Withania somnifera influences MDMA-induced hyperthermic, cognitive, neurotoxic and neuroinflammatory effects in mice

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A R T I C L E   I N F O

A B S T R A C T

Withania somnifera (WS) is utilized in Ayurvedic medicine owing to its central and peripheral beneficial properties. Several studies have accrued indicating that the recreational amphetamine-related drug $(+/-)-$ 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) targets the nigrostriatal dopaminergic system in mice, inducing neurodegeneration and gliosis, causing acute hyperthermia and cognitive impairment. This study aimed to investigate the effect of a standardized extract of *W. somnifera* (WSE) on MDMA-induced neurotoxicity, neuroinflammation, memory impairment and hyperthermia. Mice received a 3-day pretreatment with vehicle or WSE. Thereafter, vehicle- and WSE-pretreated mice were randomly divided into four groups: saline, WSE, MDMA alone, WSE plus MDMA. Body temperature was recorded throughout treatment, and memory performance was assessed by a novel object recognition (NOR) task at the end of treatment. Thereafter, immunohistochemistry was performed to evaluate in the substantia nigra pars compacta (SNc) and striatum the levels of tyrosine hydroxylase (TH), as marker of dopaminergic degeneration, and of glial fibrillary acidic protein (GFAP) and TMEM119, as markers of astrogliosis or microgliosis, respectively. MDMA-treated mice showed a decrease in TH-positive neurons and fibers in the SNc and striatum respectively, an increase in gliosis and body temperature, and a decrease in NOR performance, irrespective of vehicle or WSE pretreatment. Acute WSE plus MDMA counteracted the modifications in TH-positive cells in SNc, GFAP-positive cells in striatum, TMEM in both areas and NOR performance, as compared to MDMA alone, while no differences were observed as compared to saline. Results indicate that WSE acutely administered in combination with MDMA, but not as pretreatment, protects mice against the noxious central effects of MDMA.

1. Introduction

Withania somnifera (L.) Dunal (WS) (fam. Solanaceae), commonly known as Ashwagandha, is a medicinal plant widely used in Ayurveda, the traditional medicine heavily practiced in India and Nepal, for the treatment of various pathological conditions. Over the last decades, several experimental studies have characterized the pharmacological properties of *Withania somnifera* (L.) Dunal root extract (WSE) in models of neurodegenerative diseases, such as Alzheimer's disease (AD) [1–3] and Parkinson's disease (PD) [4–6], but also in experimental animals

exposed to drugs of abuse [7–9]. In addition, other experimental studies have demonstrated the efficacy of WSE in models of cancer, rheumatoid arthritis, stress and systemic inflammation [10–14].

AD is a neurological disorder characterized by the gradual dysfunction of limbic brain areas and gliosis [15]. These changes result in the development of the typical symptomology of AD, characterized by a progressive cognitive impairment [15]. Studies employing preclinical models of AD showed that various active constituents of WS can restore a physiological cortical and hippocampal cholinergic neurotransmission [1], reduce neuroinflammatory markers [3], and improve memory [1, 3]. Moreover, studies in patients with mild cognitive impairment revealed that WS may mitigate cognitive deficits and improve memory [2]. The mechanisms proposed for these therapeutic effects of WS include decreased nitric oxide synthesis, decreased lipid peroxidation, and increased bioavailability of antioxidant agents, such as glutathione [3].

PD is a chronic neurodegenerative disorder, characterized by a slow and progressive degeneration of dopaminergic neurons located in the substantia nigra pars compacta (SNc) that project to the striatum, mitochondrial dysfunctions, and nigrostriatal microgliosis [16]. Although neurotransmitters other than dopamine are also affected in PD, the loss of dopamine transmission within the striatum is the primary cause accounting for the characteristic motor symptoms of the disease [16]. Potential neuroprotective effects of various active constituents of WS have been studied in toxin-induced animal models of PD [4,17], together with their effects on mitochondrial dysfunctions [5,18], neuroinflammation [17], and motor impairment [6,17,18]. Moreover, studies in PD patients have revealed that WS administration caused a complete remission of treatment-refractory restless leg syndrome [19]. The mechanisms proposed for the observed therapeutic effects of WS in experimental models of PD and parkinsonian patients, similarly to those proposed for studies of AD, involve an enhanced bioavailability of antioxidant agents (i.e. glutathione) and an increased activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase [4,6,17,18].

Drugs of abuse exert their effects by activating the central reward circuits and by modifying neuronal functions, leading to neurochemical and behavioral alterations [20]. Notably, WS has been shown to counteract some of the central effects of drugs of abuse in animal models of addiction. In this regard, our group extensively investigated the influence of WSE on the effects of morphine and alcohol. Hence, administration of WSE to mice reduced the severity of the accumbal spine density alterations related to morphine-withdrawal syndrome [8], and prevented the acquisition and expression of morphine-elicited place conditioning, an established model used to assess motivation in rodents [21]. In addition, WSE has been found to prolong analgesia and to suppress hyperalgesia in morphine-treated mice [22]. Similar to what observed in morphine-treated mice, WSE administration reduced the motivation for alcohol drinking, the alcohol deprivation effect, and the reinstatement of alcohol-seeking behaviour evaluated with the operant self-administration paradigm [23]. Moreover, WSE impaired the acquisition and expression of alcohol-elicited place conditioning as well as the phosphorylation of the extracellular signal-regulated kinase in the shell of the nucleus accumbens of mice [9,24], and prevented both morphine- and alcohol-elicited mesolimbic increases in dopamine transmission in rats [7].

Prolonged exposure to drugs of abuse such amphetamine and the amphetamine-related drug (+/-)-3,4-methylendioxymethamphetamine (MDMA; "Ecstasy") may induce oxidative stress, mitochondrial dysfunctions, neurodegeneration, and neuroinflammation [25–33] in several brain areas that are typically associated with cognitive and memory deficits [20,34]. A limited number of studies have evaluated the possible beneficial influence of WS on the neurotoxic and neuroinflammatory effects elicited by amphetamine-related drugs. To the best of our knowledge, only a study by Kurapati and colleagues performed in human neuronal SK-N-MC cells [35] demonstrated neuroprotective

properties of WS, by showing that WS prevented the increase of microtubule-associated protein 2 levels, a marker of neurodegeneration, induced by methamphetamine [35].

Hence, the study of the beneficial effect of WS in experimental models of neurotoxicity induced by amphetamine-related drugs is of particular interest. In this regard, MDMA is an intriguing amphetamine-related drug to be used as model to assess the neuroprotective properties of WS. Indeed, MDMA has abuse properties and, when administered to mice, it exerts central and peripheral toxic effects that include acute hyperthermia [31,33,36], memory impairment, neurotoxicity, and glial activation in the dopaminergic nigrostriatal and mesolimbic systems [26–30,36]; all these effects could possibly be modulated by WS.

On these bases, the present study evaluated the potential beneficial effects of WSE on MDMA-induced central toxicity. To this end, we used a previously validated schedule of MDMA treatment [27,30,31] where WSE was co-administered with MDMA and given as pretreatment before WSE and MDMA co-administration. In the SNc and striatum, we evaluated the presence of degeneration of dopaminergic neurons and fibers and of astrogliosis and microgliosis. Moreover, we evaluated the effects of WSE on body temperature and correlated it with results obtained in neurons and glial cells. Finally, the effects of WSE on memory performance was assessed by means of the novel object recognition (NOR) task. Results of the study show, for the first time, a wide beneficial effects of WSE on both neurotoxic and detrimental memory effects induced by an amphetamine-related drug. These results could also be relevant to humans, since the doses of WSE and MDMA used may be regarded as moderate/high, considering studies performed in humans [37,38].

2. Materials and methods

2.1. Animals

Adult male C57BL/6J mice weighing 20–23 g at the beginning of the experiments were purchased from Charles River (Charles River, Calco, Italy). All mice were housed in groups of 4/5 per cage, with food (standard mouse chow) and water available ad libitum, in temperature- and humidity-controlled rooms under a 12-h light/dark cycle (lights on 7:00 am). All experiments were conducted in accordance with the ARRIVE guidelines [39], with the European Community directives (2010/63/EU; L.276; 22/09/2010) and with the guidelines approved by the Ethics Committee of the University of Cagliari. Experiments were designed to minimize animal pain and discomfort to the least possible extent and to reduce the number of mice used.

2.2. Drugs

WSE extract (kindly provided by Natural Remedies Pvt. Ltd., Bangalore, India; Batch No. PC/FWS1701003) was solubilized in saline and administered intraperitoneally (i.p.) in a volume of 10 ml/kg as described elsewhere [9,21,22,24]. MDMA was synthesized, solubilized, and administered by the i.p. route in a volume of 10 ml/kg, as described elsewhere [27,30,40]. The doses of WSE and MDMA were both selected on the basis of our previous studies [8,21,23,27,30,40].

2.3. Drug pretreatment and treatment

At the beginning of the experiments, mice received a 3-day pretreatment with vehicle or WSE (100 mg/kg, twice a day, i.p.) with a 12-hour interval. On the following day, both vehicle- and WSE-pretreated mice were randomly assigned to four treatment groups: 1) saline; 2) WSE (100 mg/kg, twice a day, 4-hour interval, i.p.); 3) MDMA alone (20 mg/kg, four times, 2-hour intervals, i.p.); 4) WSE (100 mg/kg, twice a day, 1 h before the first and third MDMA administration, i.p.) plus MDMA (20 mg/kg, four times, 2-hour intervals, i.p.).

2.3.1. Sacrifice and tissue preparation

Two days after the treatment, mice were deeply anesthetized and transcardially perfused. The time of sacrifice was selected based on our previous experiments showing that such a schedule of MDMA administration induces neuroinflammation and neurodegeneration in the nigrostriatal dopaminergic system of mice [31,40].

Then the brain were removed and serial coronal sections (50 μm thick) were cut from each of the two brain regions of interest at the following coordinates: from − 2.92 to − 3.52 mm (SNc) and from 1.34 to 0.74 mm (striatum), relative to bregma, according to the mouse brain atlas of Paxinos and Franklin [41].

2.3.2. Reaction protocols and Nissl staining

For diaminobenzidine visualization of TH-positive dopaminergic neurons and fibers, after pre-incubation of the sections in a blocking solution, the polyclonal rabbit anti-TH (1:1000, Millipore, Temecula, CA, USA) primary antibody was used. After the incubation with the primary antibody was completed, sections were incubated with the biotinylated secondary antibody goat anti-rabbit (1:500, Jackson ImmunoResearch Europe, Suffolk, UK) and the avidin–biotin–peroxidase complex protocol was followed [42].

For immunofluorescent staining of astroglial (GFAP-positive) and microglial (TMEM119-positive) cells, after pre-incubation of the sections in a blocking solution, the following unconjugated primary antibodies were used: monoclonal mouse anti-GFAP (1:1000, Sigma-Aldrich, Milan, Italy) and monoclonal rabbit anti-TMEM119 (1:500, Abcam, Cambridge, UK). Then, sections were incubated with the secondary antibody (AlexaFluor® 488-labeled donkey anti-mouse or anti-rabbit IgG, both 1:400, Jackson ImmunoResearch Europe, Suffolk, UK). Thereafter, sections were incubated with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride, 1:10,000, Sigma-Aldrich, Milan, Italy), to allow visualization of cell nuclei, and then mounted onto gelatine-coated glass slides. Omission of either the primary or secondary antibodies served as negative control and yielded no labeling (data not shown). An additional set of SNc sections was rinsed in water and stained with Nissl staining to evaluate cell death in this area [40].

2.3.3. Image analysis in the SNc and striatum

Stereological analysis of the total number of TH-positive neurons in the SNc was carried out, as previously described, using the software Stereologer [43].

For the analysis of TH immunoreactivity in the striatum, the density of immunoreacted fibers was determined quantitatively using the ImageJ software (U.S. National Institutes of Health, USA). The final values were expressed as a percentage of saline-treated mice [40].

For Nissl staining analysis in the SNc, images were counted manually using the ImageJ software [40].

The number of GFAP- and TMEM119-positive cells, co-labeled with the nuclear marker DAPI, was counted manually for each level of the SNc and striatum using the ImageJ software [29].

2.3.4. Temperature recording

Baseline temperature was recorded using a rectal probe (BRET-3) digital thermometer (MicroTherma 2 T Hand Held Thermometer, 2Biological Instruments, Besozzo, Varese, Italy), prior to the first drug administration (groups 1–4), to ascertain whether differences in this parameter occurred among the experimental groups. Then, temperature was recorded 1 h after each administration of either MDMA or saline.

2.3.5. NOR task

Groups 1–4 were evaluated for their NOR performance. The experimental procedure consisted of three phases: habituation to the test cage for 5 min (S0), performed the day after the completion of the drug treatments (groups 1–4); acquisition (S1) and testing (S2), performed the day after S0. Mouse performance was videotaped, and the following parameters were evaluated: a) the total amount of time spent by each mouse in exploring the objects during S1 and S2; and b) the percentage of time spent exploring the novel object over the total amount of time spent in exploring both objects (novel and familiar) during S2 [29,44]. Immediately after the completion of S2, mice were sacrificed.

2.4. Statistics

Data regarding the effects on body temperature were analyzed by means of a repeated-measures four-way analysis of variance (ANOVA) (pretreatment × treatment × effect × time). Data obtained from the NOR task and immunohistochemistry evaluations were analyzed by means of a three-way ANOVA (pretreatment × treatment × effect). ANOVAs were followed by Tukey's or Newman–Keuls post hoc test, where appropriate. Moreover, Pearson's test was used to ascertain the presence of a significant correlation between body temperature (calculated as the average value over the four drug administrations) and the intensity of dopaminergic degeneration or astroglial/microglial activation. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK, USA) or Prism (GraphPad, La Jolla, CA, USA). Results were considered significant at $p < 0.05$, and the results are expressed as mean $\pm$ standard error of the mean (S.E.M.) for every analysis performed.

3. Results

3.1. Immunoreactivity for TH in the SNc and striatum and Nissl staining in the SNc

Treatment with MDMA reduced the number of TH-positive neurons in the SNc and the density of TH-positive fibers in the striatum. In both the SNc and striatum, three-way ANOVA did not reveal significant effects of pretreatment. In the SNc, three-way ANOVA revealed significant effects of WSE ($F_{1,45} = 36.07$, $p < 0.001$) and MDMA ($F_{1,45} = 30.30$, $p < 0.001$) treatments, as well as a significant WSE treatment × MDMA treatment interaction ($F_{1,45} = 33.70$, $p < 0.001$). In the striatum, three-way ANOVA revealed a significant effect of MDMA treatment ($F_{1,46} = 56.74$, $p < 0.001$). Number of mice = SNc, 6–8 per group; striatum, 6–9 per group.

In the SNc, Tukey post hoc test revealed that the total number of TH-positive neurons was reduced by MDMA-alone administration in both vehicle- and WSE-pretreated mice compared with the respective saline group (*** $p = 0.000138$ for vehicle-pretreated mice, *** $p = 0.000175$ for WSE-pretreated mice, Fig. 1) and with the respective WSE plus MDMA-treated group (### $p = 0.000131$ for vehicle-pretreated mice, ### $p = 0.000142$ for WSE-pretreated mice, Fig. 1). In addition, MDMA induced a decrease in the number of Nissl-positive cells (−19%) compared with saline-treated mice (data not shown). No significant differences in the number of Nissl-positive cells were observed in WSE- or WSE plus MDMA-treated mice compared with saline (data not shown).

In the striatum, Tukey post hoc test revealed that the density of TH-positive fibers was reduced by MDMA-alone and WSE plus MDMA administration in both vehicle- and WSE-pretreated mice compared with the respective saline group (MDMA-alone: * $p = 0.008177$ for vehicle-pretreated mice, * $p = 0.01073$ for WSE-pretreated mice; WSE plus MDMA: * $p = 0.007466$ for vehicle-pretreated mice, *** $p = 0.0008$ for WSE-pretreated mice, Table 1).

3.2. Immunoreactivity for GFAP in the SNc and striatum

Treatment with MDMA increased the number of GFAP-positive cells in the striatum. In both the SNc and striatum, three-way ANOVA did not reveal significant effects of pretreatment. In the SNc, three-way ANOVA revealed a significant effect of MDMA treatment ($F_{1,40} = 17.64$, $p < 0.001$). In the striatum, three-way ANOVA revealed significant effects of WSE ($F_{1,58} = 5.93$, $p < 0.05$) and MDMA ($F_{1,58} = 18.41$, $p < 0.001$) treatments, as well as a significant WSE treatment × MDMA
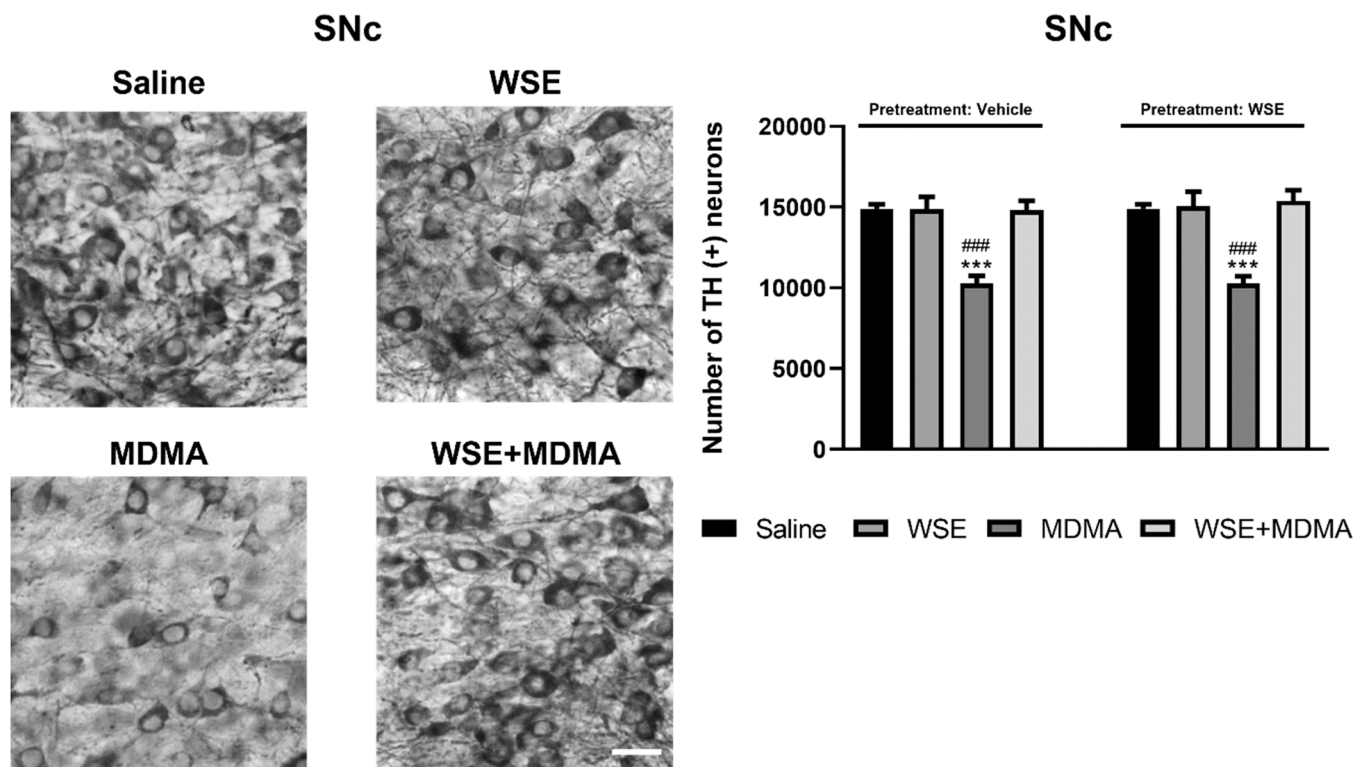
Fig. 1. Effect of administration of (+/-)−3,4-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the immuno-reactivity for tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNc) of vehicle- or WSE-pretreated mice. Number of mice = 6–8 per group. Representative sections of the SNc immunostained for TH of vehicle-pretreated mice and histogram of the number of TH-positive cells in the SNc. The total number of TH-positive neurons, calculated with stereological analysis, is expressed as mean ± S.E.M. *** p < 0.001 compared with the respective saline group; ### p < 0.001 compared with the respective WSE plus MDMA-treated group by Tukey post hoc test. Scale bar: 50 μm.

Table 1

Effect of administration of (+/-)−3,4-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the immunoreactivity for tyrosine hydroxylase (TH) in the striatum of vehicle- or WSE-pretreated mice.

Pretreatment	Treatment	TH density
Vehicle	Saline	99.99 ± 3.42
Vehicle	WSE	97.99 ± 2.74
Vehicle	MDMA	66.06 ± 9.88 *
Vehicle	WSE+MDMA	66.93 ± 7.60 *
WSE	Saline	99.69 ± 0.41
WSE	WSE	97.93 ± 4.59
WSE	MDMA	65.40 ± 9.69 *
WSE	WSE+MDMA	59.24 ± 7.09 ***

Number of mice = 6–9 per group. The values are expressed as a percentage of the respective saline group. * p < 0.05, *** p < 0.001 compared with the respective saline group.

treatment interaction ($F_{1,58}$ = 5.73, p < 0.05). Number of mice = SNc, 6 per group; striatum, 6–10 per group.

In the SNc, Tukey post hoc test failed to reveal any significant effect (Fig. 2C).

In the striatum, Tukey post hoc test revealed that the number of GFAP-positive cells was increased by MDMA-alone administration in both vehicle- and WSE-pretreated mice compared with the respective saline group (* p = 0.0402 for vehicle-pretreated mice, * p = 0.0217 for WSE-pretreated mice, Fig. 2A and B).

3.3. Immunoreactivity for TMEM119 in the SNc and striatum

Treatment with MDMA increased the number of TMEM119-positive cells in both the SNc and the striatum. In both areas, three-way ANOVA

did not reveal significant effects of pretreatment. In the SNc, three-way ANOVA revealed significant effects of WSE ($F_{1,40}$ = 4.93, p < 0.05) and MDMA ($F_{1,40}$ = 27.67, p < 0.001) treatments, as well as a significant WSE treatment × MDMA treatment interaction ($F_{1,40}$ = 5.39, p < 0.05). In the striatum, three-way ANOVA revealed a significant effect of MDMA treatment ($F_{1,39}$ = 23.31, p < 0.001). Number of mice = SNc, 6 per group; striatum, 6–10 per group.

In the SNc, Tukey post hoc test revealed that the number of TMEM119-positive cells was increased by MDMA-alone administration in vehicle-pretreated mice compared with the respective saline group (*** p = 0.0004, Fig. 3). Moreover, MDMA-alone-treated mice, pretreated with vehicle, showed a trend towards an increase in TMEM119 immunoreactivity in the SNc compared with the respective WSE plus MDMA-treated group (p = 0.063, Fig. 3).

In the striatum, Tukey post hoc test revealed that the number of TMEM119-positive cells was increased by MDMA-alone administration in both vehicle- and WSE-pretreated mice compared with the respective saline group (* p = 0.0481 for vehicle-pretreated mice, * p = 0.0292 for WSE-pretreated mice, Fig. 4).

3.4. Body temperature

Body temperature recordings, performed before the beginning of the treatments (groups 1–4), revealed no differences in the basal values of body temperature among the experimental groups. Moreover, vehicle or WSE treatment failed to induce any change in body temperature compared with basal values.

Repeated-measures four-way ANOVA revealed significant effects of MDMA treatment ($F_{1,45}$ = 431.11, p < 0.001) and number of MDMA administrations ($F_{4,180}$ = 74.76, p < 0.001). Moreover, repeated-measures four-way ANOVA revealed the following significant interactions: MDMA treatment × number of MDMA administrations
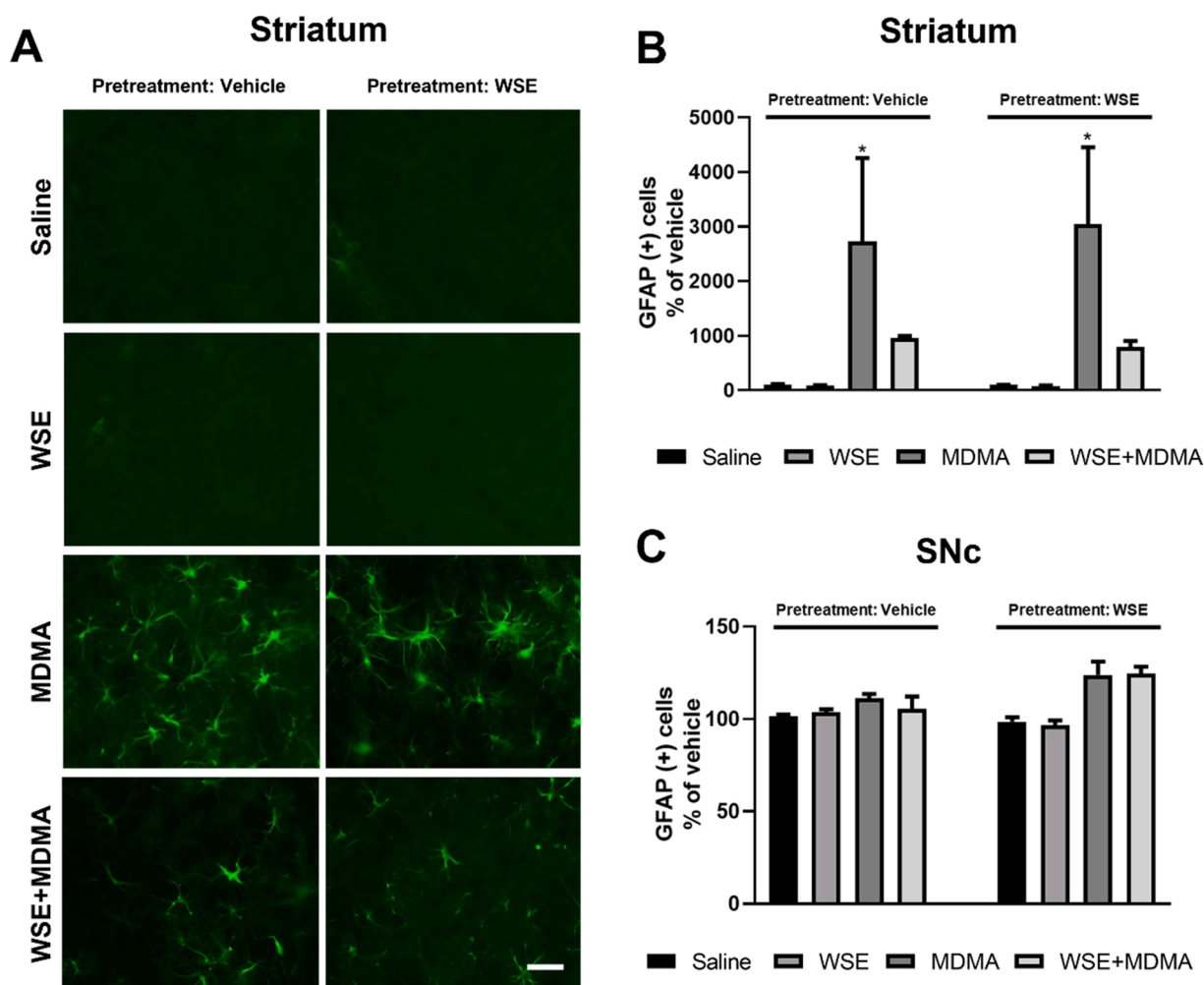
Fig. 2. Effect of administration of $(+/-)-3,4$-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the immuno-reactivity for glial fibrillary acidic protein (GFAP) in the substantia nigra pars compacta (SNc) and in the striatum of vehicle- or WSE-pretreated mice. Number of mice = 6 per group (SNc), 6-10 per group (striatum). (A) Representative sections of the striatum immunostained for GFAP and histogram of the number of GFAP-positive cells (B) in the striatum and (C) in the SNc. The number of GFAP-positive cells is expressed as mean ± S.E.M. * p < 0.05 compared with the respective saline group by Tukey post hoc test. Scale bar: 50 μm.

$(F_{4,180} = 114, p < 0.001)$; pretreatment $\times$ WSE treatment $\times$ MDMA treatment $(F_{1,45} = 6.61, p < 0.05)$; pretreatment $\times$ WSE treatment $\times$ number of MDMA administrations $(F_{4,180} = 7.67, p < 0.001)$; WSE treatment $\times$ MDMA treatment $\times$ number of MDMA administrations $(F_{4,180} = 4.64, p < 0.01)$; pretreatment $\times$ WSE treatment $\times$ MDMA treatment $\times$ number of MDMA administrations $(F_{4,180} = 6.9, p < 0.001)$. Number of mice = 7–12 per group.

Newman–Keuls post hoc revealed that MDMA-alone administration in both vehicle- and WSE-pretreated mice increased the body temperature after the first (* p = 0.036919 for vehicle-pretreated mice, *** p = 0.00075 for WSE-pretreated mice), second (*** p = 0.000033 for vehicle-pretreated mice, *** p = 0.00028 for WSE-pretreated mice), third (*** p = 0.000031 for vehicle-pretreated mice, *** p = 0.000031 for WSE-pretreated mice), and fourth (*** p = 0.000022 for vehicle-pretreated mice, *** p = 0.000035 for WSE-pretreated mice) administration, compared with the respective saline group (Table 2). Moreover, MDMA-alone-treated mice, pretreated with vehicle or WSE, showed a higher increase in body temperature after the third administration compared with the respective WSE plus MDMA-treated group (# p = 0.039584 for vehicle-pretreated mice, #p = 0.023958 for WSE-pretreated mice, Table 2). Furthermore, MDMA-alone-treated mice, pretreated with WSE, also showed a higher increase in body temperature after the fourth administration compared with the respective WSE plus

MDMA-treated group (### p = 0.000013, Table 2).

Newman–Keuls post hoc also revealed that WSE plus MDMA-treated mice, pretreated with vehicle or WSE, showed a higher increase in body temperature after the first (*** p = 0.000013 for vehicle-pretreated mice, ** p = 0.0013 for WSE-pretreated mice), second (*** p = 0.000043 for vehicle-pretreated mice, *** p = 0.000027 for WSE-pretreated mice), third (*** p = 0.000028 for vehicle-pretreated mice, *** p = 0.000036 for WSE-pretreated mice), and fourth (*** p = 0.000022 for vehicle-pretreated mice, *** p = 0.000022 for WSE-pretreated mice) administration, compared with the respective saline group (Table 2). Moreover, WSE plus MDMA-treated mice pretreated with vehicle, displayed a higher increase in body temperature after the fourth administration compared with WSE plus MDMA-treated mice pretreated with WSE ($ p = 0.003317, Table 2).

Interestingly, Pearson's test disclosed positive correlations between the increase in body temperature and both dopamine neuron degeneration and glial activation. Regarding dopamine neuron degeneration, a positive correlation was found in the SNc (Pearson's r coefficient: -0.5026; r square: 0.2527; p = 0.0123), but not in the striatum (p > 0.05). Moreover, a positive correlation was found between body temperature and astroglial cell activation in the striatum (Pearson's r coefficient: 0.9738; r square: 0.9482; p < 0.001), but not in the SNc (p > 0.05). Finally, a positive correlation was found between body
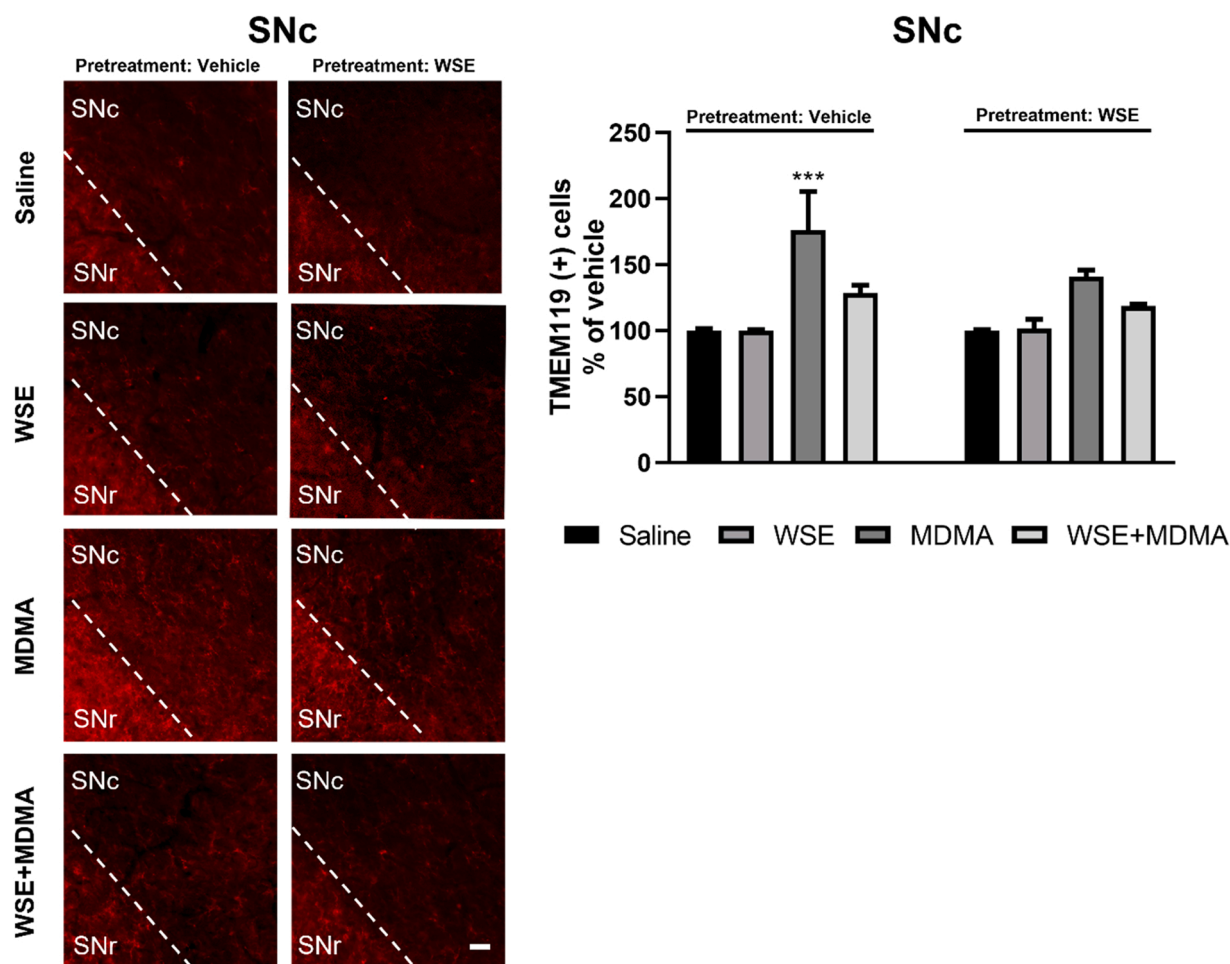
Fig. 3. Effect of administration of $(+/-)-3,4$-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the immuno-reactivity for TMEM119 in the substantia nigra pars compacta (SNc) of vehicle- or WSE-pretreated mice. Mice = 6 per group. Representative sections of the SNc immunostained for TMEM119 and histogram of the number of TMEM119-positive cells in the SNc. The number of TMEM119-positive cells is expressed as mean ± S. E.M. *** $p < 0.001$ compared with the respective saline group by Tukey post hoc test. SNr, substantia nigra pars reticulata. Scale bar: 50 μm.

temperature and microglial cell activation in the SNc (Pearson's r co-efficient: 0.5707; r square: 0.3257; p = 0.0036), but not in the striatum (p > 0.05).

3.5. NOR performance

Treatment with MDMA altered the NOR performance in both vehicle- and WSE-pretreated mice. Three-way ANOVA did not reveal significant effects of pretreatment. On the other hand, three-way ANOVA revealed significant effects of WSE ($F_{1,50} = 36.83$, p < 0.001) and MDMA ($F_{1,50} = 23.05$, p < 0.001) treatments, as well as a signifi-cant WSE treatment × MDMA treatment interaction ($F_{1,50} = 5.86$, p < 0.05). Number of mice = 6–10 per group.

Newman–Keuls post hoc test indicated that MDMA-alone adminis-tration in both vehicle- and WSE-pretreated mice impaired NOR per-formance compared with the respective saline group (*** p = 0.000694 for vehicle-pretreated mice, * p = 0.036223 for WSE-pretreated mice, Table 3) and with the respective WSE plus MDMA-treated group ([###] p = 0.000147 for vehicle-pretreated mice, [#] p = 0.015233 for WSE-pretreated mice, Table 3).

In all the NOR experiments performed, no significant differences in the total amount of time spent exploring the objects during S1 and S2 were observed among the various experimental groups (data not shown).

4. Discussion

Several preclinical and clinical findings indicate that WS may induce a series of beneficial effects in a wide range of neurological disorders [1–6]. Interestingly, rodent studies from our group have previously demonstrated the efficacy of WSE in contrasting alterations associated not only with neurological disorders [1–6], but also with the exposure to addictive drugs, such as morphine and alcohol. Consistent with this, rodents treated with WSE displayed a reduction in the behavioral and neurochemical alterations induced by morphine or alcohol administra-tion [7–9]. These findings indicate that WSE positively affects brain function and led us to hypothesize that it could also contrast the detri-mental effects elicited in the brain by amphetamine-related drugs endowed with neurotoxic and neuroinflammatory properties. The re-sults of the present study support this hypothesis, by demonstrating that adult C57BL/6J male mice treated with an acute WSE administration in combination with MDMA displayed a reduction of MDMA-induced hy-perthermia and memory deficits, as well as a decrease of MDMA-induced nigral dopamine neuron degeneration, astrogliosis, and microgliosis.

In line with earlier reports, we observed that administration of MDMA-alone decreased the total number of TH-positive neurons in the SNc [40,45], compared with mice administered either saline or WSE plus MDMA. This decrease was manifested irrespective of the pretreat-ment with WSE. These findings indicate that acute WSE may protect the SNc from the dopaminergic damage induced by MDMA, and substanti-ate our hypothesis that WSE administration may be beneficial in
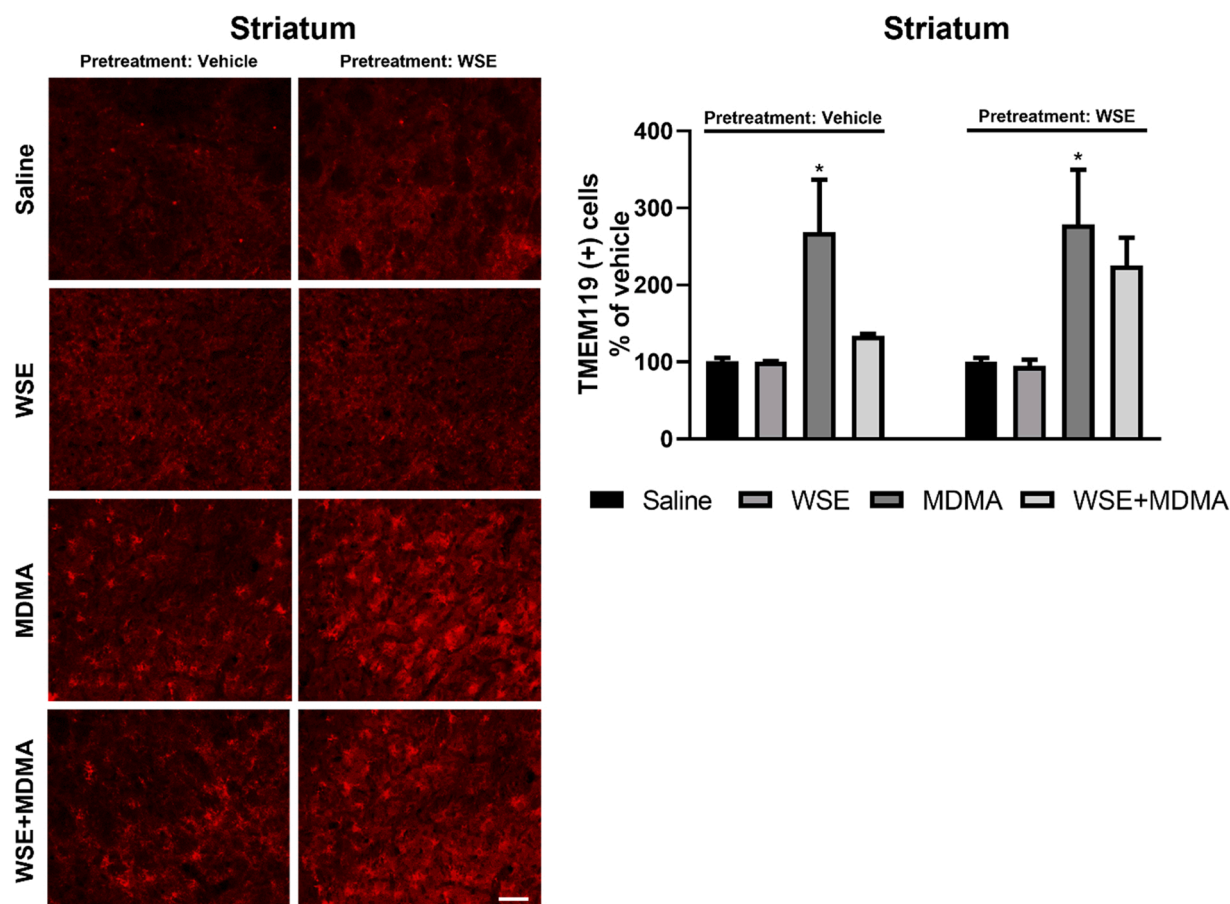
Fig. 4. Effect of administration of $(+/-)-3,4$-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the immuno-reactivity for TMEM119 in the striatum of vehicle- or WSE-pretreated mice. Number of mice = 6-10 per group. Representative sections of the striatum immuno-stained for TMEM119 and histogram of the number of TMEM119-positive cells in the striatum. The number of TMEM119-positive cells is expressed as mean ± S.E.M. * $p < 0.05$ compared with the respective saline group by Tukey post hoc test. Scale bar: 50 μm.

Table 2

Effect of administration of $(+/-)-3,4$-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) on the body temperature of vehicle- or WSE-pretreated mice.

Time	Pretreatment: Vehicle				Pretreatment: WSE			
	Saline	WSE	MDMA	WSE+MDMA	Saline	WSE	MDMA	WSE+MDMA
Basal	36.48 ± 0.14	36.5 ± 0.12	36.2 ± 0.05	36.54 ± 0.12	36.37 ± 0.1	36.08 ± 0.06	36.6 ± 0.10	36.2 ± 0.05
60'	36.56 ± 0,12	36.57 ± 0.11	37.26 ± 0.09*	37.51 ± 0.08***	36.23 ± 0.13	36.05 ± 0.11	37.36 ± 0.14***	37.26 ± 0.09**
120'	36.34 ± 0.12	36.34 ± 0.10	38.02 ± 0.18***	38.05 ± 0.08***	35.82 ± 0.21	36.6 ± 0.06	38.05 ± 0.19***	38.02 ± 0.18***
180'	36.03 ± 0.14	36.01 ± 0.13	39.2 ± 0.22***#	38.72 ± 0.13***	35.88 ± 0.14	36.58 ± 0.13	38.74 ± 0.19***#	39.2 ± 0.22***
240'	36.06 ± 0.10	36.02 ± 0.09	38.2 ± 0.09***	38.44 ± 0.25***$	35.83 ± 0.05	36.63 ± 0.08	38.92 ± 0.35***###	37.7 ± 0.15***

Number of mice = 7–12 per group. Body temperature values are reported as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the respective saline group; # $p < 0.05$, ### $p < 0.001$ compared with the respective WSE plus MDMA-treated group; $ $p < 0.05$ compared with WSE plus MDMA-treated mice pretreated with WSE, by Newman–Keuls post hoc test.

contrasting the pathological alterations that follow the exposure to amphetamine-related drugs. Moreover, these results are in line with those obtained in animal models of PD, showing that WSE protected nigral neurons from the dopaminergic neurotoxin 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) [6]. In this regard, it is interesting to note that it was previously suggested that MDMA and MPTP may share similar mechanisms of neurotoxicity, such as the selective inhibition of mitochondrial complex I in nigrostriatal dopaminergic neurons [25,46]. Indeed, the inhibition of mitochondrial complex I leads to an increased production of reactive oxygen species (ROS) responsible for oxidative stress [47] and, in turn, for neurotoxicity [26,27,48]. As mentioned in the introduction, WSE may increase the activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase [1,3,

4,6,18,49]. Hence, it could be possible that the lack of decrease in TH-positive neurons in mice treated with WSE plus MDMA may be a consequence of the WSE-elicited activation of antioxidant enzymes. The finding that TH immunoreactivity in the striatum is insensitive to the neuroprotective effects of acute WSE may depend, as suggested else-where [32], on the fact that MDMA only induces a partial depletion of dopaminergic fibers in this brain area.

To further characterize the interplay between WSE and the noxious effects of MDMA in the nigrostriatal system, we also evaluated astroglia and microglial activation. In agreement with our earlier reports [30,40], MDMA increased the number of GFAP-positive cells in the striatum, but not in the SNc. Interestingly, the MDMA-induced increase in striatal GFAP immunoreactivity was not observed in WSE plus MDMA-treated

Table 3

Effect on novel object recognition (NOR) performance of administration of $(+/-)-3,4$-methylenedioxymethamphetamine (MDMA) given alone or with *Withania somnifera* extract (WSE) in vehicle- or WSE-pretreated mice.

Pretreatment	Treatment	Time exploring the novel object (%)
Vehicle	Saline	60.80 ± 2.95
Vehicle	WSE	72.30 ± 1.17
Vehicle	MDMA	34.71 ± 4.19 ***###
Vehicle	WSE+MDMA	69.10 ± 5.15
WSE	Saline	59.85 ± 2.60
WSE	WSE	68.43 ± 4.11
WSE	MDMA	46.21 ± 1.66 *#
WSE	WSE+MDMA	58.53 ± 5.05

Number of mice = 6–10 per group. Data show the mean $\pm$ S.E.M of the percentage of time spent exploring the novel object. * $p < 0.05$, *** $p < 0.001$ compared with the respective saline group; # $p < 0.05$, ### $p < 0.001$ compared with the respective WSE plus MDMA-treated group, by Newman–Keuls post hoc test.

mice. Regarding the underpinning mechanisms mediating such effect, it was previously shown that amphetamine-related drugs with neurotoxic potential share a common mechanism of astroglial activation with neurotoxins such as MPTP, that involves the activation of Janus kinase 2–signal transducer and activator of transcription (STAT) 3 pathway [50]. STAT3, as STAT1, another member of the STAT protein family, is an important mediator of astrocyte differentiation [51]. Interestingly, Atluri and colleagues [52] recently demonstrated a downregulation of STAT1 expression mediated by WSE administration in an in vitro model of AD, with a consequent decrease in neuroinflammation. Based on this evidence, we hypothesize that administration of MDMA alone may increase STAT3 signaling in the striatum thus leading to astrogliosis. The consequent astrogliosis may contribute to nigrostriatal dopamine neuron degeneration, whereas these events may be counteracted by WSE in mice treated with WSE plus MDMA.

On the other hand, as far as microgliosis is concerned and in line with previous results obtained with other markers, such as cluster of differentiation molecule 11b [40], and ionized calcium-binding adaptor protein-1 [30], administration of MDMA alone increased the number of TMEM119-positive cells in the SNc and striatum. Interestingly, when mice were pretreated with WSE and subsequently treated with MDMA alone, the increase in TMEM119 in the SNc was not observed, suggesting an anti-inflammatory role of WSE also when given before MDMA. Finally, treatment with WSE plus MDMA, with or without WSE pretreatment, did not increase TMEM119-positive cells in the SNc and striatum. Collectively, these results may indicate that WSE treatment concomitantly with MDMA exposure is able to inhibit microglial activation, as previously suggested by in vitro studies in models of β-amyloid and lipopolysaccharide administration [53]. Importantly, WSE has also been reported to decrease the release of proinflammatory mediators, such as interleukin (IL) 1β and IL-6, tumor necrosis factor-α, and ROS production [3,53,54]. The mechanism behind the anti-inflammatory effects of WSE seems to rely on the downregulation of elements which trigger the pro-inflammatory pathways, such as nuclear factor-κB/mitogen-activated protein kinase [53,54].

Collectively, the results obtained here in glial cells substantiate the hypothesis first suggested by Thomas and colleagues [55] on a role for microglial and astroglial activation in the neurotoxicity mediated by amphetamine-related drugs. Thus, several studies have demonstrated that astroglial and microglial activation may sustain neurodegeneration in the dopaminergic nigrostriatal system [56], and this mechanism has also been proposed to participate in the neurotoxic dopaminergic damage induced by MDMA [30].

Another interesting result of this study is the positive correlation between the elevation in body temperature and dopamine neuron degeneration or microgliosis in the SNc or astrogliosis in the striatum. These results support previous data showing that hyperthermia is a

factor promoting neurotoxicity and glial activation by MDMA [31,33, 36]. Interestingly, the rise in body temperature of mice treated with MDMA alone was significantly greater after the third administration in vehicle-pretreated mice and after the third and fourth administrations in WSE-pretreated mice, compared with mice treated with WSE plus MDMA and pretreated with either vehicle or WSE. Finally, the rise in the body temperature of mice treated with WSE plus MDMA was significantly reduced after the fourth administration in WSE-pretreated mice, compared with mice treated with WSE plus MDMA and pretreated with vehicle. This may mean that WSE mediates its neuroprotective and anti-neuroinflammatory effect also by contrasting the MDMA-induced increase in body temperature. To our knowledge, this is the first study that highlights a hypothermic effect of WSE when given concomitantly with substances able to increase the body temperature, such as MDMA. Importantly, WSE alone did not significantly alter the body temperature. Further investigations will be required to fully understand the importance of and the mechanisms behind this effect.

Lastly, in the attempt to link neurochemical evaluations with memory performance, we carried out the NOR task. Mice treated with MDMA alone showed a deficit in NOR performance, as already demonstrated in a previous study from our group [29]. Interestingly, the administration of WSE plus MDMA was able to fully contrast the MDMA-induced deficit in NOR, in both vehicle- and WSE-pretreated mice. Collectively, these data further corroborate those collected in AD models [1,3] that proposed a beneficial effect of WSE in the prevention of cognitive deficits, due to the ability of WSE to restore the cortical and hippocampal cholinergic neurotransmission.

The mechanisms by which WSE may have differentially affected the neurochemical and behavioral parameters evaluated in the present study, depending on whether it was administered as pretreatment or as a concomitant treatment is difficult to define from the present data, also in consideration of the multi-target action of WSE. Nonetheless, the observation that WSE exerted differential effects on the changes of number of TH-positive neurons or of GFAP and TMEM119 cell activation depending on the temporal contiguity with the pharmacological stimulus (MDMA administration) responsible for these effects, is in agreement with our previous observation that WSE reduced the severity of the withdrawal syndrome and prevented the reduction of spine density in the shell of the nucleus accumbens when given during chronic morphine administration, but not when administered during morphine withdrawal [8]. Overall, the present study has a major strength that it focused on both the preventive effect of WSE, when WSE was given as pre-treatment, and on the acute effect of WSE, when WSE was given in combination with MDMA. At the same time, the present study has the limitation that future studies are necessary to clarify the precise mechanisms for the neuroprotective, antineuroinflammatory and anti-hyperthermic effects mediated by WSE.

5. Conclusions

Experimental studies carried out on medicinal plants are numerous and increasing. Among medicinal plants, WS has recently emerged as rich in bioactive compounds, that have been extensively demonstrated beneficial in peripheral and central pathological conditions as well as in contrasting certain behavioral effects of drugs of abuse. Importantly, certain drugs of abuse, besides their abuse liability, may cause neurodegeneration, neuroinflammation and memory deficits, as amphetamine-related drugs like MDMA. Hence, it is crucial to conduct additional research to characterize the mechanisms underlying the central neurotoxic and neuroinflammatory effects of drugs of abuse, in order to develop novel medications for treating these toxic drug effects. The present study, by demonstrating that WSE prevents dopaminergic damage, and gliosis induced by MDMA in the nigrostriatal system of mice, strongly supports the importance of the research on protective remedies against substances with neurotoxic potential and pave the way for future investigations aimed at exploring the mechanisms responsible

for MDMA-induced neurotoxicity.

Conflict of interest statement

The authors declare no actual or potential conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

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