $GABA_A$ receptor subunits were transiently expressed in HEK293T cells as previously described (Sakamoto et al., 2019). HEK293T-α1β3γ2L cells were preincubated with Gzn-OG (100 nM) for 5 min at 25 °C, followed by removal of the medium and washing with 1 x HBS (2 mL x 2). Cells with α1β3γ2L-bound Gzn-OG were then titrated with increasing concentrations of GABA (i.e., 0.01 μM-30 mM), in the absence (control) and presence of fixed concentrations of (+)-catharanthine, (-)-18-MC, (-)-ibogaine, or (-)-voacangine (dissolved in 0.1% DMSO/HBS), and the fluorescence determined by confocal laser scanning microscopy (CLSM).

Cell fluorescence imaging analysis was performed using a Carl Zeiss CLSM (LSM-800, Germany) equipped with a 63×, NA = 1.40 oil objective and a GaAsP detector. CLSM images were acquired using the 488 nm excitation derived from diode lasers (0.75 %, Gain 750 V) with control of the focus using the Definite Focus module included in LSM-800.

The fluorescence intensity of a single cell in the absence (F_o) and presence (F) of GABA was determined by enclosing ROIs. Non-specific Gzn-OG fluorescence was determined at 30 mM GABA. To calculate the apparent IC_{50} values (i.e., ligand concentration that produces half-maximal inhibition) for GABA in the absence or presence of each ligand, non-linear regression fitting of the F/F_o ratios (mean ± SEM) was performed using KaleidaGraph 4.5 (Synergy Software, Reading, PA, USA), according to the following logistic equation:

$$F/F_o = 1 - [1/(1 + (\log[GABA]/IC_{50})^{n_H}] \quad (1),$$

where [GABA] is the GABA concentration and n_H is the Hill coefficient. The calculated IC_{50}'s were transformed to apparent K_i (i.e., inhibition constant) values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50} / (1 + [Gzn\text{-}OG]) / K_d^{Gzn\text{-}OG} \quad (2),$$

where [Gzn-OG] is the initial concentration of Gzn-OG (100 nM), and $K_d^{Gzn\text{-}OG}$ is the K_d for Gzn-OG (55 nM; (Sakamoto et al., 2019).

2.5. [^{3}H]Flunitrazepam competition binding assay

Different rat brain areas as well as HEK293 cell lines expressing selective $GABA_A$ receptor subtypes were used for radioligand competition binding experiments. We decided to use rat brains instead of mouse brains, because rat brains allowed us to prepare membranes from different brain areas in enough quantities for the radioligand binding assays. HEK293 cells (American Type Culture Collection ATCC® CRL-1574TM) were first cultured in DMEM (high glucose, GlutaMAX®), supplemented with 10% FBS, 10% MEM, and 100 U/mL penicillin-streptomycin, at 37 °C and 5% CO_2. Cells were subsequently transfected with cDNAs encoding rat $GABA_A$ receptor subunits subcloned into pCI expression vectors using the calcium phosphate precipitation method (Chen and Okayama, 1987). The ratio of plasmids used for the transfection were (per 10 cm dish): 3 μg α (1, 2, 3 or 5): 3 μg β3: 15 μg γ2. The medium was changed 4-6 h after transfection. Cells were harvested by scraping, 72 h after transfection using phosphate buffered saline. After centrifugation (10 min, 3,000xg, 4 °C) cells were resuspended in 50 mM Tris/citrate buffer (pH = 7.1), homogenized with an ULTRA-TURRAX® (IKA, Staufen, Germany), and centrifuged again (10 min, 3,000xg, 4 °C). Cell pellets were stored at -80 °C until used.

To prepare $GABA_A$ receptor-containing membranes, each brain area, including the spinal cord, brain stem, prefrontal cortex, hippocampus and olfactory bulb, was first dissected from female rats (n = 9; 4 weeks old). These experiments were performed in rats rather than mice to collect membranes in

enough quantities for the radioligand binding assays. We did not perform any radioligand binding on male rats, since our behavioral results did not show any sex differences. The tissue was subsequently homogenized with an ULTRA-TURRAX for 30 s in ice-cold homogenization buffer (10 mM HEPES, 1 mM EDTA, 300 mM sucrose, and protease inhibitor cocktail, pH 7.5), and centrifuged at 45,000 × g for 30 min at 4 °C. The pellet was resuspended in wash buffer (10 mM HEPES, 1 mM EDTA, and protease inhibitor cocktail, pH 7.5), incubated on ice for 30 min and pelleted as described above. The pellet was stored at -80 °C overnight, and the next day, washed five times with 50 mM Tris/citrate buffer (pH = 7.1), and subsequently centrifuged as described above. Membrane pellets were stored at -80 °C until final use.

To determine the effect of coronaridine congeners on [^{3}H]flunitrazepam binding to membranes prepared from each brain area, 50 µM of (+)-catharanthine, (±)-18-MC [dissolved in DMSO (0.5% final concentration)], was added to the membrane suspension. In the case of (-)-voacangine, a wider range of concentrations (i.e., 1-150 µM) was used.

Membranes/cells were suspended (500 µL) and incubated for 90 min at 4 °C in 50 mM Tris/citrate buffer (pH = 7.1), containing 150 mM NaCl and 2 nM [^{3}H]flunitrazepam. Membranes/cells were filtered through Whatman GF/B filters and the filters were rinsed twice with 4 mL of ice-cold 50 mM Tris/citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after the addition of 3 mL Rotiszint eco plus liquid scintillation cocktail (Carl Roth GmbH, Austria). Specific [^{3}H]flunitrazepam binding was calculated by subtracting nonspecific binding (5 µM diazepam) from total binding (no diazepam).

2.6. Statistical Analysis

Experimental data (mean ± SEM) were analyzed by using the Prism software (GraphPad 9.5.1., Software Inc., La Jolla, CA, USA). Two-way ANOVA analysis was used to compare drug type, dose, and time of

treatments. Tukey's multiple comparison post-hoc test was used to compare differences between sexes and between drugs. The Dunnett's post-hoc test was used to compare dose- and 10-min-period treatments. Drug effects in different brain regions and transfected HEK-293 cells were analyzed by one sample Student’s t-test. Values of $p < 0.05$ were considered significantly different.

3. Results

3.1. (+)-Catharanthine and (-)-18-MC induce sedative-hypnotic activity in naïve mice in a sex-independent manner

The sedative-hypnotic effect of (+)-catharanthine and (-)-18-MC (63 and 72 mg/kg) was compared between male and female mice using the LORR test (Fig. 2). Two-way ANOVA indicated that (+)-catharanthine increases the immobility time in both male and female mice [$F (4, 45) = 30.22$; $p < 0.0001$; for both sexes] in the 63-72 mg/kg (Fig. 2A), but not 20-50 mg/kg (data not shown) ($p > 0.999$), dose range, compared to vehicle-treated animals. (-)-18-MC also increased immobility time in both male and female mice in the same dose range [$F (2, 54) = 193.7$; $p < 0.0001$; for both sexes] (Fig. 2B). Tukey's post hoc tests indicated that the sedative effects elicited by (+)-catharanthine ($p > 0.999$) and (-)-18-MC ($p > 0.723$) were not significantly different between males and females at either dose.

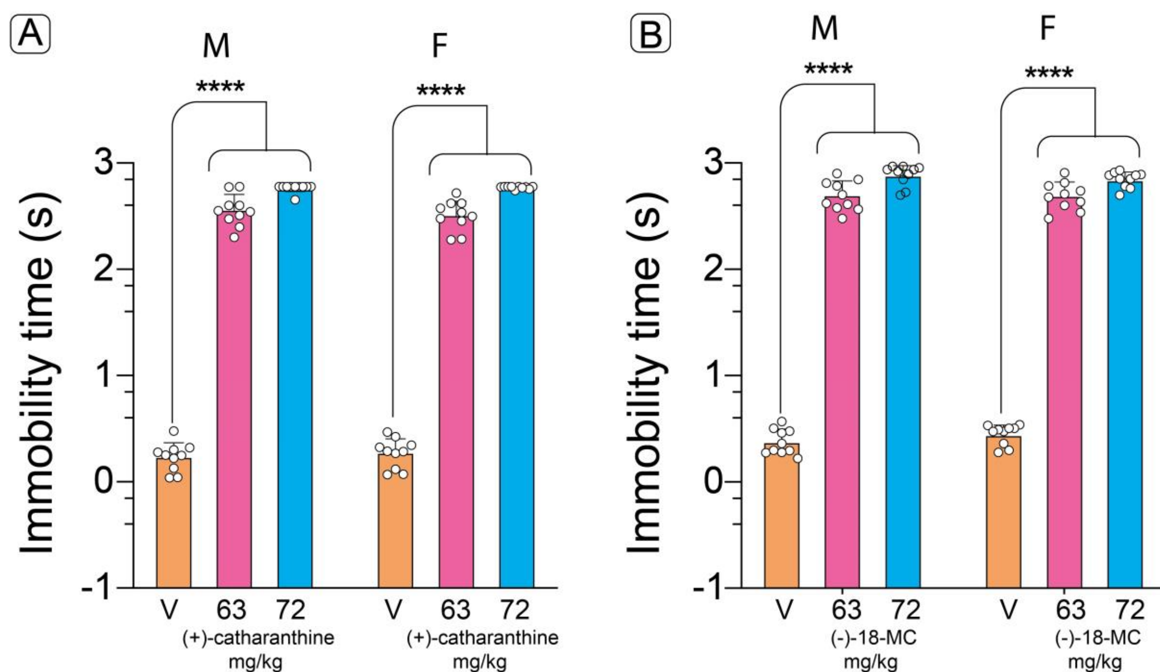

Figure 2. The comparative sedative-hypnotic activity of (+)-catharanthine and (-)-18-MC between male (M) and female (F) mice. Male and female mice (n = 10/condition) were injected (i.p.) different doses of (+)-catharanthine or (-)-18-MC (63 and 72 mg/kg), or vehicle (V), and the immobility time (i.e., sedative-hypnotic effect) subsequently determined using the loss of righting reflex (LORR) test. Previous LORR data on male mice (Arias et al., 2020a) were included for comparative purposes. (A) Two-way ANOVA analyses indicated that (+)-catharanthine increases the immobility time in both male and female mice in the 63-72 mg/kg dose range ($p < 0.0001$), compared to vehicle-treated animals. (B) Two-way ANOVA analyses indicated that (-)-18-MC increases the immobility time in both male and female mice in the same dose range ($p < 0.0001$). Tukey's post-hoc analyses indicated that the effect of (+)-catharanthine ($p > 0.999$) or (-)-18-MC ($p > 0.723$) was not significantly different between males and females at either dose. ****$p < 0.0001$.

3.2. (+)-Catharanthine and (-)-18-MC reduce locomotor activity of naïve mice in a sex-independent manner

The sedative-hypnotic effects elicited by (+)-catharanthine and (-)-18-MC are well correlated with a decrease in locomotor activity in the open field test. Previous (+)-catharanthine data on male mice (Arias et al., 2020a) were used to determine any sex dependence.

Two-way ANOVA analyses for (+)-catharanthine and (-)-18-MC showed a significant effect of treatment [(+)-catharanthine: $F(5, 324) = 29.46$, $p < 0.0001$; (-)-18-MC: $F(10, 162) = 4.704$, $p < 0.0001$], time [(+)-catharanthine: $F(5, 324) = 22.95$, $p < 0.0001$; (-)-18-MC: $F(5, 162) = 131.0$, $p <0.000$], and treatment × time interaction [(+)-catharanthine: $F(25, 324) = 3.698$, $p < 0.0001$; (-)-18-MC: $F(10, 162) = 4.704$, $p <0.0001$] (Fig. 3A,C). (+)-Catharanthine attenuated locomotor activity in both male [$F(2, 162) = 25.99$; $p < 0.0001$] and female mice [$F(2, 162) = 54.96$; $p < 0.0001$] at either 63 or 72 mg/kg, compared to vehicle-treated animals. No significant effects were observed with (+)-catharanthine in the 20-50 mg/kg dose range ($p > 0.4$) (data not shown), in agreement with previous studies in male mice (Arias et al., 2020a). Tukey's post-hoc tests showed no significant differences between males and females at active doses of (+)-catharanthine at any interval ($p > 0.994$). Since no sex-dependence was observed for (+)-catharanthine's activity, the locomotor activity of (-)-18-MC was tested only in males. (-)-18-MC attenuated locomotor activity at either 63 or 72 mg/kg [$F(2, 162) = 122.5$; $p < 0.0001$; for each dose], compared to vehicle-treated animals. Dunnett's post-hoc tests showed that the activity of (+)-catharanthine or (-)-18-MC at each 10 min-interval was statistically different from that observed in vehicle-treated mice, and that (-)-18-MC's effect lasted longer (40-50 min: $p < 0.0001$) than that for (+)-catharanthine (40-50 min: $p = 0.992$). Dunnett's post-hoc tests showed that the activity of catharanthine (Figs. 3A,B) or (-)-18-MC (C) at each 10 min-interval is statistically different from that observed in vehicle-treated mice.

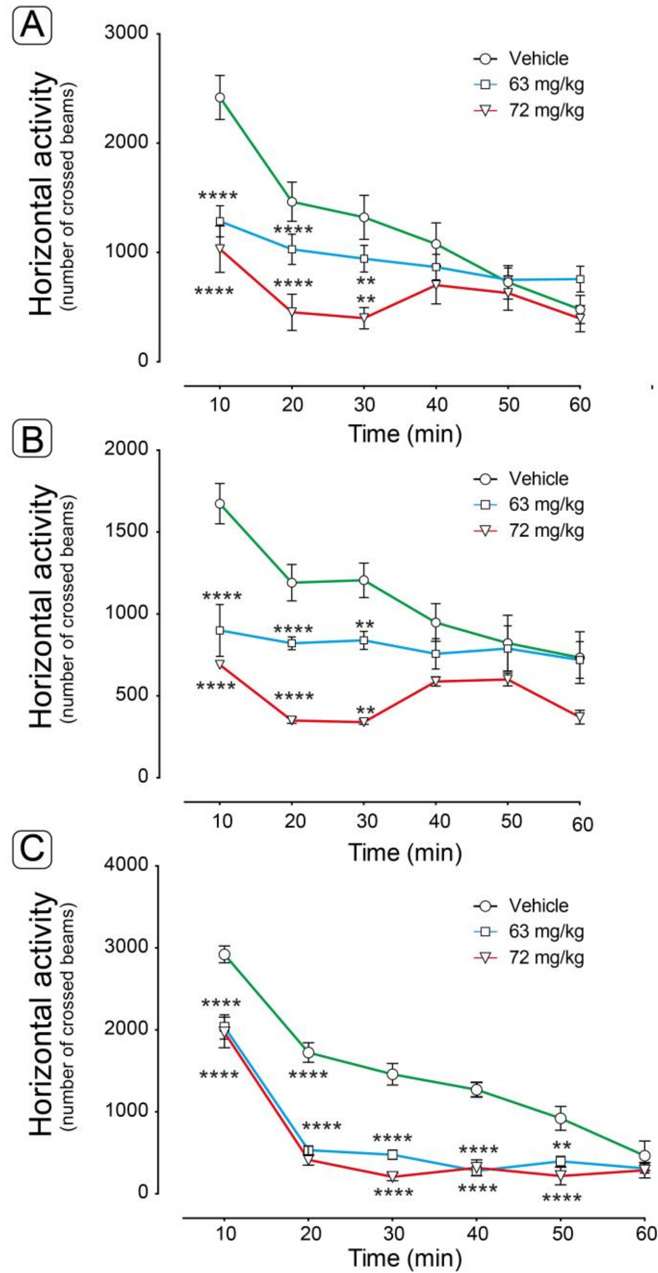

Figure 3. The Comparative locomotor activity of (+)-catharanthine and (-)-18-MC between male (A) and female (B) mice. Male and female mice (n = 10/condition) were injected (i.p.) with catharanthine or 18-MC (63 and 72 mg/kg), or vehicle, and the locomotor activity was tracked every 10 min for 1 h in the open field. Previous (+)-catharanthine data on male mice (Arias et al., 2020a) were included for comparative purposes. Two-way ANOVA for (+)-catharanthine and (-)-18-MC showed a significant effect of treatment, time, and treatment × time interaction ($p < 0.0001$). (+)-Catharanthine, at either 63 mg/kg (□) or 72 mg/kg (▽), attenuated locomotor activity in both male (A) and female mice (B) ($p < 0.0001$), compared to vehicle-treated animals (○). Tukey's post-hoc tests showed no significant locomotor activity differences between males and females ($p > 0.994$). (C) (-)-18-MC attenuated

locomotor activity in male mice at either 63 mg/kg (□) or 72 mg/kg (▽) ($p < 0.0001$). Dunnett's post-hoc tests showed that the activity of catharanthine (A,B) or (-)-18-MC (C) at each 10 min-interval is statistically different from that observed in vehicle-treated mice. ****$p < 0.0001$; **$p < 0.01$.

3.3. (-)-18-MC, but not (+)-catharanthine, induces anxiolytic-like activity in naïve, but not PTZ-treated, mice

To determine whether coronaridine congeners induce anxiolytic-like activity and decrease PTZ-induced anxiety in mice, the effect of 40 mg/kg (+)-catharanthine or (-)-18-MC was assessed on naïve and PTZ-treated (Jung et al., 2002) mice using the elevated O-maze test. Two-way ANOVA and Tukey's post hoc analyses of the acute results in naïve animals showed that 40 mg/kg (-)-18-MC significantly increases the time spent in open arms and significantly reduces the time spent in closed arms [$F (3, 72) = 34.80$; $p = 0,0085$; for both behaviors], indicating anxiolytic-like activity, while (+)-catharanthine did not produce significant effects on both behaviors [$F (3, 72) = 12.75$; $p = 0.9995$] (Fig. 4A). Repeated treatment with 20 mg/kg (-)-18-MC significantly increased the time spent in open arms and significantly reduced the time spent in closed arms with same statistical values at day 7 [$F (3, 72) = 43.77$; $p = 0.0009$] (Fig. 4B) and day 14 [$F (3, 72) = 50.69$; $p = 0.0004$] (Fig. 4C), compared to vehicle-treated naïve animals.

Two-way ANOVA analyses showed that the anxiogenic agent PTZ significantly reduces the time spent in open arms and significantly increases the time spent in closed arms [$F (3, 72) = 11.14$; $p = 0.0065$], compared to vehicle-treated animals (Fig. 4A), supporting freezing/anxiety-like behavior. However, statistical analyses indicated that neither acute (40 mg/kg) [(-)-18-MC: $F (3, 72) = 34.80$ $p = 0.107$; (+)-catharanthine: $F (3, 72) = 12.75$, $p = 0.660$] (Fig. 4A) nor repeated treatment (20 mg/kg) improved PTZ-induced anxiogenic effects at day 7 [(-)-18-MC: $F (3, 72) = 43.77$, $p = 0.471$; (+)-catharanthine: $F (3, 72) = 15.79$, $p = 606$) (Fig. 4B) or day 14 [(-)-18-MC: $F (3, 72) = 50.69$, $p = 0.609$; (+)-catharanthine: $F (3, 72) = 11.14$, $p = 0.256$] (Fig. 4C), compared to untreated animals.

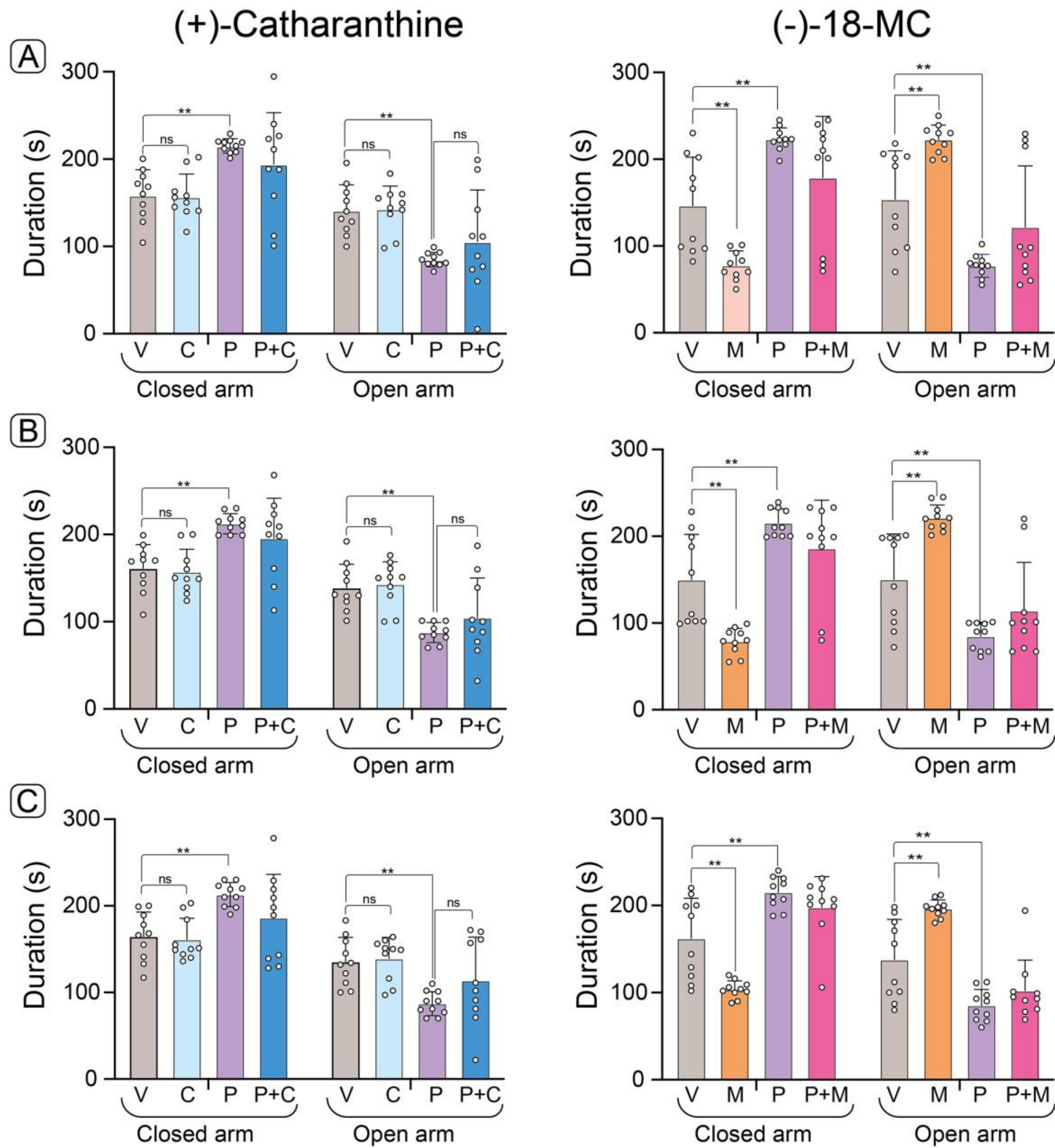

Figure 4. The comparative anxiolytic-like activity of (+)-catharanthine and (-)-18-MC after acute (A), 7 days (B), and 14 days (C) treatments on both naïve and PTZ-treated mice. Male mice (n = 10/condition) were injected (i.p.) 40 mg/kg (acute) (-)-18-MC (M) or (+)-catharanthine (C), or vehicle (V). Alternatively, mice were pretreated with PTZ (P) (20 mg/kg) for 1 h and then treated with each congener. After 15 min of the last injection, the anxiolytic-like activity was assessed using the elevated O-maze test. (A) Two-way ANOVA and Tukey's post hoc analyses of the acute results showed that (-)-18-MC ($p = 0.008$; for both behaviors), but not (+)-catharanthine ($p = 0.999$), significantly increases time spent in open arms and significantly reduces time spent in closed arms, compared to vehicle-treated mice, supporting anxiolytic-like activity. This analysis also showed that PTZ induces anxiogenic-

like activity compared to vehicle-treated mice ($p = 0.0026$), but neither (+)-catharanthine nor (-)-18-MC reversed PTZ-induced anxiogenic effects at 1 h [(-)-18-MC: $p = 0.1069$, (+)-catharanthine: $p = 0.5591$], 7 days [(-)-18-MC: $p = 0.4712$, (+)-catharanthine: $p = 0.6056$], or 14 days [(-)-18-MC: $p = 0.7787$; (+)-catharanthine: $p = 0.2526$]. (B,C) Statistical analyses also showed that 20 mg/kg (-)-18-MC, but not (+)-catharanthine ($p > 0.99$), significantly increases the time spent in open arms and significantly decreases the time spent in closed arms in naïve mice after 7 days ($p = 0.0009$) (B) and 14 days ($p = 0.0004$) (C) of treatment, respectively, compared to vehicle-treated groups. Neither (-)-18-MC nor (+)-catharanthine had significant effects on PTZ-treated animals after acute [(-)-18-MC: $p = 0.107$; (+)-catharanthine: $p = 0.660$] (A), 7 days [(-)-18-MC: $p = 0.471$; (+)-catharanthine: $p = 0.606$] (B), or 14 days [(-)-18-MC: $p = 0.609$; (+)-catharanthine: $p = 0.253$] (C) treatment, respectively, compared to untreated animals. **$p < 0.01$; ns: no statistical difference.

3.4. Both (+)-catharanthine and (-)-18-MC induced anxiolytic-like activity in mice under stressful/anxiogenic conditions

We conducted exploratory analyses using the light/dark transition test (Fig. 5A), which is commonly used to assess anxiolytic-like activity in mice under stressful/anxiogenic conditions (Bourin and Hascoët, 2003). One-way ANOVA analysis of the results showed that 40 mg/kg (+)-catharanthine [$F(2, 27) = 7.361$; $p = 0.0076$] or (-)-18-MC [$F(2, 27) = 7.361$; $p = 0.0007$] significantly increased the time spent in the bright compartment (Fig. 5B) and reduced the time spent in the dark compartment with the same statistical values (Fig. 5C), compared to vehicle-treated groups. Tukey's post hoc analyses did not provide significant differences between both drugs ($p = 0.62$).

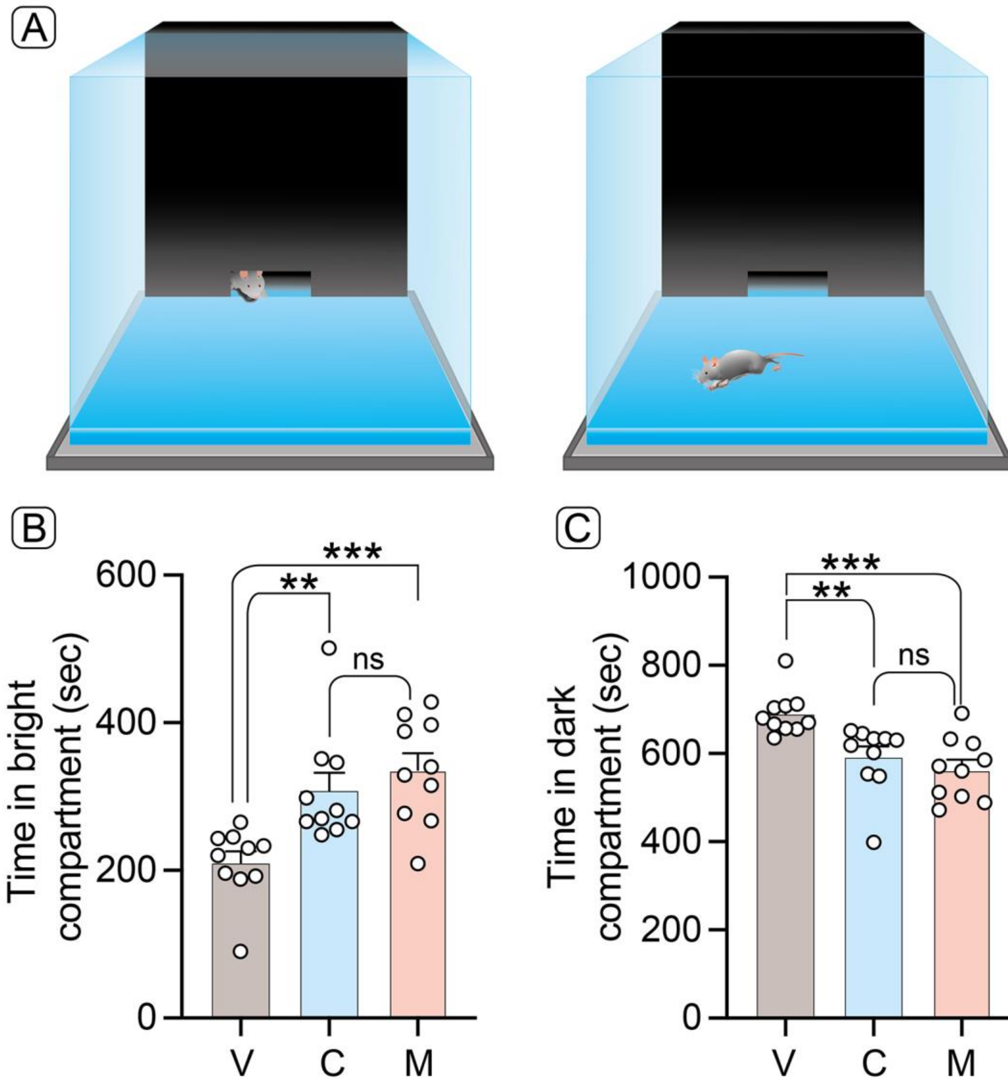

Figure 5. The anxiolytic-like activity of acute treatment with (+)-catharanthine or (-)-18-MC on mice under stressful/anxiogenic conditions using the light/dark transition test. Male mice (n = 10/condition) were injected (i.p.) 40 mg/kg (+)-catharanthine (C) or (-)-18-MC (M), or vehicle (V), and after 1 h, the anxiolytic-like activity assessed using the light/dark transition test. One-way ANOVA analysis of the results showed that (+)-catharanthine ($p = 0.0076$) and (-)-18-MC ($p = 0.0007$) significantly increase the time spent in the bright compartment (B) and reduce the time spent in the dark compartment (C), compared to vehicle-treated animals. Tukey's post hoc analysis indicated no statistical (ns) difference between both drugs ($p = 0.62$). **$p < 0.01$; ***$p < 0.001$

3.5. (+)-Catharanthine and (-)-18-MC induce anxiolytic-like activity in stressed/anxious mice

To determine whether coronaridine congeners induce anxiolytic-like activity in stressed/anxious mice, the effect of a single administration of 40 mg/kg (+)-catharanthine or (-)-18-MC was determined on mice deprived of food for 24 h (Blasco-Serra et al., 2017). Two-way ANOVA analysis was used to compare the effects of each ligand on four different NSFT parameters (Fig. 6A) at 1, 24, and 48 h, respectively. The results showed that both congeners significantly decreased the latency to approach the center of the field at 1 h [$F(2, 18) = 587.9$, $p < 0.0001$] (Fig. 6B) and 24 h [$F(2, 18) = 73.23$, $p < 0.0001$] (Fig. 6F), but not at 48 h [$F(2, 18) = 2.199$, $p > 0.144$] (Fig. 6J), and significantly reduced the latency to eat at 1 h [$F(2, 18) = 42.85$, $p < 0.0001$] (Fig. 6C) and 24 h [$F(2, 18) = 31.43$, $p < 0.0001$] (Fig. 6G), but not at 48 h [(+)-catharanthine: $F(2, 18) = 2.747$, $p = 0.250$; (-)-18-MC: $F(2, 18) = 2.199$, $p = 0.087$] (Fig. 6K), compared to vehicle-treated animals. Tukey's post hoc analyses showed that the observed effects were not significantly different between both drugs ($p > 0.8$). After animals were returned to the cage, no significant differences were found for food consumption at 1 hand 24 h [$F(2, 18) = 0.6897$; $p > 0.999$], and 48 h [$F(2, 18) = 0.188$; $p = 0.823$] (Figs. 6D,H,L, respectively) or in-cage latency to eat at 1 h[$F(2, 18) = 2.657$; $p = 0.919$], 24 h[$F(2, 18) = 0.6897$; $p > 0.999$], and 48 h [$F(2, 18) = 0.7582$; $p = 0.798$] (Figs. 6E,I,M, respectively), for both congeners compared to vehicle-treated animals.

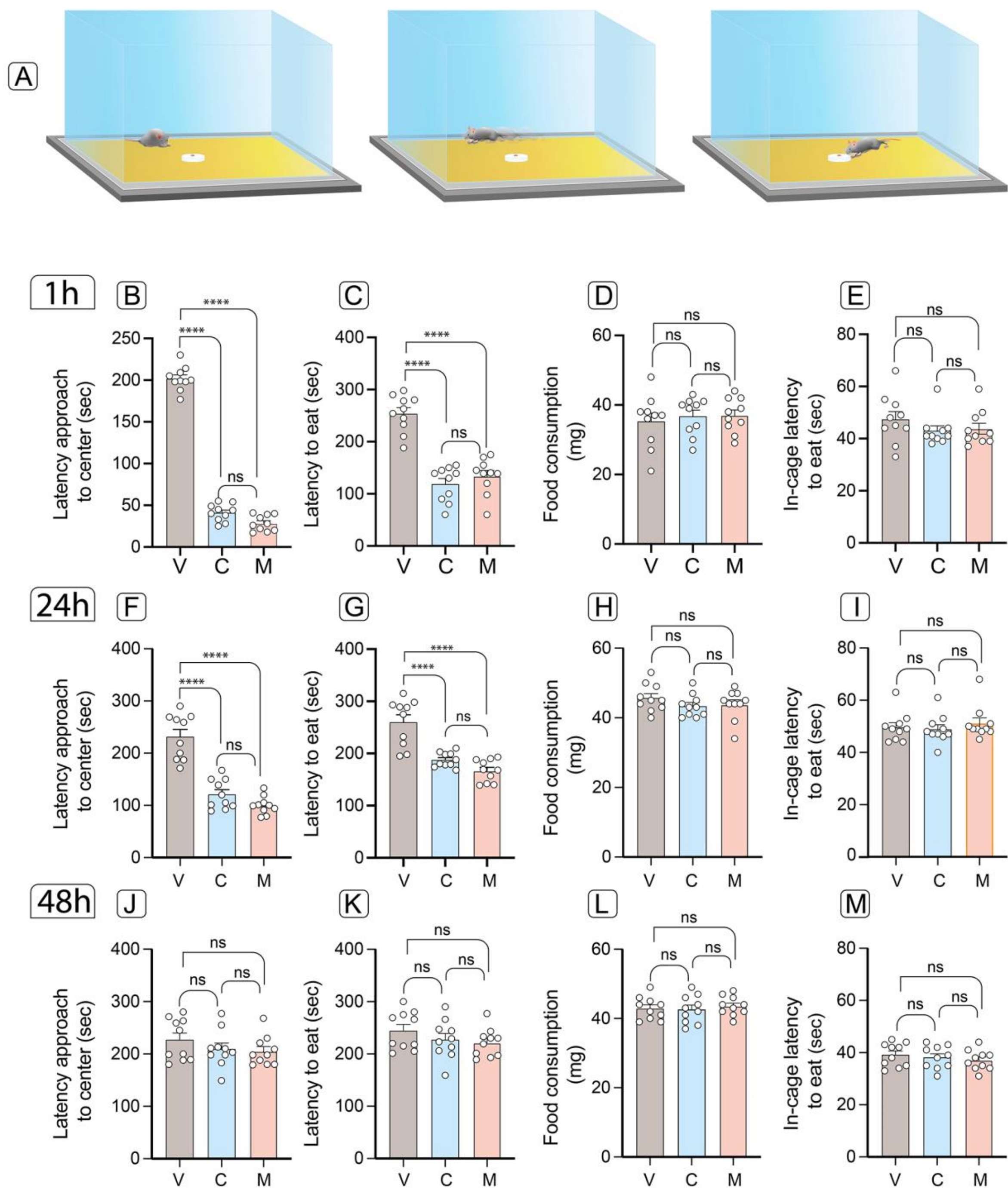

Figure 6. The anxiolytic-like activity of acute treatment with (+)-catharanthine or (-)-18-MC on stressed/anxious mice using the NFST. Male mice (n = 10/condition) were injected (i.p.) 40 mg/kg (+)-catharanthine (C) or (-)-18-MC (M), or vehicle (V), and four different behaviors assessed (A) (see details in the Methods section) at 1 h, 24 h,

and 48 h, respectively. Two-way ANOVA analyses showed that both congeners significantly increased the latency to approach the center of the field and latency to eat at 1 h (B,C) and 24 h (F,G) ($p < 0.0001$; for each condition), but not 48 h (J,K) ($p > 0.5$; for each condition), compared to vehicle-treated animals. Tukey's post hoc analyses showed that the observed effects were not significantly (ns; $p > 0.05$) different between both drugs. No significant (ns; $p > 0.05$) effects were observed for food consumption at 1 h (D), 24 h (H), or 48 h (L), and in-cage latency to eat at 1 h (E), 24 h (I), or 48 h (M), respectively, compared to vehicle-treated animals. ****$p < 0.0001$.

3.6. Coronaridine congeners increase $GABA_A$ receptor affinity for GABA

To determine the effect of coronaridine congeners on the binding affinity of GABA, α1β3γ2 $GABA_A$ receptor bound Gzn-OG fluorescence was monitored at varying concentrations of GABA in the absence and presence of either (+)-catharanthine, (-)-18-MC, (-)-ibogaine, or (-)-voacangine. Figure 7A shows fluorescence imaging of HEK293T cells with $GABA_A$ receptor-bound Gzn-OG in the absence and presence of varying concentrations of GABA, and in the absence (control) and presence of each congener at 100 μM. Increasing concentrations of GABA decreased $GABA_A$ receptor-bound Gzn-OG fluorescence, making it practically undetected at 1 mM GABA. Fluorescence differences were observed in the presence of each congener, with less intensity for (+)-catharanthine and (-)-18-MC compared to other congeners, suggesting that the former drugs are more efficient at increasing displacement of Gzn-OG by GABA.

Non-linear regression of the F/F_o plots for GABA (mean ± SEM; n = 13–21 cells/titration) in the absence or presence of each congener gave IC_{50} values that were transformed to apparent K_i values using Eq. (2). To assess the variability between GABA titrations, GABA K_i's were calculated in a daily basis, rendering similar values (i.e., 21.2 ± 2.4 μM, 15.6 ± 1.6 μM, and 16.8 ± 1.6 μM). Interestingly, GABA concentration-response curves in the presence of increasing concentrations (i.e., 10, 30, and 100 μM) of (+)-catharanthine (Fig. 7B), (-)-18-MC (Fig. 7C), and (-)-voacangine (Fig. 7E) were shifted to lower transmitter concentrations. The decrease in the GABA K_i values indicates an increase in the apparent binding affinity of GABA. The observed K_i shift between 0 (control) and 100 μM gave the following rank

of potency/efficacy: (-)-voacangine (80-fold decrease) > (+)-catharanthine (34-fold decrease) > (-)-18-MC (8-fold decrease). On the other hand, (-)-ibogaine did not induce any apparent shift in GABA titrations, instead 20% fluorescence increase was observed at 100 μM (-)-ibogaine (Fig. 7D).

We also assessed whether each congener affects the direct interaction of Gzn-OG with the orthosteric site at $GABA_A$ receptors, in the absence of GABA (Fig. 7F). The results indicated that no coronaridine congener modifies $GABA_A$ receptor-bound Gzn-OG in the 10-100 μM concentration range. These results suggest that coronaridine congeners, except (-)-ibogaine, allosterically increase the affinity of GABA for α1β3γ2 $GABA_A$ receptors in a concentration-dependent manner.

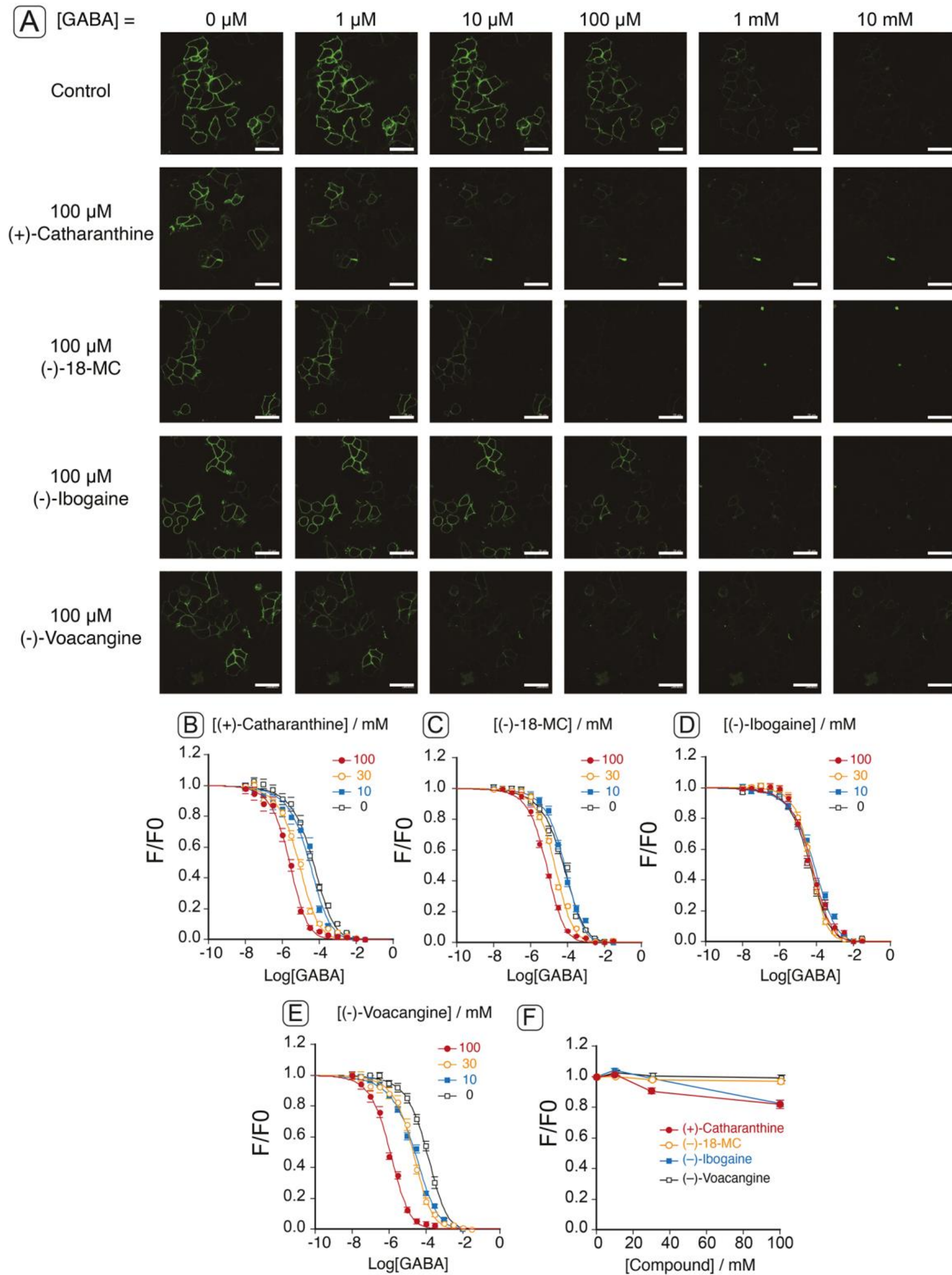

Figure 7. The effect of coronaridine congeners on binding affinity of GABA for $GABA_A$ receptors expressed in HEK293T cells using the fluorescent probe Gzn-OG. (A) Confocal images of HEK293T-α1β3γ2L cells upon addition of Gzn-OG (100 nM), in the absence or presence of increasing concentrations of GABA (1 μM-30 mM),

and in the absence (control) or presence of different concentrations of each congener. Scale bar = 40 μm. (B-E) Fluorescence intensity of HEK293T-α1β3γ2L-bound Gzn-OG in the presence over absence (F/F_o) of increasing concentrations of GABA [0.01 μM-30 mM (□)] for (+)-catharanthine (B), (-)-18-MC (C), (-)-ibogaine (D), and (-)-voacangine (E), each at concentrations of 10 (■), 30 (●), and 100 (●) μM, respectively. Non-specific binding was determined at 30 mM GABA. Non-linear regression of F/F_o *vs* [GABA] plots [eq. (1)] (mean ± SEM; n = 13–21 cells/titration) gave GABA IC_{50}'s in the absence and presence of each drug at different concentrations, which were transformed to apparent K_i values using eq. (2). (F) Effect of (+)-catharanthine (●), (-)-18-MC (○), (-)-ibogaine (■), and (-)-voacangine (□) on α1β3γ2L-bound Gzn-OG fluorescence, in the absence of GABA (mean ± SEM; n = 16 cells/titration). No congener displaced Gzn-OG from the orthosteric binding site at the used concentrations.

3.7. Coronaridine congeners do not bind to the benzodiazepine site at $GABA_A$ receptors

To demonstrate whether coronaridine congeners bind to the BZD site at different $GABA_A$ receptor subtypes, [^{3}H]flunitrazepam competition binding assays were performed using membranes prepared from different brain regions, including cerebellum, hippocampus, prefrontal cortex, brain stem, spinal cord, and olfactory bulb as well as several HEK293 cell lines expressing the respective rα1β3γ2, rα2β3γ2, rα3β3γ2, and rα5β3γ2 $GABA_A$ receptor subtype.

The results demonstrated that (+)-catharanthine did not affect [^{3}H]flunitrazepam binding to any of the brain membrane preparations at a concentration of 50 μM (Fig. 8A). Similar results were obtained with other coronaridine congeners, including (-)-ibogaine, (-)-noribogaine, and (-)-ibogamine (data not shown). One compound, (±)-18-MC (50 μM), slightly inhibited binding (one sample Student's t-test; p = 0.01-0.03) to cerebellum, prefrontal cortex, and spinal cord membranes, without affecting binding to other membranes (Fig. 8A). The highest inhibitory effect was observed in prefrontal cortex membranes (-13.5 ± 2.3 %), equivalent to that previously determined in cerebellum membranes (-13.6 ± 2.4 %) (Arias et al., 2020b).

On the contrary, 50 μM (-)-voacangine increased [^{3}H]flunitrazepam binding to cerebellum (16 ± 1 %), olfactory bulb (18 ± 3 %), and brain stem (15 ± 2 %) membranes in a statistically significant manner

($p < 0.05$-0.001), whereas the increase in prefrontal cortex, hippocampus, and spinal cord membranes was not significant (Fig. 8B). To determine the concentration-dependence of the observed increase, additional experiments were performed in cerebellum, brain stem, and hippocampus membranes, using a wider range of (-)-voacangine concentrations (1-150 μM) (Fig. 8C). The results confirmed a concentration-dependent increase. Although we could not calculate the potentiating EC_{50} values due to solubility limitations, the following apparent maximal binding percentages [compared to control (100%)] were calculated at 150 μM: cerebellum (50 ± 2 %) > brain stem (25 ± 2 %) ~ hippocampus (23 ± 2 %) (Table 1).

To determine whether the observed [^{3}H]flunitrazepam binding increase is receptor subtype-dependent, the effect of (-)-voacangine was determined on several different $GABA_A$ receptor subtypes. In this regard, (-)-voacangine increased [^{3}H]flunitrazepam binding to various $GABA_A$ receptor subtypes (Fig. 8D), with the following apparent maximal binding percentages (at 100 μM): rα3β3γ2 (85 %) > rα1β3γ2 (55 %) > rα2β3γ2 (30 %) ~ rα5β3γ2 (33 %) (Table 1).

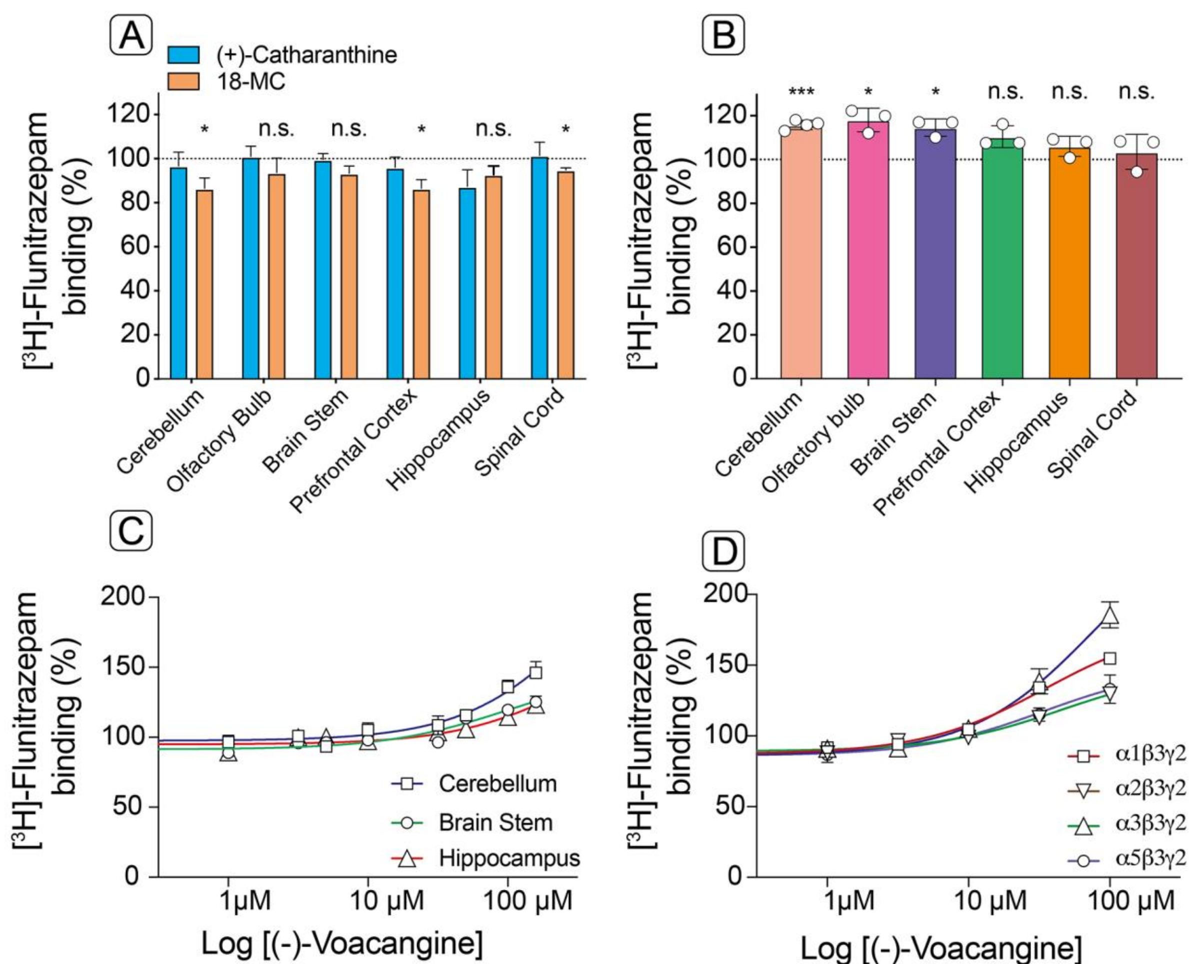

Figure 8. The effect of coronaridine congeners on [^{3}H]flunitrazepam binding to $GABA_A$ receptor-containing membranes and HEK293-$GABA_A$ receptor cells. (A,B) Effect of 50 μM (+)-catharanthine (blue), (±)-18-MC (brownish) (A), and (-)-voacangine (B) on the specific binding of [^{3}H]flunitrazepam to membranes prepared from different brain areas. One sample t-tests (mean ± SEM; n = 3-4 independent experiments) indicated that (+)-catharanthine did not affect binding (ns), whereas (±)-18-MC slightly decreased binding to the cerebellum (**$p = 0.01$), prefrontal cortex (*$p < 0.03$), and spinal cord membranes (*$p < 0.05$, each membrane), but not to other membranes (A), and (-)-voacangine increased binding to the cerebellum (***$p < 0.001$), olfactory bulb (*$p < 0.05$), and brain stem membranes (*$p < 0.05$), but not to other membranes (B). (C) In the case of (-)-voacangine, a wider range of concentrations was used (i.e., 1-150 μM) for the cerebellum (□), hippocampus (△), and brain stem (○) membranes. (D) Effect of (-)-voacangine (1-100 μM) on [^{3}H]flunitrazepam binding to HEK293 cells expressing the respective rα1β3γ2 (□), rα2β3γ2 (▽), rα3β3γ2 (△), and rα5β3γ2 (○) $GABA_A$ receptor.*$p < 0.05$; ***$p < 0.001$.

The apparent maximal binding of (-)-voacangine determined at 150 μM (C) and 100 μM (D), respectively, as well as the calculated p values, were summarized in Table 1.

Table 1. The effect of (-)-voacangine on [^{3}H]flunitrazepam binding to different $GABA_A$ receptor expressing- HEK293 cells and rat brain membranes from different areas.

$GABA_A$ receptor source	Apparent maximal binding [a] (%)	p value [b]
HEK293-rα1β3γ2 cells	55 ± 2	$p < 0.005$
HEK293-rα2β3γ2 cells	30 ± 3	$p < 0.05$
HEK293-rα3β3γ2 cells	85 ± 9	$p < 0.05$
HEK293-rα5β3γ2 cells	33 ± 10	$p < 0.05$
Cerebellum	50 ± 5	$p < 0.01$
Brain stem	25 ± 2	$p < 0.01$
Hippocampus	23 ± 2	$p < 0.01$

[a] Apparent maximal binding of (-)-voacangine [% over the control (set at 100%) in the absence of any congener] calculated at 150 μM (Fig. 8C) and 100 μM (Fig. 8D), respectively.

[b] p values were calculated by one sample t-test (difference from 100%)

4. Discussion

The main objective of the present study was to compare the sedative-hypnotic and anxiolytic-like activity of (+)-catharanthine and (-)-18-MC between male and female mice, after acute and repeated treatments. To determine the role of $GABA_A$ receptors in these behavioral effects, additional radioligand and fluorescence imaging experiments were devised.

To assess whether (+)-catharanthine and (-)-18-MC induce sedative-hypnotic effects in a sex-dependent manner, we determined the effect of both congeners on male and female mice and compared these new data with previous (+)-catharanthine results in male mice (Arias et al., 2020a). The most important conclusion from these new experiments is that both compounds induce sedative-hypnotic effects at the same dose range (63-72 mg/kg) in a time-dependent, but sex-independent, fashion (Table 2). In general, the effect of (+)-catharanthine was more pronounced after repeated (up to 14 days) treatment compared to acute (1-24 h) treatment.

Previous LORR and open field studies did not allow us to directly observe the anxiolytic-like activity of (+)-catharanthine because this type of behavior is apparent at doses that partially overlap its sedative effect (≥50 mg/kg) (Arias et al., 2020a). Thus, in this work, we used five different behavioral tests to assess the anxiolytic-like activity of (+)-catharanthine and (-)-18-MC in naïve mice and mice under stressful/anxiogenic conditions, respectively (summarized in Table 2).

Table 2. Summary of behavioral activities mediated by coronaridine congeners after acute and repeated treatments.

Test	Behavior	Treatment	(+)-Catharanthine	(-)-18-MC
LORR	Immobility time	Acute	Increase [a]	Increase [a]
Open field	Locomotor activity	Acute	Decrease [a]	Decrease [a]
Elevated O-maze	Time in open arms	Acute	No effect	Increase [a]
	Time in closed arms	Acute	No effect	Reduce [a]
	Time in open arms	Repeated	—	Increase [a]
	Time in closed arms	Repeated	—	Reduce [a]
Elevated O-maze (+PTZ)	Time in open arms	Acute	No effect	No effect
	Time in closed arms	Acute	No effect	No effect
	Time in open arms	Repeated	No effect	No effect
	Time in closed arms	Repeated	No effect	No effect
Light/dark transition	Time in dark compartment	Acute	Decrease [a]	Decrease [a]
	Time in bright compartment	Acute	Increase [a]	Increase [a]
NSFT	Latency to approach the center of the field	Acute	Decrease [a] Lasted 24 h	Decrease [a] Lasted 24 h
	Latency to eat	Acute	Decrease [a] Lasted 24 h	Decrease [a] Lasted 24 h
	Food consumption	Acute	No effect	No effect
	In-cage latency to eat	Acute	No effect	No effect

[a] One-way ANOVA analysis showed significant effects for each congener compared to vehicle-treated animals.

Using the elevated O-maze test, we determined that 40 mg/kg (-)-18-MC, but not (+)-catharanthine, induces anxiolytic-like activity in a sex-independent manner, after acute and repeated treatments. Among plausible explanations for the observed difference between (-)-18-MC and (+)-catharanthine we can include a dissimilar recruiting of serotonergic vs norepinephrinergic

neurotransmission (Arias et al., 2023b) and distinct expression of neurotrophic factors (Carnicella et al., 2010), in selective brain areas and neuronal pathways. The results showing no sex difference in the sedative-hypnotic and anxiolytic-like activity of coronaridine congeners suggest that hormonal and neurochemical differences between male and female mice are not important for the observed behavioral activities, in agreement with other GABAergic compounds (Blednov et al., 2003; Ferguson et al., 2007).

To determine the anxiolytic-like activity of coronaridine congeners under stressful/anxiogenic conditions three different behavioral tests were used (summarized in Table 2). The light/dark transition test and NSFT clearly showed that both (+)-catharanthine and (-)-18-MC induce anxiolytic-like activity in mice under stressful/anxiogenic conditions that lasted for 24 h, without modifying feeding behavior (i.e., NSFT). This would also suggest that (+)-catharanthine is effective only in anxious mice, whereas (-)-18-MC is effective in both naïve and anxious mice, likely due to a different mechanism of action (Arias et al., 2023a; Carnicella et al., 2010).

Our results also showed that neither acute nor repeated treatment with (-)-18-MC or (+)-catharanthine improves PTZ-induced mouse anxiety. The simplest reason for the observed lack of activity is based on the evidence that PTZ blocks the $GABA_A$ receptor (Huang et al., 2001). In this scenario, the potentiating activity previously determined for both congeners (Arias et al., 2022) should be also inhibited, and consequently, the resulting anxiolytic-like activity is no longer apparent. Since benzodiazepine reverses the anxiety-like status induced by PTZ (Jung et al., 2002), our results also support the notion that these congeners are not acting through the benzodiazepine site (Arias et al., 2020a) this work). Other studies showed that coronaridine congeners can induce anxiolytic or anxiogenic activity depending on the congener and animal model used. For instance, noribogaine decreased stress-induced anxiety-like behavior in zebrafish (Kalueff et al., 2017), whereas (-)-ibogaine increased anxiety in rodents after 22 h following treatment (Benwell et al., 1996).

We previously showed that (+)-catharanthine and (-)-18-MC potentiate $GABA_A$ receptors in a benzodiazepine-insensitive manner (Arias et al., 2020a). However, it was not known whether this allosteric interaction promotes changes in GABA affinity. Our current fluorescence imaging results showed that structurally different coronaridine congeners (Fig. 1) increase the binding affinity of the α1β3γ2L $GABA_A$ receptor for GABA. Additional [^{3}H]flunitrazepam competition binding results showed that (-)-18-MC slightly decreases radioligand binding to the cerebellum, prefrontal cortex, and spinal cord membranes, suggesting a small, if any, interaction with benzodiazepine sites, confirming our initial results with (+)-catharanthine (Arias et al., 2020a). On the contrary, (-)-voacangine increased [^{3}H]flunitrazepam binding to the cerebellum, olfactory bulb, hippocampus, and brain stem membranes as well as to a variety of cell-expressing $GABA_A$ receptor subtypes. These latter effects clearly state an allosteric mechanism of action rather than a competitive form of inhibition. In conclusion, subtle structural variances of the coronaridine scaffold were able to potentiate the $GABA_A$ receptor (Arias et al., 2020a), inducing long-distance conformational changes in the GABA binding site, finally improving its affinity.

The main conclusion of this work is that (+)-catharanthine and (-)-18-MC induce sedative-hypnotic and anxiolytic-like activity in naïve and stressed/anxious mice in a sex-independent manner, and that this effect lasted longer in stressed/anxious mice. The observed behavioral effects are likely mediated by a benzodiazepine-independent mechanism that increases $GABA_A$ receptor affinity for GABA, finally enhancing receptor function.

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Author contributions

Hugo R. Arias (HRA): developed the concept, wrote the manuscript. **Abdeslam Chagraoui (AC):** conducted the behavioral studies, wrote the manuscript. **Philippe De Deurwaerdère (PD)**: performed data analyses and contributed to critical comments on the manuscript and discussion. **Giuseppe Di Giovanni (GDG)**: performed data analyses and contributed to critical comments on the manuscript and discussion. **Seiji Sakamotod (SS) and Itaru Hamachi (IH)** performed the fluorescence experiments. **Petra Scholze (PS)** performed the radioligand binding experiments. **AC, PS and IH** wrote the methods and results. **HRA, PS, SS, PDD, GDG and AC** performed data analyses and contributed to critical comments on the manuscript and discussion.

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