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Myricitrin, a nitric oxide and protein kinase C inhibitor, exerts antipsychotic-like effects in animal models

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ABSTRACT

Myricitrin is a nitric oxide (NO) and protein kinase C (PKC) inhibitor that has central nervous system activity, including anxiolytic-like action. Nitric oxide inhibitors blocked the behavioral effects of apomorphine, suggesting an antipsychotic-like effect. Furthermore, PKC inhibition reduced psychotic symptoms in acute mania patients and blocked amphetamine-induced hyperlocomotion, suggesting a potential antipsychotic-like effect. The present study evaluated the effects of myricitrin in animal models that assess antipsychotic-like effects (apomorphine-induced stereotypy and climbing and the paw test) and extrapyramidal side effects (catalepsy test and paw test). Olanzapine was used as a positive control. 7-Nitroindazole (7-NI), a NOS inhibitor, and L-arginine, a NO precursor, were used to evaluate nitrergic modulation, and tamoxifen was used to test the effect of PKC inhibition. In mice, myricitrin dose-dependently and olanzapine blocked the stereotypy and climbing induced by apomorphine at doses that did not induce catalepsy. 7-Nitroindazole also blocked apomorphine-induced stereotypy and climbing, which were reversed by L-arginine pretreatment. L-arginine only attenuated the effects of myricitrin on apomorphine's effects. Tamoxifen also blocked apomorphine-induced stereotypy and climbing. In the paw test in rats, myricitrin and olanzapine increased hindlimb retraction time at doses that did not affect forelimb reaction time, whereas haloperidol affected both parameters at the same dose. Myricitrin did not induce catalepsy in the bar test. Tamoxifen did not affect hindlimb retraction time or forelimb retraction time, whereas 7-NI significantly increased hindlimb reaction time. Thus, myricitrin exhibited an antipsychotic-like profile at doses that did not induce catalepsy, and this effect may be related to nitrergic action.

1. Introduction

Antipsychotics are effective drugs for controlling psychotic symptoms in some mental disorders, such as schizophrenia. However, both typical (e.g., haloperidol) and atypical (e.g., clozapine and olanzapine) antipsychotics exert troublesome side effects that decrease adherence to treatment and affect the quality of life of patients (Novick et al., 2009; Serretti and Chiesa, 2011; Tandon et al., 2008). Furthermore, schizophrenia patients refractory to current antipsychotics are unlikely to respond to new antipsychotics that target the dopamine D_2 receptor (Stone et al., 2010). Thus, new antipsychotics drugs are necessary.

Nitric oxide (NO) can positively modulate dopaminergic neurotransmission (Fig. 1), since it appears to block dopamine reuptake and to facilitate postsynaptic activation (Bernstein et al., 2005; Del Bel and Guimarães, 2000; Fujiyama and Masukos, 1996; Hong et al., 2005; Pires et al., 2003; West et al., 2002), and drugs that inhibit NO may have potential antipsychotic effects (Bernstein et al., 2005; West et al., 2002; Wiley, 1998). 7-Nitroindazole (7-NI) and N^G-nitro-L-arginine (L-NOARG) are specific and nonspecific NO synthase inhibitors, respectively, and attenuate the disruption of prepulse inhibition induced by methylphenidate and phencyclidine, proposed models of antipsychotic action (Geyer and Ellenbroek, 2003; Issy et al., 2009;

Abbreviations: DA, dopamine; DAT, dopamine transporter; FRT, forelimb retraction time; Glu, glutamate; HRT, hindlimb retraction time; nNOS, neuronal nitric oxide synthase; L-NAME, N^g-nitro-L-arginine methyl ester hydrochloride; L-NOARG, N^G-nitro-L-arginine; 7-NI, 7-Nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; NMDA, N-methyl-D-aspartate; SNAP, S-nitroso-N-acetylpenicillamine; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

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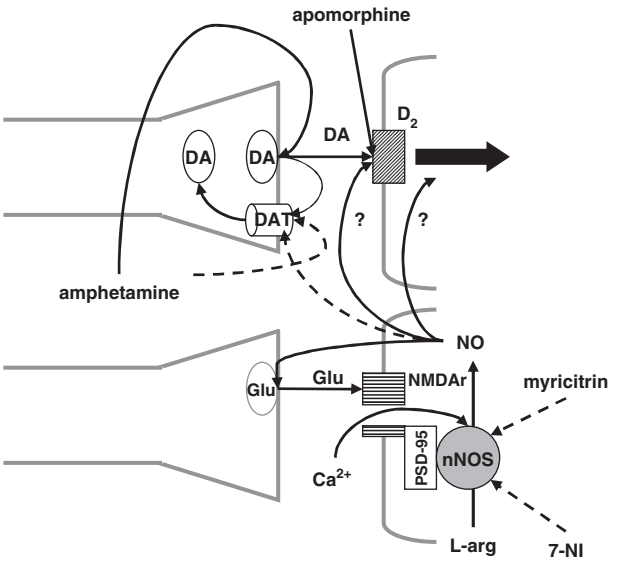

Fig. 1. Effects of nitric oxide (NO) on dopamine (DA) transmission. (1) Glutamate (Glu) activates N-methyl-D-aspartate receptors (NMDAr) in NO neurons, opening calcium channels and leading to Ca^{2+} influx. (2) Increased intracellular Ca^{2+} activates neuronal nitric oxide synthase (nNOS), generating NO from L-arginine (L-arg). (3) Nitric oxide diffuses to the extracellular space, inhibiting the dopamine transporter (DAT), which reduces DA reuptake and increases extracellular DA. (4) Dopamine activates postsynaptic D_2 receptors. (5) Nitric oxide also directly facilitates D_2 activation or postsynaptic transduction mechanisms, increasing DA transmission. (6) Nitric oxide also increases Glu release, which exacerbates its effect on NO production. Apomorphine acts at postsynaptic D_2 receptors, and amphetamine stimulates presynaptic DA release and decreases DA reuptake. Myricitrin and 7-NI block NO synthesis, reducing the facilitatory effect of NO on DA transmission. Solid line: activation; dashed line: inhibition.

Salum et al., 2008; Wiley, 1998). Furthermore, in reserpine-treated mice, L-NOARG and 7-NI attenuated the increase in locomotor activity induced by D_1 and D_2 agonists (Starr and Starr, 1995). Locomotor hyperactivity induced by dopamine agonists is another animal model for the study of antipsychotic-like action (Geyer and Ellenbroek, 2003). Clinical studies found changes in NO synthase (NOS)-containing interneurons in frontal and limbic cortices in schizophrenia patients (Akbarian et al., 1993a,b). Moreover, the addition of methylene blue, which blocks NO activity on soluble guanylyl cyclase, to neuroleptic treatment modestly improved psychotic symptoms (Deutsch et al., 1997).

Nitric oxide inhibition has also been associated with catalepsy. Nitric oxide synthase inhibitors induced catalepsy in mice (Del Bel et al., 1998, 2002, 2005), an effect that was antagonized by pretreatment with L-arginine, a NO precursor (Del Bel et al., 2005; Marras et al., 1995; Narkevich et al., 2005). The effects of NO on prepulse inhibition and catalepsy may involve dopaminergic mediation because the cataleptic effect of a NOS inhibitor was reversed by apomorphine, a dopamine agonist, and NO appeared to increase dopamine in the striatum (Del Bel et al., 2005). Furthermore, haloperidol attenuated the increase in NO levels induced by neonatal ventral hippocampus lesion, an animal model of schizophrenia (Negrete-Diaz et al., 2010).

Based on the hypothesis that protein kinase C (PKC) inhibition is an important target for mood stabilizers, tamoxifen, a PKC inhibitor, was tested in clinical trials for the treatment of acute episodes of mania (Bebchuk et al., 2000; DiazGranados and Zarate, 2008; Kulkarni et al., 2006; Yildiz et al., 2008; Zarate et al., 2007). The controlled studies with tamoxifen monotherapy showed a consistent antimanic effect (Yildiz et al., 2008; Zarate et al., 2007), which corroborated the initial hypothesis of PKC inhibition. Moreover, tamoxifen blocked amphetamine-induced hyperlocomotion, an animal model of mania (Einat et al., 2007; Sabioni et al., 2008). However, behavioral changes (e.g., hyperlocomotion) induced by amphetamine administration are also proposed as an animal model of antipsychotic-like effects (Bardin et al., 2006; Depoortere et al., 2003; Leite et al., 2008; Porsolt et al., 1993, 2010), and mania patients can show psychotic symptoms (Goodwin and Jamison, 2007). Kulkarni et al. (2006) and Yildiz et al. (2008) observed a reduction in psychotic symptoms in mania patients treated with tamoxifen. Moreover, chronic administration of clozapine and haloperidol reduced PKC activity and levels in discrete brain areas (e.g., hippocampus and cortex), which could be relevant to the antipsychotic and antimanic effects of these drugs (Dwivedi and Pandey, 1999). Interestingly, chronic clozapine and haloperidol decreased mRNA and protein levels of the PKC α and ε isozymes (Manji and Lenox, 1999). Thus, PKC inhibitors may also have antipsychotic-like effects and may be an interesting target for new antipsychotic drugs.

Myricitrin is a flavonoid extracted from some plants (Pouteria gender, *Manilkara zapota, Eugenia uniflora*, among others) and has anxiolytic and antinociceptive effects. Myricitrin increases the percentage of entries into and time spent on the open arms of the elevated plus maze, although this effect presents a bell-shaped curve, decreasing at high doses (Fernandez et al., 2009). Myricitrin also has antinociceptive action in models of acute and chronic pain (Meotti et al., 2006a, b, 2007a, b). For example, myricitrin decreases paw edema and reduces allodynia caused by intraplantar administration of complete Freund's adjuvant or induced by partial constriction of the sciatic nerve (Meotti et al., 2006a). Myricitrin appears to inhibit NO synthesis and reduce iNOS overexpression induced by lipopolysaccharide (Chen et al., 2000). Pretreatment with L-arginine prevented the antinociceptive effect of myricitrin in an abdominal writhing model induced by acetic acid (Meotti et al., 2006b). Myricitrin also blocked the effects of PMA, a PKC activator, administration on licking behavior and activation of PKC_α and PKC_ε (Meotti et al., 2006b). Additionally, myricitrin may act on small-conductance calcium-gated potassium channels and inhibit calcium influx. Furthermore, it also inhibits p38 MAPK phosphorylation induced by interleukin-1β and tumor necrosis factor α (Meotti et al., 2007b).

Several animal behavioral models have been used to evaluate antipsychotic-like drugs, and, among them, behavioral changes induced by direct and indirect dopaminergic agonists are frequently used (Geyer and Ellenbroek, 2003; Porsolt et al., 2010). The behaviors observed include stereotypy (e.g., repetitive sniffing, rearing, gnawing, licking, and head bobbing) and climbing (i.e., when a mouse climbs the walls of cage and holds the wire mesh with two to four paws). Apomorphine-induced climbing has been mainly related to mesolimbic dopaminergic activity, whereas stereotypy induced by apomorphine appears to involve striatal D_2 receptors (Porsolt et al., 1993, 2010). In response to apomorphine, the preferential blockade of climbing over stereotypy is hypothesized to indicate an atypical antipsychotic profile (Geyer and Ellenbroek, 2003). In the paw test, which was developed to differentiate typical and atypical antipsychotic drugs, rats treated with test drugs are placed on a platform with four holes (one for each paw), and the time to withdraw the forelimbs and hindlimbs from the holes is the independent variable (Cools et al., 1995; Ellenbroek et al., 1987; Ellenbroek and Cools, 1988). The paw test was validated with several antipsychotic and non-antipsychotic drugs, showing good predictive validity (Cools et al., 1995; Ellenbroek et al., 1987; Ellenbroek and Cools, 1988). Catalepsy is considered the relative incapacity of an animal to change an unusual posture imposed by the experimenter (Porsolt et al., 1993). This test is a useful procedure for assessing potential extrapyramidal motor side effects (Bardin et al., 2006; Porsolt et al., 1993, 2010). In the present study, apomorphine-induced climbing and stereotypy and the paw test were used to predict potential antipsychotic effects, and catalepsy was used to predict extrapyramidal side effects. In these models, typical and atypical antipsychotics were hypothesized to exert differential behavioral profiles. Typical antipsychotics block stereotypy and climbing induced by apomorphine, induce catalepsy, and impair forelimb and hindlimb retraction time in the paw test at the same dose. Atypical

antipsychotics are more efficient at blocking apomorphine-induced climbing, do not induce catalepsy (or induce it at only high doses), and impair hindlimb retraction time at a lower dose than is necessary to impair forelimb retraction time (Cools et al., 1995; Ellenbroek et al., 1987; Ellenbroek and Cools, 1988; Geyer and Ellenbroek, 2003; Porsolt et al., 1993, 2010).

Based on the aforementioned considerations, myricitrin, similar to other NOS inhibitors, was hypothesized to exert antipsychotic-like effects. Therefore, the objective of the present study was to evaluate the effects of myricitrin in animal models of antipsychotic action (i.e., apomorphine-induced stereotypy and climbing in mice and the paw test with rats) and extrapyramidal side effects (i.e., catalepsy test and paw test) at doses that do not alter locomotor activity (Bardin et al., 2006; Costall et al., 1978; Depoortere et al., 2003; Echeverry et al., 2007; Geyer and Ellenbroek, 2003; Leite et al., 2008; Porsolt et al., 1993; Protais et al., 1976). Furthermore, although monotherapy is the first treatment option for schizophrenia, several patients do not respond satisfactorily to this approach, so a second drug is often added to the pharmacotherapy. Thus, the present study also evaluated the effects of a myricitrin and olanzapine combination on stereotypy and climbing induced by apomorphine.

2. Methods

2.1. Animals

Animals were male adult Swiss albino mice (30–40 g, $n=511$) and male Wistar rats (250–350 g, total $n=93$) from our own breeding stock. They were kept in groups in polypropylene cages ($41\times34\times16$ cm; 4–5 rats per cage and 20 mice per cage) with wood shavings as bedding, under controlled conditions of light (12 h/12 h light/dark cycle, lights on at 7:00 a.m.) and temperature (22 ± 2 °C). The animals had free access to water and food throughout the experiment. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experiments, Biological Sciences Sector, Federal University of Parana (protocol number 385).

2.2. Drugs and drug administration

Myricitrin was obtained from leaves of *Eugenia uniflora* (known as Brazilian cherry tree) collected at Daniela Beach, Florianópolis. It was isolated by the Department of Chemistry, Federal University of Santa Catarina, and identified by spectral analysis (i.e., ^{1}H nuclear magnetic resonance, ^{13}C nuclear magnetic resonance) and compared with the published spectra (Agrawal, 1989). The chemical purity was >98%, determined by high-performance liquid chromatography. Myricitrin was dissolved in Tween 80 followed by saline and administered intraperitoneally (i.p.) at doses of 5, 10, and 30 mg/kg.

Olanzapine (1, 5, and 10 mg/kg; Zyprexa, Eli Lilly, São Paulo, Brazil), haloperidol (0.25, 0.5, and 1.0 mg/kg; Haldol, Jansen-Cilag, São Paulo, Brazil), L-arginine (200, 400, and 600 mg/kg; Sigma, USA), and tamoxifen (1.0 mg/kg; Sigma) were dissolved in saline and administered i.p. Apomorphine (1.0 mg/kg; Sigma) was dissolved in distilled water and administered subcutaneously (s.c.). 7-Nitroindazole (3 and 6 mg/kg; Sigma) was dissolved in corn oil and maintained in ice until administration. For the test of apomorphine-induced stereotypy, 7-NI was administered intracerebroventricularly (i.c.v.) in a constant volume of 2 μl per site using the free-hand method (Laursen and Belknap, 1986; Sabioni et al., 2008). In the paw test, 7-NI was administered i.p.

All drugs were prepared freshly just before the experiments. Drugs were administered in a constant volume of 1 ml/kg for rats and 10 ml/kg for mice (unless otherwise specified). The doses were based on previous studies (Bardin et al., 2006; Del Bel et al., 1998; Issy et al., 2009; Meotti et al., 2006a, 2006b; Protais et al., 1976; Sabioni et al., 2008). Olanzapine and haloperidol were chosen because they are typical and atypical, respectively, antipsychotic drugs commonly used clinically and frequently used as a positive control in preclinical studies (e.g., Bardin et al., 2006; Cools et al., 1995). Tamoxifen is the only PKC inhibitor used clinically that has good penetration in the central nervous system (Bebchuk et al., 2000; Yildiz et al., 2008; Zarate et al., 2007).

2.2.1. Free-hand method for i.c.v. administration

The free-hand method for i.c.v. administration was based on previous studies (Baretta et al., 2001; Laursen and Belknap, 1986; Sabioni et al., 2008). Briefly, 15 min before apomorphine administration, the mice received light anesthesia with enflurane. A hypodermic needle (0.4 mm outer diameter) attached to a cannula linked to a 10 μl Hamilton syringe was inserted through the skull (1 mm to the right or left of a midpoint in a line drawn through the anterior base of the ears). This needle was lowered 2 mm into the mouse's brain. 7-Nitroindazole or its vehicle was administered in a constant volume (0.2 μl) over a period of 25 s, and the needle was left in place for an additional 25 s to allow for diffusion. After the experiments, the mice were sacrificed by deep anesthesia, and their brains were dissected and macroscopically examined. Mice that exhibited substantial bleeding or misplaced injection sites were discarded from the analysis. This occurred with 6 mice in the experiment with 7-NI and with 10 mice in the experiment with 7-NI plus L-arginine (equally distributed across groups).

2.3. Behavioral tests

2.3.1. Apomorphine-induced stereotypy and climbing

Mice were isolated in wire-mesh cages ($30\times15\times19$ cm; vertical bars 1 cm apart, 2 mm diameter) for 30 min and then treated with myricitrin (5 or 10 mg/kg, i.p.), olanzapine (10 mg/kg, i.p.), or saline. After 30 min, they were injected with apomorphine (1 mg/kg, s.c.) and observed for 90 min at 10 min intervals for signs of stereotypy based on the following scale: 0 (asleep or stationary), 1 (active), 2 (active with predominantly stereotyped sniffing and rearing), 3 (stereotyped sniffing with bursts of licking and/or gnawing and biting), 4 (continual licking and/or gnawing of cage grids; Chinen et al., 2006).

In the same mice, climbing behavior was also assessed for 90 min at 10 min intervals according to the following scale: 0 (no climbing), 1 (climbing with two forepaws only), 2 (climbing with four paws; Marcais et al., 1978; Protais et al., 1976). Total scores were calculated by summing the scores recorded for each 10 min interval.

To determine the participation of NO and PKC, we evaluated the effects of tamoxifen (1 mg/kg, i.p.) and 7-NI (3 and 6 mg/kg, i.c.v.) on apomorphine-induced climbing and stereotypy. The protocol was the same as previously described for the experiments with olanzapine and myricitrin, but 7-NI was administered i.c.v.

L-arginine pretreatment was used to evaluate the role of NO in the effects of myricitrin in the apomorphine tests. First, we evaluated the ability of 200, 400, and 600 mg/kg L-arginine (i.p.) to reverse the effects of 7-NI in the apomorphine tests to establish the minimum effective dose, which was found to be 200 mg/kg. Mice were then pretreated with 200 mg/kg L-arginine (i.p.). Ten minutes later, they received myricitrin (10 mg/kg, i.p.) or saline, followed 30 min later by apomorphine treatment (1 mg/kg, s.c.). The animals were then observed for stereotypy and climbing as described earlier.

2.3.2. Catalepsy test

Catalepsy was measured using the bar test (Bardin et al., 2006; Pires et al., 1996) in two situations: (1) saline pretreatment before administration of the test drug (myricitrin or olanzapine) or saline and (2) haloperidol (0.25 or 1.0 mg/kg) pretreatment before administration of the test drug or saline. Mice were habituated to the test room for 30 min and then treated with haloperidol (0.25 or 1.0 mg/kg, i.p.) or saline. After 30 min, the mice received myricitrin (10 or 30 mg/kg, i.p.), olanzapine (10 mg/kg, i.p.), or saline. Thirty

minutes after the second treatment, the mice were tested in the bar test. Each mouse was individually placed on a table with its forepaw positioned on a cylindrical glass bar (diameter, 0.4 cm; width, 5 cm) at a height of 4 cm. The time that the mouse maintained both forepaws on the bar was recorded three times, with a 1 min intertrial interval. The maximum time of each trial was 30 s, and the summation of the three trials was calculated.

2.3.3. Paw test

The paw test was performed according to Ellenbroek and colleagues (Cools et al., 1995; Ellenbroek et al., 1987, 1996, 2001; Ellenbroek and Cools, 1988). Briefly, the apparatus was a wooden platform (30×30 cm, elevated 20 cm above the floor) that contained four holes, two with a 4 cm diameter for the forelimbs and two with a 5 cm diameter for the hindlimbs (Ellenbroek and Cools, 1988). The rats were individually housed 24 h before the test. For habituation, they were placed in the test room 1 h before the test. The paw test was performed 30 min after drug or vehicle administration (1 h for haloperidol and its vehicle). The rat was held behind the forelimbs. Its hindlimbs were gently placed in the respective holes, and then the forelimbs were introduced to the other holes. The latencies for the rat to withdraw its forelimbs (forelimb retraction time [FRT]) and hindlimbs (hindlimb retraction time [HRT]) were recorded in three trials each, conducted 10 min apart. The minimum retraction time was 1 s, even when the rat immediately withdrew its paw or did not permit its forelimbs to be placed in the hole. The average FRT and HRT (mean of three trials) of each rat were then calculated and analyzed (Ellenbroek et al., 2001).

2.3.4. Locomotor activity

Spontaneous locomotor activity was measured in activity chambers (40×20×26 cm) equipped with three photocells on the walls (10 cm apart). The lateral walls were made of wood, the floor was made of wired mesh, and the top was made of dark green Plexiglas. The level of illumination on the floor of the apparatus was 20 lx. The mice were individually placed in the activity chambers, and the numbers of beam interruptions were cumulatively recorded for a period of 20 min (Camarini et al., 1995).

2.4. Statistical analysis

The total apomorphine-induced stereotypy scores, climbing scores, catalepsy (with vehicle pretreatment), and locomotor activity data were analyzed by one-way analysis of variance (ANOVA), with treatment as a factor, followed by the Newman–Keuls test. Catalepsy (haloperidol pretreatment and treatment factors) scores was analyzed by two-way ANOVA followed by the Newman–Keuls test. The latencies to withdraw the limbs in the paw test were analyzed by a nonparametric Kruskal–Wallis ANOVA followed by the multiple comparison test. All statistical analyses were performed using Statistica 7.0 software (Statsoft) or Prism 5.0 software (GraphPad). Statistically significant differences were set at $p<0.05$.

3. Results

3.1. Stereotypy

Olanzapine (10 mg/kg) blocked apomorphine-induced stereotypy (Fig. 2). The total stereotypy score was reduced by olanzapine ($F_{7,59}=122.71$, $p<0.001$; Fig. 2). Apomorphine-induced stereotypy was also blocked by 10 mg/kg myricitrin (Fig. 2). The total stereotypy score was dose-dependently blocked by myricitrin, and a lower dose (5 mg/kg) did not induce any significant effects. At the doses tested, myricitrin alone did not induce stereotypy.

7-Nitroindazole had a dose-response effect on total apomorphine-induced stereotypy scores. A high 7-NI dose (6 mg/kg) blocked

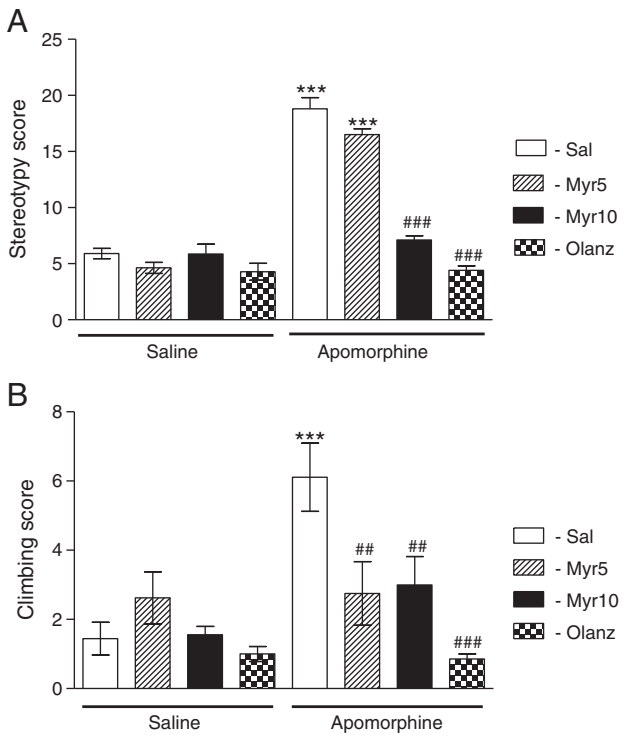

Fig. 2. Effects of myricitrin (5–10 mg/kg) and olanzapine (10 mg/kg) on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. Data are expressed as mean + SEM ($n=7$–10 per group). $^{***}p<0.001$, compared with control (saline + saline); $^{###}p<0.001$, compared with apomorphine + saline. Myr5, 5 mg/kg myricitrin; Myr10, 10 mg/kg myricitrin; Olanz, 10 mg/kg olanzapine.

stereotypy, and the effects of a low dose were not different from the vehicle + apomorphine group ($F_{5,33}=62.09$, $p<0.001$; Fig. 3). Tamoxifen (1.0 mg/kg) also blocked apomorphine-induced stereotypy ($F_{3,32}=118.00$, $p<0.001$; Fig. 4).

Pretreatment with L-arginine (200–600 mg/kg) reversed the effect of 7-NI ($F_{15,80}=26.38$, $p<0.001$; Fig. 5) on apomorphine-induced stereotypy (total score), but the reversal of the effect of myricitrin (10 mg/kg) was only partial ($F_{7,44}=110.44$, $p<0.001$; Fig. 6). Although myricitrin significantly reduced the total stereotypy score compared with the apomorphine + saline group, the effect still differed significantly from the saline + saline group.

A significant effect of co-administration of subeffective doses of myricitrin (5.0 mg/kg) and olanzapine (1.0 mg/kg) was observed on apomorphine-induced stereotypy ($F_{7,40}=165.100$, $p<0.0001$; Fig. 7). Myricitrin + olanzapine co-administration reduced stereotypy compared with the other groups treated with apomorphine (all $p<0.001$). However, all of the apomorphine-treated groups (including myricitrin + olanzapine) were different from the control group (saline + saline + saline; all $p<0.05$). Thus, the myricitrin + olanzapine combination only partially blocked apomorphine-induced stereotypy.

3.2. Climbing

Myricitrin (5 and 10 mg/kg) and olanzapine inhibited the total score of apomorphine-induced climbing ($F_{7,58}=6.89$, $p<0.001$; Fig. 2). 7-Nitroindazole at the high dose (6 mg/kg; $F_{5,33}=2.62$, $p<0.02$) and tamoxifen ($F_{3,32}=3.64$, $p<0.02$) reduced the total score of apomorphine-induced climbing (Figs. 3 and 4, respectively). Pretreatment with L-arginine (200–600 mg/kg) reversed the effects of 7-NI ($F_{15,95}=3.63$, $p<0.001$; Fig. 5), but only partially inhibited the effect of myricitrin ($F_{7,44}=2.65$, $p<0.02$; Fig. 6).

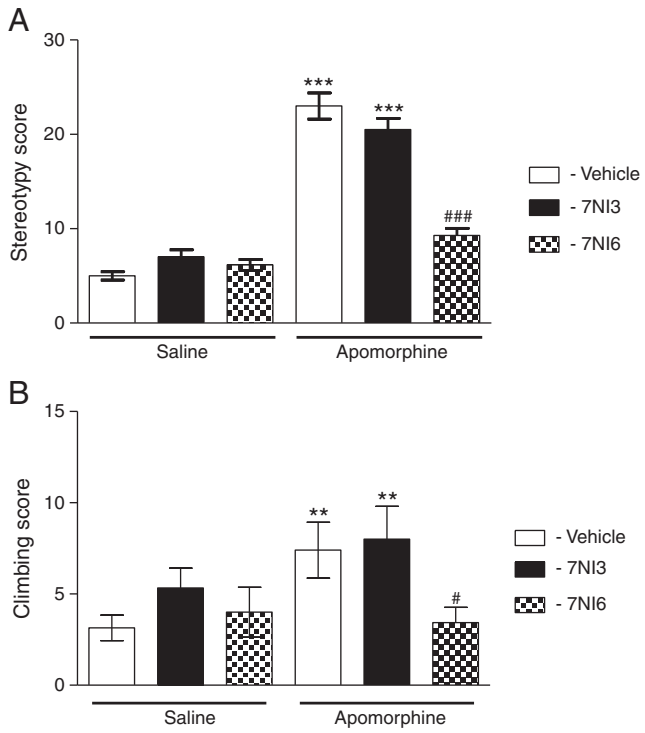

Fig. 3. Effects of 7-nitroindazole (7-NI, 3–6 mg/kg) on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. Data are expressed as mean + SEM ($n = 7$–10 per group). $^{**}p<0.01$, $^{***}p<0.001$, compared with control (saline + vehicle); $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, compared with apomorphine + vehicle. 7NI3, 3 mg/kg 7-NI; 7NI6, 6 mg/kg 7-NI.

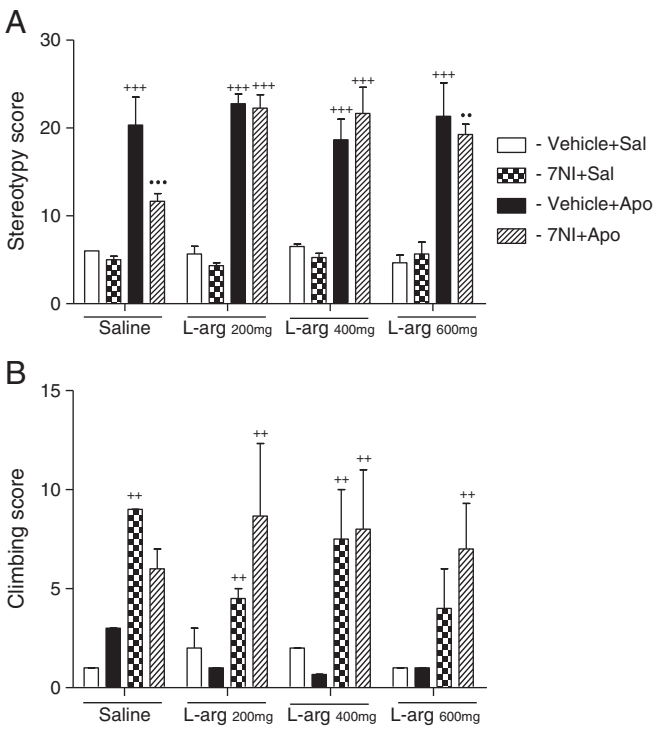

Fig. 5. Effect of pretreatment with L-arginine (L-arg, 200, 400, and 600 mg/kg) on the effect of 6 mg/kg 7-NI on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. Data are expressed as mean + SEM ($n = 7$–10 per group). $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, compared with vehicle + saline; $^{\bullet\bullet}p<0.01$, $^{\bullet\bullet\bullet}p<0.001$, compared with vehicle + apomorphine at the same dose of L-arginine.

Myricitrin + olanzapine co-administration had a significant effect on apomorphine-induced climbing ($F_{7,40} = 7.084$, $p<0.001$; Fig. 7). However, apomorphine induced climbing only in the saline + saline and olanzapine + saline groups (both $p<0.01$ compared with controls). Thus, although myricitrin + olanzapine blocked the effect of apomorphine ($p<0.05$) compared with saline + saline + apomorphine, this

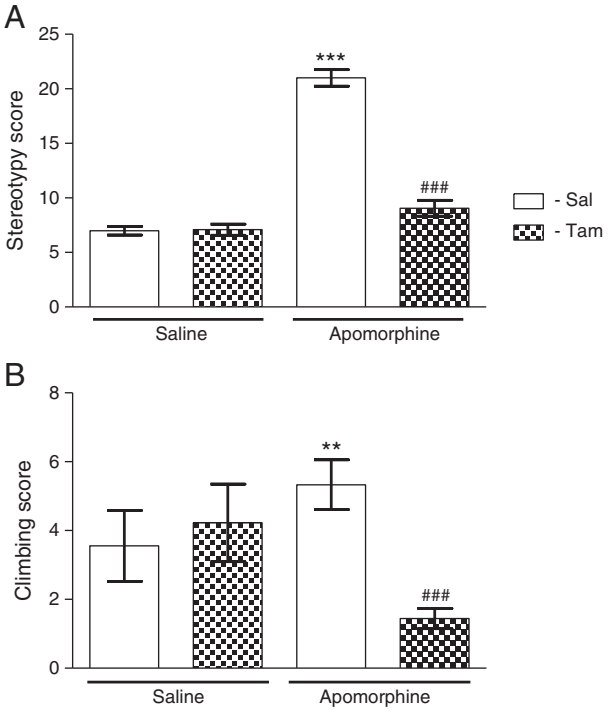

Fig. 4. Effects of tamoxifen (Tam, 1 mg/kg) on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. Data are expressed as mean + SEM ($n = 7$–10 per group). $^{**}p<0.01$, $^{***}p<0.001$, compared with control (saline + saline); $^{\#\#\#}p<0.001$, compared with apomorphine + saline.

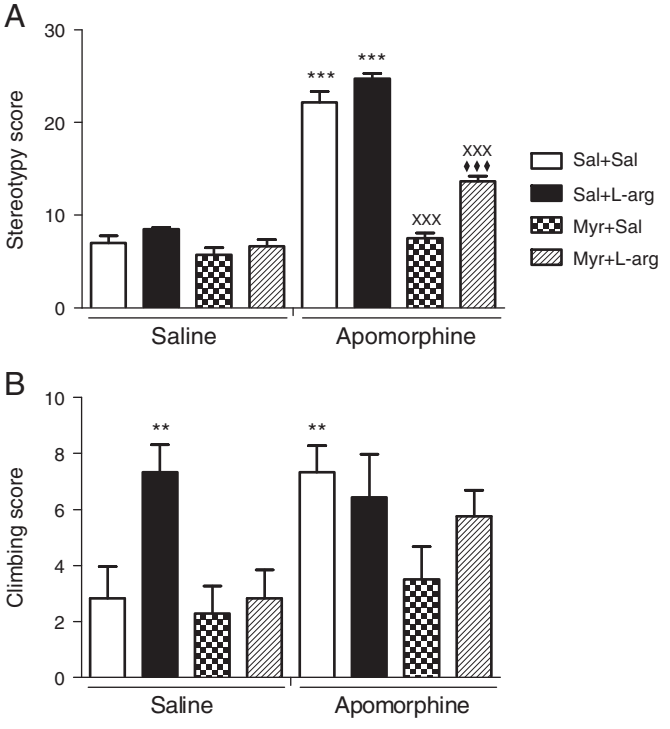

Fig. 6. Effect of pretreatment with L-arginine (L-arg, 200 mg/kg) on the effect of 10 mg/kg myricitrin on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. $^{**}p<0.01$, $^{***}p<0.001$, compared with control (saline + saline + saline); $^{XXX}p<0.001$, compared with saline + saline + apomorphine.

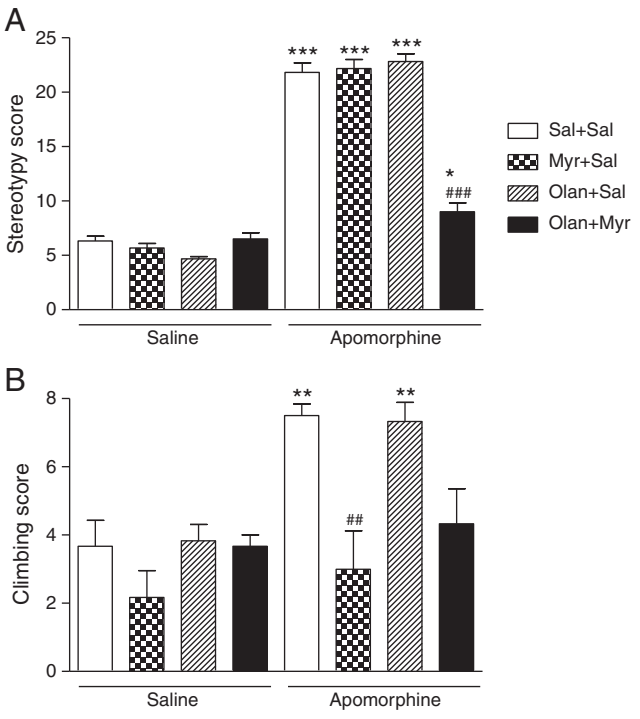

Fig. 7. Effects of co-administration of myricitrin (5.0 mg/kg) and olanzapine (1.0 mg/kg) on (A) apomorphine-induced stereotypy scores and (B) apomorphine-induced climbing scores. Data are expressed as mean + SEM ($n = 6$ per group). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with control (saline + saline); $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, compared with apomorphine + saline.

effect was already seen when myricitrin was administered alone ($p<0.05$) compared with saline + saline + apomorphine.

3.3. Paw test

As shown in Fig. 8A, haloperidol impaired FRT and HRT at 1 mg/kg compared with saline (FRT: $H_{2,18} = 7.12$, $p<0.05$; HRT: $H_{2,18} = 12.49$, $p<0.01$). As shown in Fig. 8, olanzapine impaired HRT at 5 and 10 mg/kg ($H_{3,20} = 15.80$, $p<0.001$), doses that did not modify FRT ($H_{2,18} = 3.00$, $p>0.05$). As shown in Fig. 8C, myricitrin 10 and 30 mg/kg impaired HRT only (FRT: $H_{2,29} = 0.00$, $p>0.05$; HRT: $H_{2,29} = 16.01$, $p<0.001$). Tamoxifen did not have any effect in the paw test (Fig. 8D), and 7-NI at a dose of 6 mg/kg impaired HRT only (HRT: $H_{2,21} = 14.09$, $p<0.001$; FRT: $H_{2,21} = 0.00$, $p>0.05$; Fig. 8D).

3.4. Catalepsy test

The ANOVA showed a significant effect of haloperidol pretreatment ($F_{2,96} = 108.43$, $p<0.001$) but no effect of treatment ($F_{3,96} = 2.57$, $p = 0.059$) and no treatment interaction ($F_{6,96} = 1.04$, $p>0.05$). Haloperidol dose-dependently increased catalepsy time (all $p<0.001$). Myricitrin and olanzapine alone did not increase the latency for the mice to withdraw their forepaws from the bar ($F_{3,32} = 1.00$, $p>0.05$; Fig. 9).

3.5. Locomotor activity

At the doses tested, myricitrin did not alter spontaneous locomotor activity ($F_{2,24} = 1.17$, $p>0.20$; mean ± SEM: saline, 88 ± 9; myricitrin 10 mg/kg, 75 ± 6; myricitrin 30 mg/kg, 72 ± 7; $n = 8$–10/group).

4. Discussion

Myricitrin is hypothesized to exert a positive effect in animal models used in the search for putative antipsychotic-like drugs. The predictive validity of these animal models is based on a comparison of the test drug with a well-established antipsychotic drug such as haloperidol or olanzapine. Considering that both conventional and atypical antipsychotics blocked apomorphine-induced climbing and stereotypy (Bardin et al., 2006; Costall et al., 1978; Depoortere et al., 2003) and affect HRT in the paw test (Ellenbroek et al., 1987), the activity of myricitrin in these models suggests that it could be a putative antipsychotic-like drug for the positive symptoms of schizophrenia. Importantly, these effects were seen at doses that did not affect spontaneous locomotor activity or induce catalepsy. This

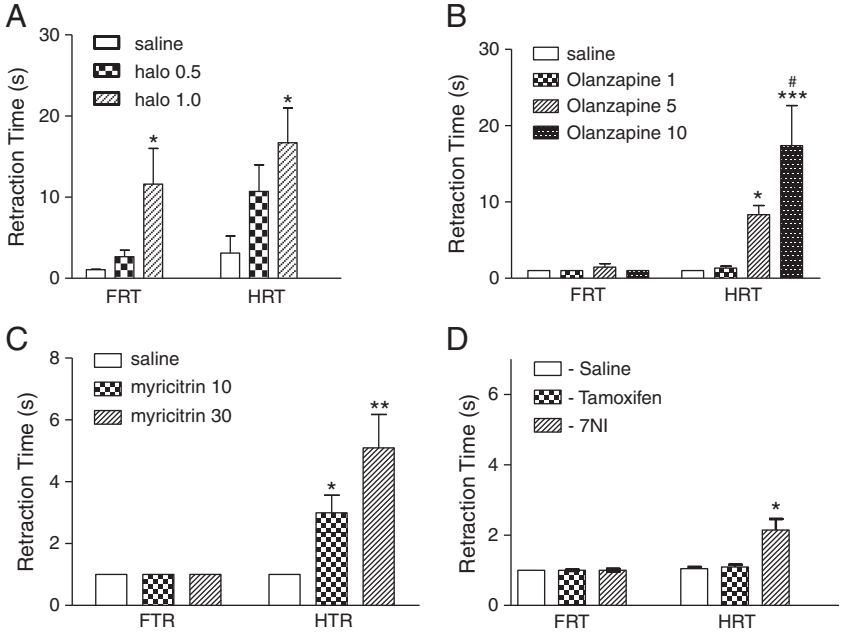

Fig. 8. Effects of (A) 0.5 and 1.0 mg/kg haloperidol, (B) 1, 5, and 10 mg/kg olanzapine, (C) 10 and 30 mg/kg myricitrin, and (D) 6.0 mg/kg7-NI and 1.0 mg/kg tamoxifen or saline on forelimb retraction time (FRT) and hindlimb retraction time (HRT) in the paw test. Data are expressed as mean + SEM (n = 7–10 per group). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with saline; $\#p<0.05$, compared with olanzapine (1 and 5 mg/kg).

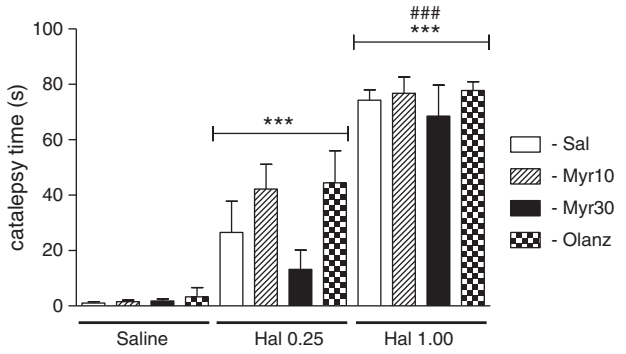

Fig. 9. Effects of olanzapine, myricitrin, or saline in the catalepsy (bar) test, with and without haloperidol pretreatment (0.25 and 1.0 mg/kg, i.p.). Sal, saline; Myr10, 10 mg/kg myricitrin; Myr30, 30 mg/kg myricitrin; Olanz: 10 mg/kg olanzapine. Data are expressed as mean + SEM ($n = 9$–10 per group). $^{***}p<0.001$, compared with saline; $^{###}p<0.001$, compared with 0.25 mg/kg haloperidol.

conclusion was supported by the behavioral profile of myricitrin observed in the present study, which was similar to olanzapine.

7-Nitroindazole, a specific neuronal NO inhibitor, also reduced apomorphine-induced stereotypy and climbing, which is consistent with previous studies of methylphenidate-induced disruptions of prepulse inhibition (Issy et al., 2009). The effect of 7-NI was reversed by L-arginine, suggesting mediation by NO inhibition. L-arginine only partially reversed the myricitrin-induced blockade of apomorphine-induced stereotypy, which also suggests NO mediation (Fig. 1). The effect of L-arginine on apomorphine-induced climbing was less clear; although a similar pattern was observed, no significant effect was seen. This result is complicated by L-arginine's effect on climbing, which may have been attributable to an increase in NO, which increases synaptic dopamine by facilitating its release and blocking its reuptake (Del Bel et al., 2005; West et al., 2002). However, L-arginine would be expected to potentiate the effect of apomorphine, but no difference was found between the saline + apomorphine group and L-arginine + apomorphine group. Altogether, the present results suggest that both nitrergic and non-nitrergic actions contribute to the antipsychotic-like effects of myricitrin. Supporting the proposed involvement of nitrergic and non-nitrergic mechanisms in myricitrin-induced inhibition of apomorphine-induced stereotypy, tamoxifen, a PKC inhibitor, also inhibited this behavior, indicating the PKC inhibition also exerts an antipsychotic-like effect in these models.

Although an antidopaminergic effect of drugs that inhibit NO transmission, such as myricitrin and 7-NI, is predictable based on previous studies (Bernstein et al., 2005; Del Bel et al., 1998, 2002, 2005; Issy et al., 2009; Salum et al., 2006; West et al., 2002; Wiley, 1998), these studies demonstrated an indirect effect mediated by changes in synaptic dopamine levels via facilitation of its release or uptake inhibition. The present results with apomorphine indicate an additional effect on D_2 receptor activation or an intracellular postsynaptic mechanism. Administration of S-nitroso-N-acetylpenicillamine (SNAP), a NO donor, reversed the inhibitory effect of 3-(R)-2-carboxypiperazin-4-propyl-1-phosphonic acid (CPP) on apomorphine-induced climbing, whereas N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), a NO inhibitor, decreased the facilitatory effect of N-methyl-D-aspartate (NMDA) on apomorphine-induced climbing (Hong et al., 2005). This direct positive effect of NO on dopamine receptors was previously suggested by Hong et al. (2005). Thus, in addition to a decrease in the NO inhibitory effect on dopamine uptake, additional mechanisms related to postsynaptic D_2 receptor activation may be involved in the antipsychotic-like effect of myricitrin and other NO inhibitors (e.g., 7-NI; Fig. 1). However, the present results contrast with Salum et al. (2006), whom did not find any effect of NO inhibitors on prepulse inhibition disruption induced by dopamine agonists, including apomorphine.

The paw test is an animal model of antipsychotic-like action and extrapyramidal side effects that has good pharmacological predictive validity (Ellenbroek and Cools, 1988; Geyer and Ellenbroek, 2003). The results with haloperidol and olanzapine confirm previous findings, in which typical antipsychotics enhanced FRT and HRT at the same dose, whereas atypical antipsychotics increased FRT at a higher dose than the one that increased HRT (Cools et al., 1995; Ellenbroek et al., 1996, 2001; Ellenbroek and Cools, 1988). Myricitrin had an atypical antipsychotic profile because it increased HRT at a lower dose than the one that increased FRT. This result is supported by the lack of an effect of myricitrin in the catalepsy test at a dose that blocked the effects of apomorphine. Tamoxifen did not change retraction times in the paw test, suggesting that PKC inhibition cannot fully explain the antipsychotic-like effects of myricitrin.

Although 7-NI had a significant effect on HRT, this effect appeared to be smaller (nearly 2 s) than olanzapine and other antipsychotics (10–20 s). However, this effect may indicate NO activity, which is consistent with other animal models (Del Bel et al., 1998, 2005) and the present apomorphine-induced stereotypy and climbing results.

The myricitrin + olanzapine combination showed promising results. Subeffective doses of these drugs were able to block the stereotypy induced by apomorphine when co-administered. Conversely, no conclusion can be drawn from the analysis of climbing behavior because the myricitrin dose used for this experiment already blocked climbing induced by apomorphine. This result was also observed in the first experiment in the present study. Altogether, these results indicate that myricitrin exhibits an effect profile that is similar to atypical antipsychotic drugs. For apomorphine-induced behaviors, a higher dose is necessary to block stereotypy than to block climbing (Geyer and Ellenbroek, 2003), which is consistent with the effects of myricitrin in the paw test and on catalepsy.

The dose of myricitrin used in the present study did not induce catalepsy or alter spontaneous locomotor activity, which is consistent with previous studies that used a similar dose range (Fernandez et al., 2009; Meotti et al., 2006a). No specific or systematic study has investigated the potential side effects of myricitrin. However, the studies cited earlier did not find any overt signs of toxicity after acute administration. Meotti et al. (2006a) did not observe a decrease in locomotor activity, changes in body temperature, or any other adverse side effects of myricitrin administration (30–100 mg/kg). No sedative effects were seen in the elevated plus maze, locomotor activity test, and horizontal wire test (Fernandez et al., 2009).

The overall behavioral profile of myricitrin was similar to the atypical antipsychotic olanzapine in models that evaluated positive symptoms (i.e., apomorphine-induced stereotypy and climbing and the paw test) and catalepsy (i.e., catalepsy test and paw test). Considering the behavioral tests employed here, atypical antipsychotics are proposed to have less of an effect on apomorphine-induced stereotypy, FRT in the paw test, and catalepsy than typical antipsychotics (Bardin et al., 2006; Geyer and Ellenbroek, 2003). Both typical and atypical antipsychotics are effective in apomorphine-induced climbing and HRT in the paw test. The present results partially support this proposal. Olanzapine also blocked apomorphine-induced stereotypy. Interestingly, these results are consistent with the idea that striatal D_2 blockade is correlated with improvements in positive symptoms in schizophrenia patients (Agid et al., 2007), and stereotypy induced by dopamine agonists is related to D_2 receptor activation in the striatum (Porsolt et al., 1993, 2010). Moreover, climbing behavior was suppressed by striatal lesions (Protais et al., 1976), and a correlation was found between D_2 striatal receptor blockade and apomorphine-induced climbing (Assié et al., 2006). However, although the present results suggest an atypical profile for myricitrin, the models used here did not evaluate the negative symptoms of schizophrenia (Porsolt et al., 2010), which is an important aspect of atypical antipsychotics.

5. Conclusion

In conclusion, myricitrin showed antipsychotic-like effects in animal models (i.e., apomorphine-induced climbing and stereotypy and the paw test) at doses that did not induce catalepsy or alter locomotor activity, suggesting that myricitrin may be a potential drug treatment for the positive symptoms of schizophrenia.

Conflict of interest

All authors declare that they have no conflicts of interest.

Contributors

Roberto Andreatini and Adair RS Santos proposed the study; Marcela Pereira, Maria ABF Vital and Roberto Andreatini designed most of the study protocol; Marcela Pereira, Diego Correia, Isadora P Siba, and Lea R Chioca conducted all experiments; Moacir G Pizzolatti isolated and identified myricitrin; all authors contributed for data analysis and interpretation, wrote the manuscript and approved the final article.

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