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Research paper

Pregnenolone for the treatment of L-DOPA-induced dyskinesia in Parkinson's disease

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ABSTRACT

Growing preclinical and clinical evidence highlights neurosteroid pathway imbalances in Parkinson's Disease (PD) and L-DOPA-induced dyskinesias (LIDs). We recently reported that 5α-reductase (5AR) inhibitors dampen dyskinesias in parkinsonian rats; however, unraveling which specific neurosteroid mediates this effect is critical to optimize a targeted therapy. Among the 5AR-related neurosteroids, striatal pregnenolone has been shown to be increased in response to 5AR blockade and decreased after 6-OHDA lesions in the rat PD model. Moreover, this neurosteroid rescued psychotic-like phenotypes by exerting marked antidopaminergic activity. In light of this evidence, we investigated whether pregnenolone might dampen the appearance of LIDs in parkinsonian drug-naïve rats.

We tested 3 escalating doses of pregnenolone (6, 18, 36 mg/kg) in 6-OHDA-lesioned male rats and compared the behavioral, neurochemical, and molecular outcomes with those induced by the 5AR inhibitor dutasteride, as positive control.

The results showed that pregnenolone dose-dependently countered LIDs without affecting L-DOPA-induced motor improvements. Post-mortem analyses revealed that pregnenolone significantly prevented the increase of validated striatal markers of dyskinesias, such as phospho-Thr-34 DARPP-32 and phospho-$ERK_{1/2}$, as well as D_1-D_3 receptor co-immunoprecipitation in a fashion similar to dutasteride. Moreover, the antidyskinetic effect of pregnenolone was paralleled by reduced striatal levels of BDNF, a well-established factor associated with the development of LIDs. In support of a direct pregnenolone effect, LC/MS-MS analyses revealed that striatal pregnenolone levels strikingly increased after the exogenous administration, with no significant alterations in downstream metabolites.

All these data suggest pregnenolone as a key player in the antidyskinetic properties of 5AR inhibitors and highlight this neurosteroid as an interesting novel tool to target LIDs in PD.

1. Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of the nigrostriatal pathway. Hallmark of pathology is the onset of severe motor symptoms, with gait and balance dysfunctions, resting tremors, and bradykinesia among others (Balestrino and Schapira, 2020). Despite many efforts directed at halting the degenerative process, the treatment is currently symptomatic.

The dopamine (DA) precursor L-DOPA (L-3,4-dihydroxyphenylalanine) still represents the gold standard therapy for PD, with tremendous impact on the patient's quality of life (Nagatsua and

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Sawadab, 2009). However, the progressive dopaminergic degeneration disrupts the fine-tuned regulation of L-DOPA-derived DA release, leading to swings in its extracellular concentration and consequent pulsatile stimulation of sensitized striatal DA receptors (Chase, 1998; Bibbiani et al., 2005; Olanow et al., 2006). As a result, over the years, patients under chronic L-DOPA medication display burdensome motor effects, named L-DOPA-induced dyskinesias (LIDs), a spectrum of motor complications featured by atypical, stereotyped, and purposeless hyperkinetic movements. About 90% of patients eventually experience some degree of LIDs after the first decade of L-DOPA treatment, with significant complications in disease management (Ahlskog and Muenter, 2001; Turcano et al., 2018). Despite the huge number of preclinical and clinical studies aimed at targeting LIDs, the NMDA receptor antagonist amantadine is the only FDA-approved medication for treating these motor complications. However, its efficacy is not devoid of common adverse effects, including confusion, paranoia, and hallucinations (Oertel et al., 2017; Elmer et al., 2018; Mehta et al., 2021). Hence, there is a compelling unmet need for new and more effective medications to treat the troublesome side effect of chronic L-DOPA medications while extending its beneficial effects.

Several studies have shown that neurosteroids, a class of endogenous steroids that are synthesized and active in the brain, modulate dopaminergic signaling within the striatum (Nuwayhid and Werling, 2003; Sánchez et al., 2010; Frau et al., 2015). Of note, these endogenous mediators are dysregulated in the brain, cerebrospinal fluid (CSF), and plasma of PD patients and PD animal models (di Michele et al., 2003; Luchetti et al., 2010; Melcangi et al., 2012).

In particular, the changes in neurosteroid biosynthesis appear to involve alterations of the 5AR and 3α-HSD pathways. Accordingly, two clinical studies reported significant reductions in plasma and CSF concentrations of the 5α- and 3α-reduced progesterone derivatives-THP and -DHP, respectively (di Michele et al., 2003). Moreover, in the 6-hydroxydopamine (6-OHDA) rodent model of PD, the striata of parkinsonian rats exhibited specific reductions not only of the 5AR-reduced metabolite DHP but also of pregnenolone (PREG) (Melcangi et al., 2012).

Accordingly, we have recently reported that inhibition of 5α-reductase (5AR), a critical enzyme in the synthesis of neurosteroids, exerts significant antidyskinetic effects in a rat model of PD (Frau et al., 2017a; Fanni et al., 2019). This effect was paralleled by the normalization of the phosphorylation levels of extracellular regulated kinases 1/2 ($ERK_{1/2}$) and the DA and cAMP-regulated phosphoprotein (DARPP-32), whose increased levels are well-established correlates of dyskinesia (Pavón et al., 2006; Santini et al., 2012). Moreover, we found a reduced interaction of DA D_1-D_3 receptors (D_1-D_3R), previously reported to be correlated with the severity of LIDs in parkinsonian rats (Farré et al., 2015; Fiorentini et al., 2015; Guitart et al., 2014; Solís and Moratalla, 2018; Solís et al., 2017). 5AR inhibitors have been instrumental in unraveling that neurosteroids play a role in the development of dyskinesias. Nonetheless, it would be of great relevance to identify which neurosteroid(s) underlies their antidyskinetic properties, in an attempt to find more selective treatments with reduced side effects. Interestingly, we previously reported a reduction in PREG concentration in the striata of 6-OHDA-lesioned rats (Melcangi et al., 2012). Moreover, inhibition of 5AR produces a robust increase in enzyme substrates, especially PREG (Frau et al., 2017b; Frau et al., 2015). PREG has also been shown to rescue several conditions characterized by the overactivation of dopaminergic signaling, such as psychotic-like states, drug addiction, and stress-related conditions (Frau et al., 2019; Vallée, 2016; Wong et al., 2015; Wong et al., 2012).

Considering the above observations, we investigated the effects of PREG in drug-naïve 6-OHDA-lesioned rats to evaluate its ability to dampen the development of LIDs and prevent the associated molecular alterations.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats ($n = 103$, 275–300 g, Envigo, Italy) were housed 3–4 per cage, under a 12-h light/dark cycle (light on from 7:00 to 19:00) in a controlled environment of temperature and humidity, and a regimen of ad libitum food and water.

The present study was carried out in accordance with the European Directive (EU 2010/63) and Italian D.Lgs (2014/26) and approved by the local ethical committee (OPBA) and the Italian Ministry of Health (n. 629/2020-PR).

2.2. Drugs

Animals underwent general anesthesia with a mixture (20:1) of fentanyl (Fentanest, Pfizer) plus the alpha-2 selective agonist medetomidine-chlorhydrate (Domitor, Orion Pharma) with a dose of 5 mL/kg (intraperitoneal, i.p.). In order to reach a complete lesion of the nigrostriatal tract, rats received a unilateral injection of 16 μg/4 μL of 6-OHDA-HCl (Santa Cruz Biotechnology, CAS 28094–15-7) dissolved in a solution of 0.1% of ascorbic acid in saline. 20 min before the surgery, animals were injected i.p. with the SSRI citalopram (0.25 mg/kg/mL) to avoid lesion of serotonergic neurons. After the surgery, anesthesia awakening was promoted by atipamezole (Antisedan, Orion Pharma) [0.31 mg/kg, subcutaneously (s.c.)]. L-DOPA methyl-ester (6 mg/kg, Carbosynth, UK) and benserazide (6 mg/kg, Carbosynth, UK) were daily prepared, dissolved in saline (0.9% NaCl) as a single solution, and given s.c..

PREG (Carbosynth, UK) was administered s.c. at the doses of 6, 18 or 36 mg/kg, whereas dutasteride (DUTA) was given i.p. at the dose of 15 mg/kg. Both drugs were formulated as a suspension in 5% Tween80 and 95% saline.

2.3. Stereotaxic surgery

All the animals were deeply anesthetized and placed in the stereotactic frame, before receiving a unilateral injection in the right Medial Forebrain Bundle (MFB), according to the following coordinates: AP -4.4, ML -1.2, AP -7.9 from the dura mater surface (Paxinos and Watson, 2006) with the tooth bar adjusted at −2.4.

16 μg of 6-OHDA (4 μg/μL) were locally delivered with a Hamilton syringe at a rate of 1 μL/min. The syringe was kept in place for further 2 min before slowly being retracted.

After the surgery, animals were placed in clean cages and daily monitored until they reached a complete recovery. Animals were allowed to recover for 3 weeks before the beginning of the behavioral tests (see Fig. S1.A for the experimental design).

2.4. Behavior analyses

2.4.1. Stepping test

The stepping test was performed after a 5-day training to assess the motor impairment on day −2 (prior to L-DOPA treatment) and day 20 (of L-DOPA treatment). The outcome of the first test was used to allocate animals into well-balanced groups, while the last time point allowed us to estimate possible interference of PREG on L-DOPA therapeutic effect. The test consists in the evaluation of the number of adjusting steps of an animal held by the operator with only one forelimb free to move, alternatively. The unrestrained paw was free to slide over a smooth surface of 90 cm in 5 s in two directions, as previously described (Tronci et al., 2013). The rating is expressed as percentage of the left forepaw use by means of two consecutive trials of the forehand and backhand directions. On day 20, the test was repeated at two time points: before L-DOPA administration (time 0, baseline), and 150 min later, when the beneficial effect of L-DOPA was still present, but dyskinesia wore off.

Testing at earlier time points was not possible due to the marked dyskinetic behavior.

2.4.2. Dyskinesia assessment

Abnormal Involuntary Movements (AIMs) were assessed at different time points to evaluate the antidyskinetic effect of PREG over time, according to a well-established procedure (Cenci and Lundblad, 2007; Lundblad et al., 2002; Tronci et al., 2013). Each animal was tested for 60 s every 20 min for the entire course of dyskinesias (120–140 min). AIMs were classified as forelimb, orolingual and axial dyskinesia, based on their topographical distribution. The score was assigned from 0 to 4 depending on how long the AIM subtype manifested (0: absent; 1: occasional, i.e., present <50% of the time; 2: frequent, i.e., present >50% of the time; 3: continuous but interrupted by strong sensory stimuli and 4: continuous, not interrupted by strong sensory stimuli). Total AIMs score is calculated as the sum of the three components.

2.4.3. Locomotor activity

The locomotor activity was assessed by 2 horizontal orthogonal sets of 16 photocells with infrared light beams (Omnitech Digiscan Animal Activity Monitor, Columbus, OH, USA). On the day of the test, animals were placed individually in 41x41x30 cm transparent plastic cages, immediately after the L-DOPA injection. Total distance and horizontal activity were monitored for 120 min and scored every 10 min for a total of 12 sessions.

2.5. Post-mortem evaluations

Animals were sacrificed 60 min after the last L-DOPA administration (100 min after PREG, DUTA, or Veh treatment) and brains were rapidly dissected. The striata were frozen in dry ice for subsequent biochemical studies and neurosteroid quantifications, while midbrains were immediately postfixed for immunohistochemical studies.

2.5.1. Immunohistochemistry

In order to appraise the extent of the lesion, midbrains were processed, and tyrosine hydroxylase (TH) immunoreactivity verified within the substantia nigra. Microtome-sectioned slices (40 μm) were incubated overnight with a rabbit anti-TH antibody (1:2000, Merck AB152). The day after, a secondary biotinylated anti-rabbit antibody (1:200, Vector BA-1000) was used over 1 h of incubation. Avidin-biotin complex (Vector PK6100) was exploited to amplify the reaction, and 3′3-diaminobenzidine chromophore was used in developing the color reaction. Only rats displaying a loss of TH^+ neurons higher than 80% in the right substantia nigra were included in the study (see supplementary figs. S1·C and S1·D).

2.5.2. Neurosteroids quantification

The levels of neurosteroids, such as PREG, progesterone (PROG), dihydro-progesterone (DHP), tetrahydro-progesterone (THP), iso-allopregnanolone, dehydroepiandrosterone (DHEA), testosterone (TESTO), dihydrotestosterone (DHT), 3α-diol, 17β-Estradiol were evaluated in the rat striatum. Tissues were extracted and purified as previously described (Pesaresi et al., 2010; Caruso et al., 2008). $^{13}C_3$-17β-Estradiol (2 ng/sample) $^{13}C_5$– PROG (0.4 ng/sample) and $^{13}C_2$ D_2– PREG (10 ng/sample) were used as internal standards.

The analysis was conducted by liquid chromatography (LC) supplied of Surveyor liquid chromatography Pump Plus and Surveyor Autosampler Plus (ThermoElectron Co., San Jose, CA, USA) with a linear ion trap - mass spectrometer (LTQ, ThermoElectron Co, San Jose, CA, USA) operated in positive atmospheric pressure chemical ionization (APCI+). The chromatographic separation was achieved with a Hypersil Gold column C18 (100 × 2.1 mm, 3 μm; ThermoFisher Scientific) was maintained at 40 °C. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0,1% formic acid in methanol (mobile phase B). Gradient elution was as follows: 0–1.50 min 70% A, 30% B; 1.50–2.00 min 55% A, 45%B; 2.00–3.00 min. 55% A, 45% B; 3.00–35.00 min. Linear gradient to 36% A, 64% B; 35.00–40.00 min. 25% A, 75% B; 41.00–45.00 min. 1% A, 99% B; 45.00–45.20 min. 70% A, 30% B and 45.40–55.00 min equilibrate with 70% A and 30% B. 25 μL sample was injected at a flow rate of 0.250 mL/min. The divert valve was set at 0–8 min to waste, 8–45 min to source and 45–55 min to waste. The injector needle was washed with MeOH/Water 1/1 (v/v).

Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using standards. Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) peaks were appraised using software Excalibur® release 2.0 SR2 (ThermoElectron Co, San Jose, CA, USA).

2.5.3. Western blotting

Proteins (30 μg) were separated on 4–15% TGX Stainfree Criterion precast gels (Bio-Rad Laboratories, Inc., Hercules, CA) and then transferred to nitrocellulose membranes (Scheggi et al., 2020). The membranes were incubated with the following primary antibodies: anti-D_1R and D_3R (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, #SC-33660, 1:1000 and #SC-9114, 1:500), anti-phospho p44/42 MAPK (p- $ERK_{1/2}$; Thr202/Tyr204, Cell SignalingTechnology; #4370, 1:1000), anti-p44/42 MAPK ($ERK_{1/2}$, Cell Signaling Technology, #4695, 1:1000), anti-phospho-Thr34 DARPP-32, DARPP-32 (Cell Signaling Technology, #12438; 1:1000 and #2306, 1:1000), and anti-BDNF (Abcam, ab216443). Specific antibody binding was detected by chemiluminescence with the ChemiDoc XRS+ Imager (Bio-Rad Laboratories). Samples from each treatment group were immunoblotted and analyzed together. To control for equal loading for D_1R and D_3R, $ERK_{1/2}$, DARPP-32, and BDNF expression, blots incubated with antibodies were stripped and reprobed using anti-β-actin (Sigma-Aldrich, St.Louis, MO, USA); for phospho- $ERK_{1/2}$ and phospho-Thr34 DARPP-32, blots were stripped and reprobed using anti total ERK or anti total DARPP-32 antibody, respectively. Bands were quantified in arbitrary units and normalized using the software Image Lab (Bio-Rad Laboratories) using β-actin, ERK, and DARPP-32 as loading controls.

2.5.4. Co-immunoprecipitation

Immunoprecipitation was performed using monoclonal D_1R antibody against the last 123C-terminal amino acids of rat DA D_1 receptor (D_1R) (SC-33660, Santa Cruz Biotechnology) as previously detailed (Fanni et al., 2019; Scheggi et al., 2020). Antibodies were coupled to protein A Dynabeads (Invitrogen) using 5 μg antibody by rotating the mixture for 10 min at room temperature. The immunoprecipitates were separated on a 4–15% TGX Criterion precast gel as previously described (Scheggi et al., 2020). The membranes were incubated with polyclonal anti-D_3R antibody (SC-9114, Santa Cruz Biotechnology). Chemiluminescence was detected and quantified with the ChemiDoc Imaging System (Bio-Rad Laboratories). Samples from control and treated rats were run on the same immunoblots and then analyzed together. Relative to contralateral striata, 5 randomly chosen samples have been subjected to molecular analysis, as this n generally provides reliable results, as reported previously (Fanni et al., 2019).

Band intensities of co-immunoprecipitated D_3 receptors (D_3R) were normalized to the band intensities of immunoprecipitated D_1R. Band intensities were quantified using Image Lab software/Gel Doc XRS+ system; values, expressed as arbitrary units, were then calculated as percentage of the Vehicle + Saline group values.

2.6. Experimental design

The timeline of the experiment is shown in Fig. S1.A. Due to the high number of animals, experiments were conducted in two rounds with the same AIMs scoring intervals; for this reason, here we present the pooled behavioral data. The 36 mg/kg dose was investigated during a second round of experiments as we questioned whether the antidyskinetic effect already reached a plateau with the 18 mg/kg pregnenolone dose.

Therefore, animals of the first round of experiments underwent LC-MS/MS analyses for the neurosteroid level quantifications, while animals of the second round underwent molecular investigations. For this reason, the effect of 36 mg/kg dose is only available for molecular analyses.

Animals underwent dopaminergic lesions as indicated above. Three weeks after surgical procedures, the stepping test was performed to establish the impact of the lesion on the contralateral forelimb functionality, as a measure of motor impairment (day −2, see Fig. S1. B). Thus, animals were randomly distributed into 6 balanced groups based on the stepping score. 6-OHDA-lesioned rats received single daily administrations with L-DOPA (plus the peripheral decarboxylase inhibitor benserazide) in combination with either a different PREG dose (6, 18, 36 mg/kg, s.c.), DUTA (15 mg/kg, i.p.), as a positive control, or vehicle. One more group of 6-OHDA-lesioned rats was treated with saline and vehicle (Veh) to evaluate the effect of the lesion per se.

PREG or DUTA (or Veh) were administered 40 min before L-DOPA injection, with a pretreatment 24 h before the beginning of L-DOPA administration. The doses of PREG and DUTA were chosen based on previous studies (Frau et al., 2019; Vallée et al., 2014; Fanni et al., 2019). The treatments were administered for 3 weeks, to ascertain that no tolerance developed after repeated daily administrations.

AIMs were scored once or twice a week, starting from day 1, for a total of 5 time points. The stepping test (day 20) and the locomotor activity test (days 18–19, only available for the second set of animals) were carried out to detect a possible negative impact of PREG on the therapeutic efficacy of L-DOPA, as described.

On the 23rd day of treatment, animals were sacrificed 60 min after the last L-DOPA administration (and 100 min after the last PREG, DUTA, or Veh treatment). Brains were collected to perform striatal molecular analyses, and neurosteroids quantification, whereas midbrains were postfixed and analyzed to verify the extent of the lesion by immunohistochemistry (see Fig.S1·C and S1·D).

2.7. Statistical analyses

Based on the experimental design, behavioral data were analyzed by repeated-measures two-way ANOVA, with treatment (PREG, DUTA, or Veh) and time (days or minutes) as independent factors. Ordinary one-way ANOVA or Kruskal-Wallis tests were used for between-group studies of both molecular and neurosteroid quantifications. Tukey's, Dunnett's, or Dunn's post hoc tests were carried out for multiple post hoc comparisons. Mann-Whitney or Student's *t*-test were applied when appropriate. Pearson correlation coefficient was exploited to assess any association between continuous variables. Alpha error probability was set at $p < 0.05$.

3. Results

3.1. Effect of pregnenolone on the development of LIDs

Previous data from our group indicated that pharmacological inhibition of 5AR was paralleled by a marked increase in endogenous PREG and PROG levels in rats (Frau et al., 2017b; Frau et al., 2019). Similar observations on PREG levels were recently obtained in the lesioned striata of dyskinetic rats (unpublished). Therefore, in order to address whether PREG might be responsible for the dampening effect on LIDs seen with 5AR inhibitors (Fanni et al., 2019; Frau et al., 2017a), in this study we tested the effect of 3 escalating doses of PREG on the onset of LIDs and compared the outcome with the effect of the positive control DUTA, as we have previously demonstrated its ability to dampen dyskinesias by acting on the neurosteroid pathway (Fanni et al., 2019; Frau et al., 2017a).

As shown in Fig.1, all the tested PREG doses effectively reduced LIDs over time (treatment x time interaction, F (16, 276) = 2.765, $p = 0.0004$; main effect of time, F (4, 276) = 173.5, $p < 0.0001$; and treatment, F (4, 69) = 12.18, $p < 0.0001$). Interestingly, the 18 mg/kg dose was effective

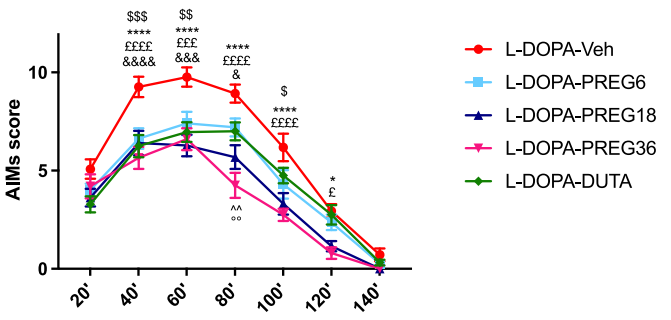

Fig. 1. Effect of pregnenolone on LIDs development over 22 days of treatment. Scores are shown as the sum of axial, limb, and orolingual Abnormal Involuntary Movements (AIMs) on each session rated. Values represent mean ± SEM for each experimental group (L-DOPA-Veh, $N = 19$; L-DOPA-PREG6, $N = 15$; L-DOPA-PREG18, $N = 16$; L-DOPA-PREG36, $N = 10$; L-DOPA-DUTA, $N = 14$). Day 1: *, $p < 0.05$ L-DOPA-Veh vs L-DOPA-PREG18; ç, $p < 0.05$ L-DOPA-DUTA vs L-DOPA-PREG18; Day 4: *, $p < 0.05$ vs L-DOPA-PREG18; £££, $p < 0.001$ vs L-DOPA-PREG36; °°, $p < 0.01$ L-DOPA-PREG36 vs L-DOPA-DUTA; ^, $p < 0.05$ vs L-DOPA-PREG6; Day 8: $$, $p < 0.01$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.0001$ vs L-DOPA-PREG36; &, $p < 0.05$ vs L-DOPA-DUTA; Day 15: $, $p < 0.05$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.0001$ vs L-DOPA-PREG36; &&, $p < 0.01$ vs L-DOPA-DUTA; Day 22: $$$, $p < 0.001$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.0001$ vs L-DOPA-PREG36; &&&, $p < 0.001$ vs DUTA (Tukey's multiple comparison post hoc test).

from the 1st day of L-DOPA treatment ($p = 0.0342$ vs L-DOPA-Veh and $p = 0.0362$ vs L-DOPA-DUTA). By contrast, as reported in our previous study (Fanni et al., 2019), the antidyskinetic effects of DUTA became significant after about 1 week. Overall, the lowest dose of PREG reduced LIDs akin to the effect of DUTA, whereas the 18 and 36 mg/kg doses exerted a greater effect. Importantly, no tolerance developed to PREG effects, as full efficacy was still observed up to the 22nd day of treatment ($p = 0.0007$, L-DOPA-Veh vs L-DOPA-PREG6, $p = 0.0003$ L-DOPA-Veh vs L-DOPA-DUTA, and $p < 0.0001$, L-DOPA-Veh vs L-DOPA-PREG18 and vs L-DOPA-PREG36).

Next, we analyzed the time course of LIDs during the last session of dyskinesias assessment (Fig.2). Two-way ANOVA highlighted a time x treatment interaction (F (24, 414) = 2.876, $p < 0.0001$), and a main

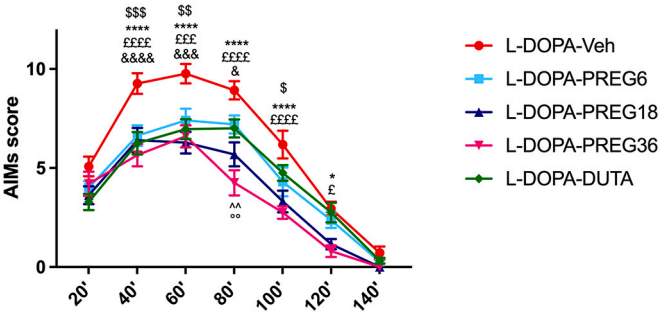

Fig. 2. Effect of pregnenolone on LIDs time-course at the last dyskinesia session.
Ratings are shown as the total AIMs score (L-DOPA-Veh, N = 19; L-DOPA-PREG6, N = 15; L-DOPA-PREG18, N = 16; L-DOPA-PREG36, N = 10; L-DOPA-DUTA, N = 14). 40′: $$$, $p < 0.001$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.0001$ vs L-DOPA-PREG36; &&&&, $p < 0.0001$ vs L-DOPA-DUTA; 60′: $$, $p < 0.01$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; £££, $p < 0.001$ vs L-DOPA-PRE36; &&&, $p < 0.001$ vs L-DOPA-DUTA; 80′: ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.001$ vs L-DOPA-PREG36; &, $p < 0.05$ vs L-DOPA-DUTA; ^^, $p < 0.01$ L-DOPA-PREG36 vs L-DOPA-PREG6; °°, $p < 0.01$ L-DOPA-PREG36 vs L-DOPA-DUTA; 100′: $, $p < 0.05$ vs L-DOPA-PREG6; ****, $p < 0.0001$ vs L-DOPA-PREG18; ££££, $p < 0.0001$ vs L-DOPA-PREG36; 120′ *, $p < 0.05$ vs L-DOPA-PREG18; £, $p < 0.05$ vs L-DOPA-PREG36 (Tukey's multiple comparisons post hoc test).

effect of time (F (6, 414) = 220.1, p < 0.0001) and treatment (F (4, 69) = 10.90, p < 0.0001). As expected, the L-DOPA-Veh group reached the peak in AIMs score between 40 and 80 min; by contrast, PREG and DUTA-treated animals showed a reduction of LIDs already at 40 min after L-DOPA administration (p = 0.0006 L-DOPA-Veh vs L-DOPA-PREG6, and p < 0.0001 L-DOPA-Veh vs L-DOPA-PREG18, vs L-DOPA-PREG36 and vs L-DOPA-DUTA). The antidyskinetic effects of DUTA and of the low dose of PREG were significant up to the 100 min time point, while those produced by the higher doses persisted longer. Moreover, the L-DOPA-PREG36 group showed a marked reduction in LIDs at 80 min, which was greater than that observed in the L-DOPA-PREG6 and L-DOPA-DUTA groups (p = 0.0013, and p = 0.0040 respectively) at this time point.

3.2. Effect of pregnenolone treatment on Axial, Limb, and Orolingual components

AIMs were scored as the sum of 3 components: Axial, Limb, and Orolingual dyskinesias (ALO), reflecting a different topographical distribution.

To investigate whether the subtypes were individually and/or differentially affected by PREG treatment, the single components were analyzed. Orolingual movements resulted the least affected (Fig.3A), with only a trend toward reduction (treatment x time interaction: F (16, 276) = 1.645, p = 0.0574; main effect of treatment: F (4, 69) = 13.36, p < 0.0001; and time: F (4, 276) = 104.2, p < 0.0001). By contrast, as shown in Fig.3B, PREG administration dose-dependently dampened limb dyskinesia (interaction time x treatment: F (16, 276) = 2.796, p = 0.0003)). Statistical analysis detected a main effect of treatment (F (4, 69) = 11.34, p < 0.0001) and time (F (4, 276) = 155.4, p < 0.0001). As previously reported for the cumulative AIMs score, the 18 and 36 mg/kg doses exerted a significant antidyskinetic effect from day 1 (p = 0.0205 L-DOPA-Veh vs L-DOPA-PREG18 and p = 0.0067 L-DOPA-Veh vs L-DOPA-PREG36). On the same day, all PREG-treated groups displayed only minor limb scores compared with L-DOPA-DUTA rats (p = 0.0067 vs L-DOPA-PREG6, p = 0.0015 vs L-DOPA-PREG18, and p = 0.0005 vs L-DOPA-PREG36).

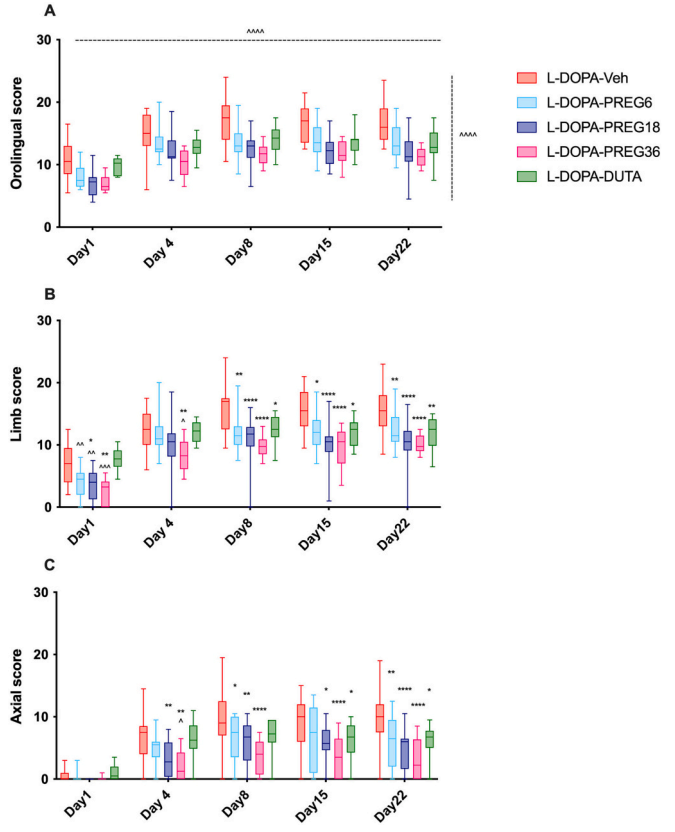

Fig. 3. Effect of pregnenolone treatment on ALO components.
Ratings for individual LIDs components: orolingual, limb, and axial score (L-DOPA-Veh, N = 19; L-DOPA-PREG6, N = 15; L-DOPA-PREG18, N = 16; L-DOPA-PREG36, N = 10; L-DOPA-DUTA, N = 14). A) Main effects of treatment and time, ^^^^, Two-way ANOVA for repeated measures. B) *, p < 0.05, **, p < 0.01, ****, p < 0.0001 vs L- DOPA-Veh; ^, p < 0.05, ^^, p < 0.01, ^^^, p < 0.001 vs L-DOPA-DUTA, Tukey's multiple comparisons post hoc test. C) *, p < 0.05, **, p < 0.01, ****, p < 0.0001 vs L-DOPA-Veh; ^, p < 0.05 vs L-DOPA-DUTA, Tukey's multiple comparisons post hoc test.

Interestingly, the antidyskinetic effect was particularly pronounced against axial dyskinesia (Fig.3C), which can be regarded as recapitulating the dystonic component of human dyskinesias, with a reduction of about 65% on day 22 with the highest PREG dose (p < 0.0001 vs L-DOPA-Veh) (main effect of time: F (4, 276) = 100.6, p < 0.0001; main effect of treatment: F (4, 69) = 7.545, p < 0.0001; interaction time x treatment: F (16, 276) = 3.105, p < 0.0001).

Overall, except for day 1, all the dyskinesia components were similarly affected by the lowest dose of PREG and DUTA, while the dampening effect on LIDs was higher as the PREG dose used increased.

3.3. Pregnenolone treatment shows no adverse effect on L-DOPA-induced therapeutic effects

The ideal antidyskinetic drug should not affect the efficacy of L-DOPA on motor disabilities; therefore, animals were subjected to the locomotor activity test and the stepping test to assess any possible interference of PREG treatment on L-DOPA therapeutic effect.

General motor activity, as indexed by horizontal and vertical activity, was carried out on days 18 and 19 of treatment to verify that PREG administration did not impact locomotor behavior, producing any sedative effect.

The results revealed that all groups had a similar locomotor activation pattern for the entire session (120 min from L-DOPA injections), suggesting no adverse effects of PREG and DUTA treatment on L-DOPA-induced motor activation for both the parameters measured (Fig. 4A, two-way ANOVA – time: F (3.247, 103.9) = 30.89, p < 0.0001; treatment: F (4, 32) = 0.5058, p = 0.7318; time x treatment: F (44, 352) = 0.7520, p = 0.8757; Fig. 4B, two-way ANOVA – time: F (5.423, 173.5) = 11.63, p < 0.0001; treatment: F (4, 32) = 0.6732, p = 0.6154; time x treatment: F (44, 352) = 1.021, p = 0.4408). As predicted, L-DOPA-Veh treated animals displayed improved performance in both horizontal activity and total distance parameters compared to the Sal-Veh group (Fig. 4A, two-way ANOVA- time: F (3.525, 45.82) = 13.25, p < 0.0001; treatment: F (1,13) = 23.68, p = 0.0003; time x treatment: F (11, 143) = 6.778, p < 0.0001; and Fig. 4B, two-way ANOVA- time: F (3.196, 41.55) = 3.327, p = 0.0263; treatment: F (1, 13) = 12.32, p = 0.0038; time x treatment: F (11, 143) = 2.247, p = 0.0150).

The stepping test was performed on day 20. The test was repeated at two time points, before L-DOPA administration (time 0, as baseline) and 150 min later (Fig. 4C).

Within all groups, some rats showed a partial recovery in the left forepaw functionality at the baseline (time 0) compared to the test used for screening the animals after the lesion. For this reason, all animals with >20% of steps with their left forepaws were excluded from the test (L-DOPA-Veh, n = 2; L-DOPA-PRE6, n = 3; L-DOPA-PRE18, n = 3; L-DOPA-PRE36, n = 1; L-DOPA-DUTA, n = 3).

Although a partial reduction was seen with the mid PREG dose, two-way ANOVA detected no difference in the percentage of the contralateral forepaw use among the groups (main effect of time: F (1, 57) = 49.76, p < 0.0001; main effect of treatment: F (4, 57) = 2.332, p = 0.0667; interaction time x treatment: F (4, 57) = 2.122, p = 0.0898).

It is important to highlight that despite the highest therapeutic efficacy of L-DOPA was expected between 40 and 100 min after drug administration, the stepping test could not be performed at these time

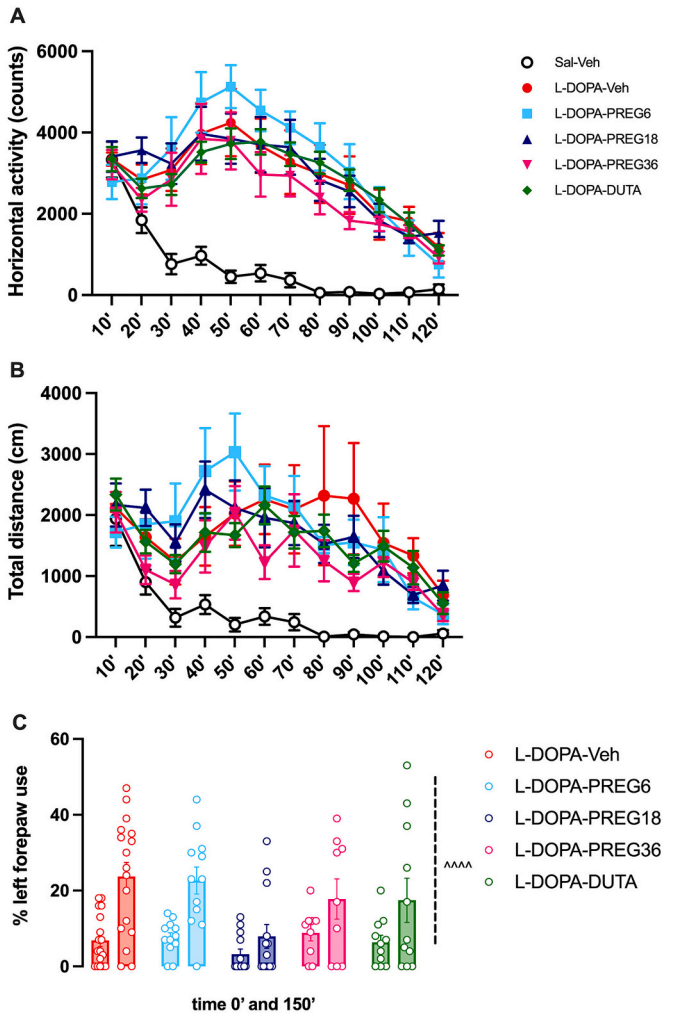

Fig. 4. Pregnenolone treatment shows no adverse effect on L-DOPA-induced therapeutic effects

A) Time course of the spontaneous horizontal activity of dyskinetic rats (L-DOPA-Veh, $N = 7$; L-DOPA-PREG6, $N = 5$; L-DOPA-PREG18, $N = 8$; L-DOPA-PREG36, $N = 10$; L-DOPA-DUTA, N = 7; Sal-Veh, N = 8) and B) total distance traveled, after pregnenolone and dutasteride pretreatment. Locomotor activity was recorded for 120 min immediately after L-DOPA injections. Each point represents mean value ± SEM. (L-DOPA-Veh, N = 7; L-DOPA-PREG6, N = 5; L-DOPA-PREG18, N = 8; L-DOPA-PREG36, N = 10; L-DOPA-DUTA, N = 7; Sal-Veh, N = 8). C) Effect of pregnenolone on L-DOPA-induced motor improvements on forelimb use. Data are normalized by means of the percentage of the ipsilateral forepaw stepping score and represented as mean value ± SEM. ^^^^, $p < 0.0001$Main effect of time, Two-way ANOVA (L-DOPA-Veh, $N = 17$; L-DOPA-PREG6, $N = 12$; L-DOPA-PREG18, $N = 13$; L-DOPA-PREG36, $N = 9$; L-DOPA-DUTA, $N = 11$).

points, as biased by the dyskinetic behavior. This may account for the significant but partial L-DOPA-induced improvement in the performance of the test shown by all groups.

3.4. Striatal neurosteroid quantification after pregnenolone administration

To verify whether PREG treatment effectively increased its striatal level, animals were sacrificed 1 h after L-DOPA administration (100 min after PREG injection) on day 23. Analyses of striatal neurosteroid levels were performed by LC-MS/MS (Fig. 5). PREG-treated rats showed a dose-dependent striking increase in striatal PREG levels compared with L-DOPA and vehicle-treated animals (Kruskal-Wallis, $p < 0.0001$; $p = 0.0385$ L-DOPA-Veh vs L-DOPA-PREG6; $p < 0.0001$ L-DOPA-Veh vs L-DOPA-PREG18). These data demonstrate that the formulation of PREG used was highly effective in increasing striatal PREG levels. Moreover, the analysis showed that most of the other neurosteroids appeared unaffected by PREG administration, at least at the time point analyzed here (100 min post-PREG administration), except for isoallopregnenolone (main effect of treatment, Isoallopreg: Kruskal-Wallis, $p = 0.0104$. PROG: Kruskal-Wallis, $p = 0.9909$. DHP: Kruskal-Wallis, $p = 0.1794$. THP: Kruskal-Wallis, $p = 0.3938$. DHEA: Kruskal-Wallis, $p = 0.3534$. TESTO: Kruskal-Wallis, $p = 0.1843$. DHT: Kruskal-Wallis, $p = 0.6514$. 3a-diol: Kruskal-Wallis, $p = 0.1888$. 17β-Estradiol: Kruskal-Wallis, $p = 0.7972$).

3.5. Effect of pregnenolone and dutasteride on striatal markers of dyskinesia

Finally, we evaluated whether the antidyskinetic effects of PREG were paralleled by molecular changes in well-established markers of dyskinesias. In fact, we have previously reported that DUTA effectively counteracted the L-DOPA-induced increase in striatal phospho- $ERK_{1/2}$ and phospho-Thr-34 DARPP-32 levels. This effect was accompanied by a reduction of D_1-D_3R heteromers, which are regarded to be implicated in the aberrant D_1R signaling in LIDs.

The analysis by one-way ANOVA of phospho-Thr-34 DARPP-32 levels ($F(5, 38) = 5.249$, $p = 0.0009$) followed by post hoc analysis found that L-DOPA treated animals developed a marked increase in phospho-Thr-34 DARPP-32 levels ($p = 0.0002$ compared to the Sal-Veh control group). Notably, the administration of DUTA, and PREG at both 18 and 36 mg/kg prevented this increase ($p = 0.0056$, $p = 0.0028$ and $p = 0.0024$ respectively, compared to the L-DOPA-Veh group) (Fig. 6A). Similarly, the analysis by one-way ANOVA of $ERK_{1/2}$ phosphorylation levels showed a significant difference between the experimental groups ($F(5, 38) = 3.219$, $p = 0.0161$), and post hoc analysis by the Dunnett's test demonstrated that dyskinetic animals (L-DOPA-Veh group) displayed higher levels of phospho-$ERK_{1/2}$ compared with those observed in the Sal-Veh control group ($p = 0.0064$). DUTA or PREG administration, both at 6 and 18 mg/kg dose, prevented the L-DOPA-induced increase in phospho- $ERK_{1/2}$ levels ($p = 0.0082$, $p = 0.0380$ and $p = 0.0287$ respectively, compared to the L-DOPA-Veh group, Fig. 6B). Overall, these results confirm that the development of dyskinesias induced by repeated treatment with L-DOPA in hemiparkinsonian rats is associated with increased D_1R signaling and that the concomitant treatment with either DUTA or PREG can prevent the development of these maladaptive signaling alterations.

Next, we analyzed the expression of DA D_1 and D_3R. Western blotting revealed that D_1 and D_3R were not individually modified by the treatments (D_1R: $F(5, 38) = 1.731$, $p = 0.151$; D_3R: $F(5, 38) = 0.783$, $p = 0.568$) (Fig. 6C, D). However, both DUTA and PREG appeared to alter D_1-D_3R interaction. In fact, the analysis by one-way ANOVA showed a significant difference between the experimental groups in the expression levels of D_3R associated with D_1R ($F(5, 38) = 4.051$, $p = 0.0048$). As expected, post hoc comparison by Dunnett's test revealed that repeated L-DOPA treatment increased the levels of heteromeric complexes ($p = 0.0206$, compared to the Sal-Veh group). Interestingly, co-administration of PREG at 18 or 36 mg/kg with L-DOPA decreased the formation of D_1-D_3R complexes induced by repeated L-DOPA administration ($p = 0.0058$ and $p = 0.0153$, respectively) as previously seen and confirmed here for DUTA ($p = 0.0025$, compared to the L-DOPA-Veh group) (Fig. 6E). No differences were seen in the above markers in the intact striata (S2 supplementary figure).

3.6. Effect of pregnenolone on striatal BDNF levels

In an attempt to shed light on the mechanism accounting for the protective effect of PREG against dyskinesia, we investigated the impact of chronic treatment on striatal BDNF levels.

Indeed, previous data suggested BDNF as an intriguing possible

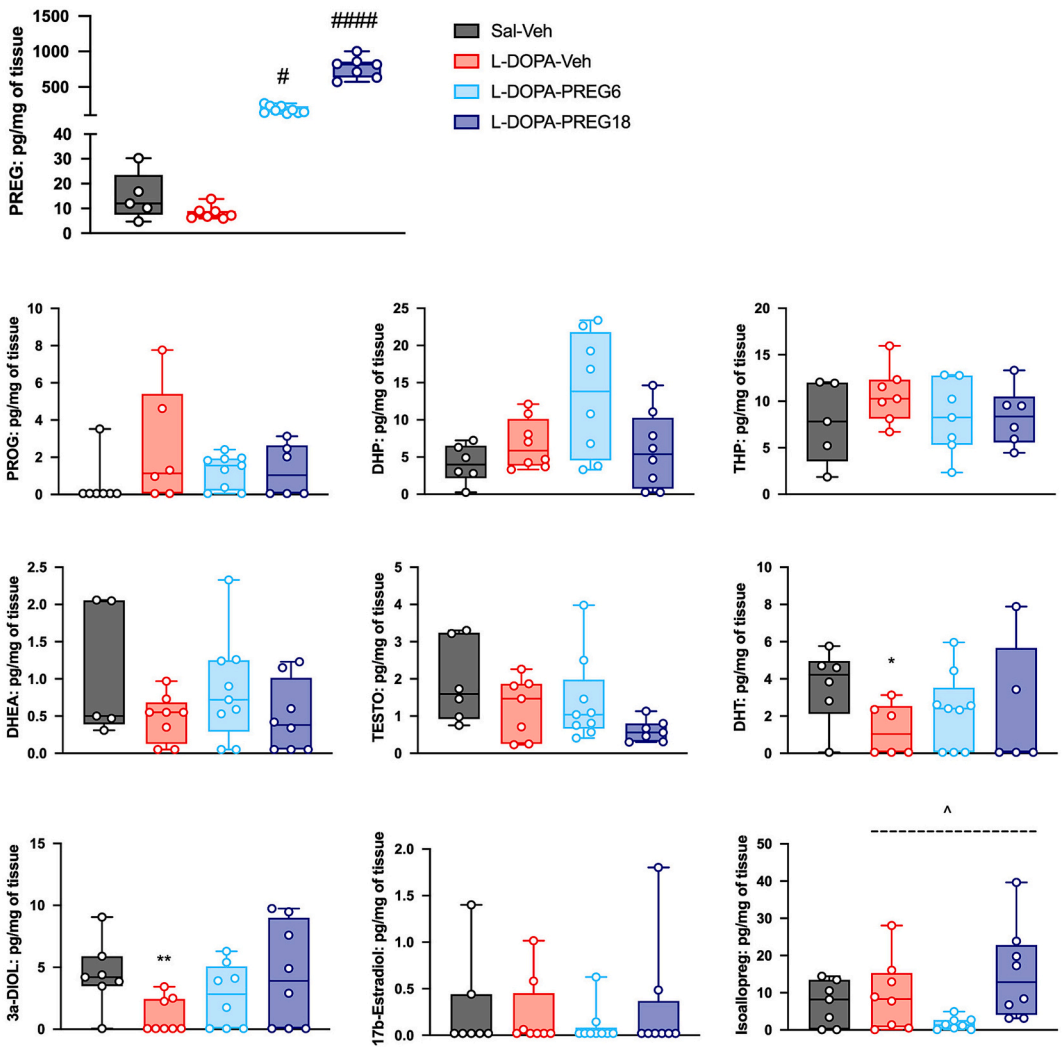

Fig. 5. Neurosteroid striatal levels after pregnenolone administration.
^, $p < 0.05$, Kruskal Wallis test; *, $p < 0.05$, and **, $p < 0.01$ vs Sal-Veh, Mann-Whitney test; #, $p < 0.05$, ####, $p < 0.0001$ vs L-DOPA-Veh, Dunn's multiple comparisons post hoc test (L-DOPA-Veh, $N = 6–8$; L-DOPA-PREG6, $N = 7–9$; L-DOPA-PREG18, $N = 5–8$; Sal-Veh, $N = 5–7$).

player in dyskinesia as its levels resulted increased upon chronic L-DOPA treatment (Okazawa et al., 1992; Guillin et al., 2001; Rylander et al., 2010; Leino et al., 2018). Moreover, we recently demonstrated that viral vector overexpression of striatal BDNF levels exacerbates LIDs in 6-OHDA-lesioned rats via D_1-D_3 heteromeric complexes (Scheggi et al., 2020).

Interestingly, as shown in Fig. 7A, PREG dose-dependently elicited a dampening effect on L-DOPA-induced BDNF upregulation (One-way ANOVA, $F(5,38) = 3.159$, $p = 0.0176$), suggesting a possible link between the antidyskinetic properties of PREG, D_1-D_3 heteromers, and BDNF. This effect appears to be specific to the DA-depleted striatum, as the intact left striatum displays no changes in BDNF levels among the groups (Fig. S3). Conversely, proBDNF levels were not affected by any treatment (One-way ANOVA, $F(5,38) = 1.546$, $p = 0.1988$).

In further confirmation of an interplay between BDNF and D_1-D_3 heteromeric complexes, we found that BDNF striatal levels positively correlate with the expression of D1-D3 co-immunoprecipitates (Fig. 7B).

4. Discussion

In the present study, we show for the first time that chronic PREG administration dose-dependently counteracts the development of LIDs in 6-OHDA-lesioned rats without affecting the therapeutic efficacy of L-DOPA. Moreover, the antidyskinetic effect of PREG was accompanied by inhibition of the rise of dyskinesia markers, such as phospho-Thr-34 DARPP-32 and phospho-$ERK_{1/2}$, as well as by reduced striatal BDNF levels and D_1-D_3R co-immunoprecipitates, akin to the effects of the 5AR inhibitor DUTA. Therefore, our data suggest that restoring PREG levels efficaciously counterbalances the alterations in striatal D_1R-related signaling induced by chronic L-DOPA in 6-OHDA hemiparkinsonian rats.

In a view of a repurposing drug strategy, we recently reported (and confirmed here) that 5AR inhibitors, such as finasteride and DUTA, significantly reduce LIDs in parkinsonian rats (Fanni et al., 2019; Frau et al., 2017a). The antidyskinetic effect of DUTA was paralleled by the reduction of well-established striatal markers of dyskinesia, while it did not affect L-DOPA-induced improvements in motor deficits (Fanni et al., 2019). These interesting properties might suggest a possible clinical application of our findings since 5AR inhibitors are used in clinical settings for the treatment of benign prostatic hyperplasia (BPH) (Salisbury and Tadi, 2022). However, the potent and irreversible inhibition of both 5AR isoforms by DUTA, hampers all the 5AR downstream pathways, leading to decreased levels of several neurosteroids, raising concerns for possible detrimental consequences. Indeed, clinicians report both central and peripheral adverse effects after 5AR inhibition in patients with BPH or androgenetic alopecia, such as depression, suicidal

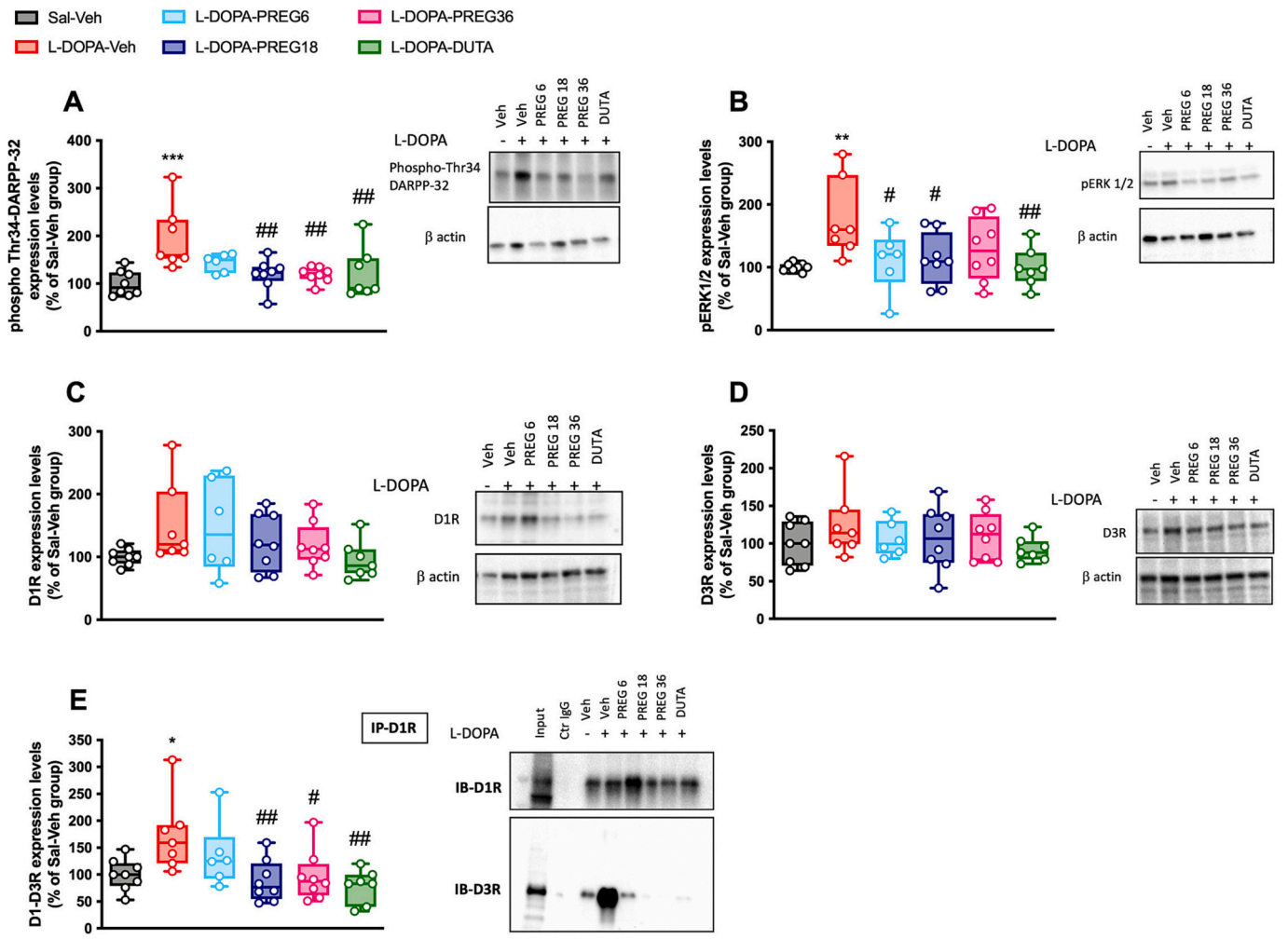

Fig. 6. Effect of pregnenolone on striatal markers of dyskinesia.
A) ***, $p < 0.001$ vs Sal-Veh, ## $p < 0.01$ vs -L-DOPA-Veh; B) **, $p < 0.01$ vs Sal-Veh; #, $p < 0.05$, and ##, $p < 0.01$ vs -L-DOPA-Veh; E) *, $p < 0.05$ vs Sal-Veh, #, $p < 0.05$, and ##, $p < 0.001$ vs L-DOPA-Veh; One-way ANOVA, Dunnett's multiple comparisons post hoc test (L-DOPA-Veh, $N = 7$; L-DOPA-PREG6, $N = 6$; L-DOPA-PREG18, $N = 8$; L-DOPA-PREG36, $N = 8$; L-DOPA-DUTA, $N = 7$; Sal-Veh, $N = 8$).

ideation, anxiety, sexual dysfunctions, and insulin resistance, raising further concerns for applications in PD patients (Diviccaro et al., 2019). Thus, understanding which neurosteroid(s) might be implicated in the antidyskinetic effect of 5AR inhibitors becomes crucial in the attempt to retain the therapeutic efficacy and reduce the side effects, paving the ground for clinical application.

Among the neurosteroids implicated in the pathway of the 5AR, converging preclinical and clinical evidence points to the substrate PREG as a possible key player in the antidyskinetic properties of 5AR inhibitors. First, we have previously reported that 6-OHDA lesion resulted in a specific reduction of striatal PREG concentration (Melcangi et al., 2012). Second, in parallel to the depletion of the metabolites, the inhibition of 5AR led to a robust increase in its related substrates in the striatum, especially PREG (Frau et al., 2017b; Frau et al., 2015). Third, this neurosteroid has shown marked antidopaminergic activity, similar to the 5AR inhibitor DUTA; in fact, both drugs rescued the hyperactivity, stereotyped behavior, and prepulse inhibition deficits underlying striatal hyperdopaminergia (Frau et al., 2019; Frau et al., 2015; Devoto et al., 2012; Bortolato et al., 2008; Wong et al., 2012). Hence, the present findings support previous evidence indicating modulating properties of PREG on dopamine neurotransmission and point out this neurosteroid as an interesting drug candidate also for treating LIDs in PD.

Importantly, as assessed by LC-MS/MS, the exogenous administration of PREG in our animals was highly effective in increasing its striatal levels, while most of the other neurosteroids were unaffected, which may represent a significant advantage compared with the 5AR inhibitors. The lack of modifications in PROG, TESTO, and the downstream 17β-Estradiol levels supports the idea that the antidyskinetic effect of PREG does not stem from sex-related steroid actions and the hypothesis that the effect of PREG is not sex-specific.

The conversion of cholesterol into PREG within the mitochondria is the first rate-limiting step of the neurosteroid pathway (Reddy, 2010; Vallée, 2016; Liang and Rasmusson, 2018). Therefore, PREG is not only the precursor of the neurosteroids related to the 5AR pathway but also of all other classes of steroids (Reddy, 2010; Vallée et al., 2014; Tuem and Atey, 2017). Although numerous investigations have reported beneficial effects of PREG in both animal models and patients (Ritsner et al., 2010; Vallée et al., 2014; Frau et al., 2019; Tomaselli and Vallée, 2019), they cannot rule out the involvement of downstream metabolites. However, while the main downstream metabolites were not modified in our neurochemical analyses, due to technical limitations we could not ascertain a possible involvement of the sulfated neurosteroids, such as PREG sulfate and DHEA sulfate. Indeed, besides being a substrate of the 3β-HSD, PREG and its CYP17A1-derived metabolite DHEA are also converted into their sulfated forms (PREGS and DHEAS) by sulfotransferase. PREGS and DHEAS modulate the activity of DA receptors, mainly by indirect mechanisms. Specifically, they act as agonists on NMDAR and exert an antagonistic activity on GABA-A R (Reddy, 2010).

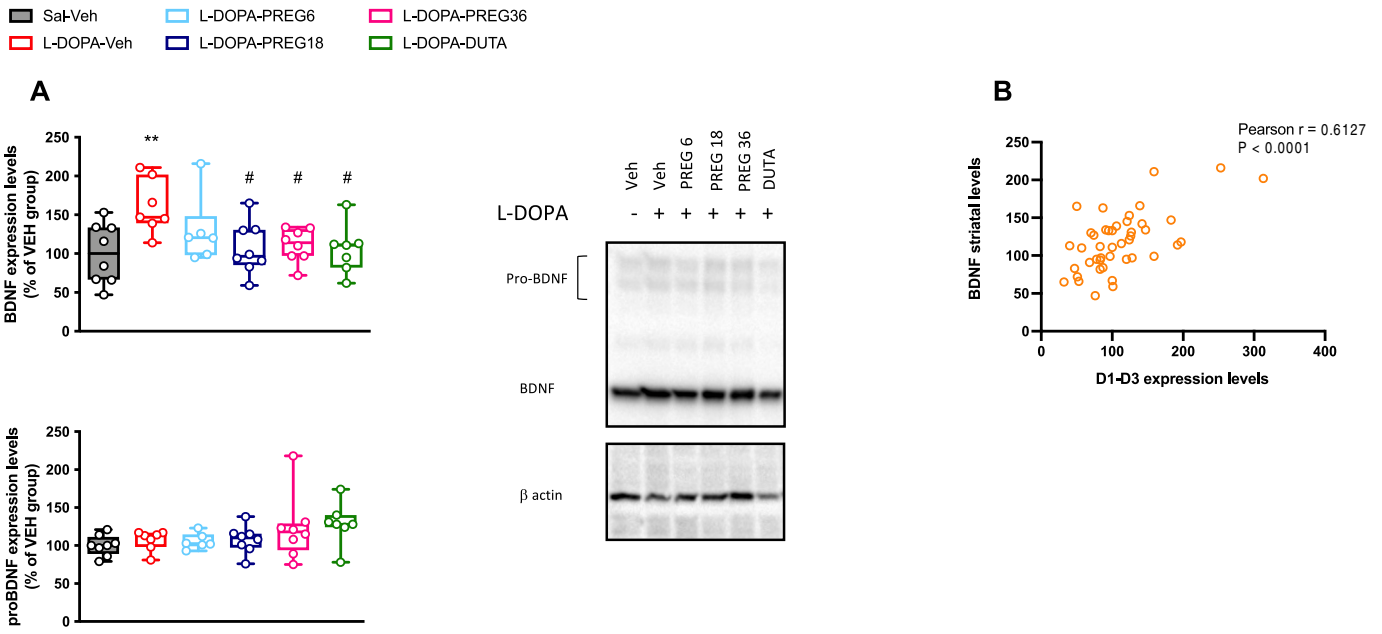

Fig. 7. BDNF and proBDNF expression levels after pregnenolone treatment.
A) Variations of BDNF and proBDNF expression levels in 6-OHDA-lesioned rats daily treated either with Veh, L-DOPA, L-DOPA plus pregnenolone, or L-DOPA plus dutasteride. **, $p < 0.01$ vs Sal-Veh; #, $p < 0.05$ vs L-DOPA-Veh (Dunnett's multiple comparison test). B) Positive correlation between striatal BDNF levels and D1-D3R co-immunoprecipitates (r2:0.3753, $p < 0.0001$) (L-DOPA-Veh, N = 7; L-DOPA-PREG6, N = 6; L-DOPA-PREG18, N = 8; L-DOPA-PREG36, N = 8; L-DOPA-DUTA, N = 7; Sal-Veh, N = 8).

The modulatory action of PREGS on NMDAR (Malayev et al., 2002; Chopra et al., 2015) may be important to regulate both the striatal outputs and the cortico-striatal afferents since glutamate efflux was found to be reduced ipsilateral to the lesion, whereas GABA levels were increased in both hemispheres of chronic L-DOPA-treated rats (Lindenbach et al., 2016). These data are relevant as the involvement of NMDA and GABA transmissions in dyskinesia is well established (Bastide et al., 2015). Moreover, PREG, DHEA, and their sulfates are all ligands of σ1R (Maurice et al., 2006; Maurice and Su, 2009) that in turn might modulate DA signaling (Nuwayhid and Werling, 2003; Maurice and Su, 2009; Sambo et al., 2018). In fact, Navarro and co-workers found that σ_1R forms heteromers with both D_1 and D_2R. In this context, σ_1 ligands by acting on D_1-σ_1 dimers may change the downstream signaling, potentiating the PKA/DARPP32 pathway while inhibiting MAPK/ERK (Navarro et al., 2010, 2013). In addition, PREG has also inhibited the increase in $ERK_{1/2}$ signaling through its negative allosteric modulation on CB_1R (Vallée et al., 2014).

Of note, several neurosteroids have been reported to play a role in neuroinflammation. In line with this evidence, PREG modulated the targeted degradation of two key pro-inflammatory signals, TIRAP and TLR2, leading to suppression of TNFα and IL-6 secretion (Murugan et al., 2019). The anti-inflammatory properties shown by PREG, although not investigated in this study, may play a role in its antidyskinetic effect. Indeed, a wide body of evidence reported a role of neuroinflammation in LIDs development. In fact, L-DOPA administration has been reported to produce increased levels of pro-inflammatory cytokines, such as IL-1β and TNFα (Barnum et al., 2008; Mulas et al., 2016).

Although more investigations are warranted to clarify the mechanisms of PREG action and the involvement of other metabolites, our findings demonstrate a solid rationale for the use of this neurosteroid for dyskinesia. Indeed, PREG was effective in dampening LIDs development over a 3-week administration and no tolerance was observed. Instead, PREG consistently reduced AIMs over the 22 days of treatment, with the most pronounced antidyskinetic effect observed at the last session rated. When analyzing the time course of AIMs at the last scored session, we detected a significant reduction of LIDs between 40 and 80 min after L-DOPA, when L-DOPA-induced AIMs are present at their utmost. This effect might greatly impact clinical applications since peak-dose dyskinesias are one of the most frequent and troublesome types of dyskinesia (Fabbrini et al., 2007; Prashanth et al., 2011).

Dyskinesias are a complex ensemble of hyperkinetic motions, mainly choreiform and dystonic movements usually involving limbs, trunk, and orofacial muscles (Prashanth et al., 2011), which can be reproduced in the rat model of LIDs (Cenci and Lundblad, 2007). The antidyskinetic effect exerted by PREG was particularly pronounced on axial movements, which is considered to recapitulate the dystonic component of human dyskinesia. In fact, it has been reported that choreiform and dystonic elements might rely on different signaling patterns (Andreoli et al., 2021).

Of note, as previously shown for DUTA, the reduced expression of LIDs was paralleled by the normalization of well-established markers of dyskinesia, such as phospho-$ERK_{1/2}$ and phospho-DARPP-32 (at threonine 34). Importantly, we found that, while D_1 or D_3R were not individually modified by the treatments, PREG prevented the L-DOPA-induced increase of D_1-D_3R co-immunoprecipitation, in a fashion similar to DUTA. This result is particularly relevant since a wide body of evidence highlighted the role of D_1-D_3 heterodimers in the development of LIDs (Fiorentini et al., 2008; Fiorentini et al., 2015; Solís and Moratalla, 2018; Lanza et al., 2018; Moreno et al., 2022). Accordingly, it has been hypothesized that the bond of D_3R to D_1R prevents D_1R internalization and potentiates its signaling cascade at striatal neurons (Fiorentini et al., 2008; Marcellino et al., 2008; Solís et al., 2017).

Compelling evidence reports that D_3R expression is under the control of the neurotrophic factor BDNF (Guillin et al., 2001). Interestingly, chronic treatment with L-DOPA has been linked to increased synthesis of this neurotrophin, which in turn accounts for some of the maladaptive alterations occurring in LIDs (Okazawa et al., 1992; Guillin et al., 2001). We recently reported that viral vector-induced overexpression of the rat BDNF gene at striatal neurons of parkinsonian rats leads to significant worsening of dyskinesia development elicited by a direct and selective D_1R agonist (Scheggi et al., 2020). Of note, we showed that BDNF mediates an increased availability of striatal D_3R and that the D_1R stimulation seems mandatory to recruit D_3R at the plasma membrane, forming D_1-D_3 complexes (Scheggi et al., 2020). Our molecular assays revealed

that PREG countered the L-DOPA-induced increase in striatal BDNF levels, suggesting that PREG might influence D_1-D_3R co-expression by reducing striatal BDNF rise. Indeed, striatal BDNF levels were found to be positively correlated to the level of D_1-D_3 receptor co-immunoprecipitates in our animals, further reinforcing the hypothesis of a link between BDNF and D_1-D_3 heteromers in dyskinesia.

Emerging preclinical and clinical evidence indicate that PREG might be used as medication for psychotic symptoms and antipsychotic-induced extrapyramidal symptoms in schizophrenia, drug abuse, and stress disorders, all conditions characterized by DA-related alterations (Wong et al., 2012; Wong et al., 2015; Frau et al., 2019; Ritsner et al., 2010; Ritsner et al., 2014; Kreinin et al., 2017; Tomaselli and Vallée, 2019). Importantly, our data indicated no alterations in sex-related steroids after PREG treatment. TESTO, DHT, or PROG levels were not modified after chronic treatment, implying a potential advantage compared to DUTA, avoiding the adverse effects observed in some subset of patients under chronic 5AR inhibitor treatment.

Future studies are warranted to understand whether the anti-dyskinetic effect of PREG extends to already established dyskinesias and can also be observed in female subjects. However, in support of this scenario, we have already demonstrated that 5AR inhibitors are equally effective in L-DOPA-naïve and -primed rats; moreover, the anti-dyskinetic effect observed in female rats was not dissimilar to that seen in males (Frau et al., 2017a).

In conclusion, whereas further work is needed to clarify the precise mechanisms by which neurosteroids affect LIDs, this is the first report showing a direct effect of PREG on this troublesome side effect of L-DOPA chronic treatment and its ability to modulate striatal BDNF levels and D_1-D_3R interaction.

Declaration of Competing Interest

Authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2023.114370.

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