

ORIGINAL ARTICLE

Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knockout mice through the AKT/GSK3 β pathway

P Wong^{1,2}, Y Sze¹, CCR Chang¹, J Lee^{3,4} and X Zhang^{1,5,6}

Pregnenolone sulfate, an endogenous neurosteroid in the central nervous system, is a positive allosteric modulator of the NMDA receptor, and plays a role in the modulation of learning and memory. Here, we study the actions of pregnenolone sulfate using the dopamine transporter knockout (DAT-KO) mice, which exhibit endophenotypes that recapitulate certain symptoms of schizophrenia, including the psychomotor agitation, stereotypy, prepulse inhibition (PPI) deficits and cognitive impairments. We found that acute treatment with pregnenolone sulfate normalized the hyperlocomotion and stereotypic bouts, and rescued the PPI deficits of DAT-KO mice. In addition, long-term treatment with pregnenolone sulfate rescued the cognitive deficits of DAT-KO mice in the novel object recognition and social transmission of food preference tests. We also showed that pregnenolone sulfate normalized behavioral abnormalities in MK801-treated wild-type mice, whereas pregnenolone, its precursor, only partially rescued MK801-induced behavioral abnormalities. This indicates that there are distinct mechanisms of action between pregnenolone sulfate and pregnenolone, and the involvement of NMDA receptor signaling in the action of pregnenolone sulfate. Moreover, we found that acute treatment with pregnenolone sulfate increased the phosphorylation levels of striatal AKT and GSK3 β in DAT-KO mice, and that long-term treatment with pregnenolone sulfate increased expression levels of NR1 subunit of the NMDA receptor in hippocampus. Thus, pregnenolone sulfate was able to rescue the behavioral anomalies of DAT-KO mice through the NMDA receptor-mediated, AKT/GSK3 β signaling pathway.

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INTRODUCTION

Schizophrenia is a debilitating mental disorder, which affects ~1% of the world population.¹ Understanding the pathophysiology of schizophrenia is the key for its effective diagnosis and treatment. The dopamine hypothesis is one of the more established theories of schizophrenia to date, and is supported by clinical observations of amphetamine-induced psychosis and dopamine D2 receptor-mediated antipsychotic treatment.^{2,3} Although antipsychotics are commonly used to treat positive symptoms of schizophrenia, there are few effective tools that can be used to combat the cognitive deficits and negative symptoms of schizophrenia.^{4,5}

Another prominent hypothesis suggests that the hypofunction of glutamatergic NMDA receptors is an underlying cause of schizophrenia.^{6–9} This hypothesis arose from clinical observations that NMDA receptor antagonists, such as ketamine and phenylcyclidine, can induce schizophrenia-like symptoms,^{10,11} and that schizophrenia patients exhibit decreased levels of NMDA receptors.¹² Given that NMDA receptors regulate dopaminergic neurotransmission, the dopaminergic and glutamatergic models are not mutually exclusive, and hypofunction of NMDA receptors may in fact be responsible for the abnormal dopamine activity observed in schizophrenia.⁸ These two neurotransmitter systems also converge on many levels, one of which is the AKT/GSK3 β pathway through dopamine D2 receptor signaling¹³ or NMDA/

PI3K signaling.¹⁴ AKT phosphorylation regulates various downstream molecules, including GSK3 β .¹⁵ A constitutively active kinase that has many downstream targets that modulate synaptic plasticity, GSK3 β plays an important role in learning and memory.¹⁶ Dysfunction of the AKT/GSK3 β signaling pathway has been implicated in schizophrenia.^{17,18}

In addition to antipsychotics, there have been recent advances in the treatment of schizophrenia using alternative pharmacological agents,¹⁹ such as neurosteroids.^{20,21} Neurosteroids are synthesized in the central nervous system, and have effects on anxiety, cognition and memory.^{22–26} Altered levels of neurosteroids such as pregnenolone (Preg) and dehydroepiandrosterone have been observed in patients with schizophrenia,²⁷ and administration of Preg has been shown to rescue certain schizophrenia symptoms in proof-of-concept, randomized controlled clinical trials.²⁰ Recently, it has been shown that Preg can rescue schizophrenia-like behavior in dopamine transporter knockout (DAT-KO) mice.²⁸ DAT-KO mice have behavioral manifestations that mirror the positive and negative symptoms associated with schizophrenia,^{29–31} suggesting that the DAT-KO mouse is an ideal mouse model for use to study schizophrenia. In addition, elevated GSK3 β activity through dopamine D2 receptor has also been observed in DAT-KO mice,¹³ thus recapitulating the

¹Program in Neuroscience and Behavioral Disorders, Duke-NUS Graduate Medical School Singapore, Singapore, Singapore; ²Department of Pharmacology, Neuroscience Phenotyping Core, National University of Singapore, Singapore, Singapore; ³Office of Clinical Sciences, Duke-NUS Graduate Medical School Singapore, Singapore, Singapore; ⁴Research Division, Institute of Mental Health, Singapore, Singapore; ⁵Department of Physiology, National University of Singapore, Singapore, Singapore and ⁶Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA. Correspondence: Dr X Zhang, Program in Neuroscience and Behavioral Disorders Duke-NUS Graduate Medical School Singapore, 8 College Road, Singapore 169857, Singapore.

E-mail: xiaodong.zhang@duke-nus.edu.sg

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impaired AKT/GSK3 β signaling pathway in schizophrenia patients.^{17,18}

As Preg can be converted to its soluble form of pregnenolone sulfate (PregS), PregS may also have the potential to alleviate symptoms of schizophrenia. Moreover, PregS is a known positive allosteric NMDA receptor modulator,^{32–36} and may exert its effects by ameliorating NMDA receptor hypofunction. In this study, we propose that PregS possesses a distinct mechanism of action from its precursor, Preg, and is able to rescue schizophrenia-like behavior in DAT-KO mice by modulating AKT/GSK3 β signaling via the NMDA receptor. Therefore, PregS may be a potential alternative therapeutic agent in the treatment of schizophrenia.

MATERIALS AND METHODS

Animals

Male and female, wild-type (WT) and DAT-KO mice (8–10 weeks) were bred from C57BL/6J DAT heterozygous mice. Genotyping was carried out as previously described.²⁸ Mice were housed in a specific pathogen-free environment, with *ad libitum* access to food and water, on a 12-h light/dark cycle (lights on at 0700 h). Animal procedures were approved by the Institutional Animal Care and Use Committees of NUS and Duke-NUS Graduate Medical School Singapore, in accordance with national guidelines for the care and use of laboratory animals for scientific purposes.

Drug preparation and administration

PregS (Steraloids, Newport, RI, USA) was first dissolved in ethanol and diluted in autoclaved water to a final ethanol concentration of 0.6%. PregS or 0.6% ethanol vehicle was injected intraperitoneally with a 5 ml kg⁻¹ injection volume. Preg (60 mg kg⁻¹, Sigma-Aldrich, St. Louis, MO, USA) was administered as a suspension in peanut oil either intraperitoneally (for acute injections) or subcutaneously (for long-term injections) with a 5 ml kg⁻¹ volume. Controls were injected with peanut oil vehicle. For long-term injections, PregS was administered for 10 consecutive days, while Preg was administered for 14 consecutive days.

Open field activity

Locomotor activity was recorded using an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH, USA) under ~180 lux illumination. The mice were placed into the chamber for 30 min to obtain baseline activity, followed by an injection (intraperitoneally) with vehicle or PregS at 40 mg kg⁻¹ or 80 mg kg⁻¹, then returned to the chamber for 120 min. Activity was measured in terms of total distance traveled, rearing as vertical activity and stereotypy as the numbers of repeated beambreaks with intervals of < 1 s.

PPI of acoustic startle

The mice were injected (intraperitoneally) with vehicle, or PregS at 20 mg kg⁻¹ or 40 mg kg⁻¹ and were placed into startle chambers (SR-LAB, San Diego Instruments, San Diego, CA, USA) for 10 min of acclimatization. The mice were then exposed to a series of trials as previously described,²⁸ with three different prepulse stimuli, which were 4, 8 or 12 dB above the white-noise background. Prepulse inhibition (PPI) responses were calculated as percentage scores for each prepulse intensity, where % PPI = [1 – (prepulse trials/startle-only trials)] × 100.

Novel object recognition test

The mice were injected (intraperitoneally) with vehicle or PregS at 40 mg kg⁻¹ daily for 10 days. On the next day, the novel object recognition test was carried out as previously described.²⁸ In brief, they were trained with identical 'familiar' objects for 5 min and then assessed for short-term, long-term and remote memory, which were conducted 20 min, 24 h and 14 days after training, respectively. All the tests were video recorded and scored using the TopScan Behaviour Analyzing system (CleverSys, Reston, VA, USA). Preference score was calculated as (Time spent with novel object – Time spent with familiar object)/(Total time spent with both objects).

Social transmission of food preference test

Mice were injected (intraperitoneally), with vehicle or 40 mg kg⁻¹ PregS, daily for 10 days. After the last injection, the animals were food deprived for 16–18 hours before testing.²⁸ The familiar diet was the standard mouse diet (5LJ5, LabDiet, St. Louis, MO, USA) mixed with 1% of ground oregano (McCormick, Hunt Valley, MD, USA). Novel diets for short-term, long-term and remote memory tests were flavored with 1% ground thyme, marjoram or cumin, respectively. The mice were then tested at 20 min, 24 h and 14 days after exposure to assess for short-term, long-term and remote memory, respectively. Preference scores were calculated as (Amount of familiar diet consumed – Amount of novel diet consumed)/(Total amount of both diets consumed).

Western blotting

Tissues of striatum and hippocampus were rapidly dissected and homogenized at 4 °C in lysis buffer (20 mM Tris; pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100). Thirty micrograms of protein extracts were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). Immunoblots of striatal samples were probed with the following primary antibodies: AKT (1:10 000); phospho-AKT (Thr308) (1:2000); GSK3 β (1:30 000); phospho-GSK3 β (1:5000); GAPDH (1:30 000; Cell Signaling Technology, Beverly, MA, USA). Immunoblots of hippocampal samples were probed with antibodies against NR1 (1:4000; Synaptic Systems, Gottingen, Germany) and Actin (1:20 000; Merck Millipore, Billerica, MA, USA). Image J software (National Institutes of Health, USA) was used for the densitometric analyses.

Statistical analysis

R statistical program (R Foundation for Statistical Computing, Vienna, Austria) was used for data analyses. Data were expressed as means \pm s.e.m. and $P < 0.05$ was considered statistically significant. A two-way analysis of variance was used for open field cumulative activities, and null and startle activity in PPI. Mixed factorial design analysis of variance was used to analyze PPI, novel object recognition and social transmission of food preference tests. For all tests, genotype and treatment were between-subject factors; while inhibition across prepulse intensities for PPI, and test sessions for both cognitive tests were within-subject factors. A *t*-test was used to analyze the preference scores in the novel object recognition and social transmission of food preference tests versus '0', and for densitometric analyses. Bonferroni-corrected pair-wise comparisons were used as *post hoc* tests.

RESULTS

PregS normalizes hyperlocomotor activities in DAT-KO mice

WT and DAT-KO mice were treated with vehicle or PregS (40 and 80 mg kg⁻¹) and tested for locomotor activities. The locomotor, rearing and stereotypic activities were aggregated over baseline (0–30 min) and the post-injection (31–150 min) periods. The cumulative baseline activity showed a significant main effect of genotype, but no significant main effect of treatment nor genotype by treatment interaction, for locomotion, rearing and stereotypical activity ($F_{1,59} = 234$; 129; and 541, respectively, $P_s < 0.05$). As has been previously shown,³⁰ DAT-KO mice had significantly higher cumulative baseline activities than WT mice (Figures 1a to c). For the cumulative post-injection period, there were significant main effects of genotype (locomotion: $F_{1,59} = 117$; rearing: $F_{1,59} = 35.8$; stereotypy: $F_{1,59} = 234$, $P_s < 0.001$) and treatment (locomotion: $F_{2,59} = 41.9$; rearing: $F_{2,59} = 14.4$; stereotypy: $F_{2,59} = 31.9$, $P_s < 0.001$), with a significant genotype by treatment interaction (locomotion: $F_{2,59} = 35.3$; rearing: $F_{2,59} = 11.1$; stereotypy: $F_{2,59} = 10.4$, $P_s < 0.001$). Pair-wise comparisons showed that the activities of WT mice were unaffected by 40 or 80 mg kg⁻¹ of PregS (Figures 1d to f). However, relative to vehicle controls, the activities of DAT-KO mice were partially suppressed with 40 mg kg⁻¹ PregS ($P_s < 0.005$) and further suppressed with 80 mg kg⁻¹ PregS ($P_s < 0.001$). Activities of DAT-KO mice treated with 80 mg kg⁻¹ PregS were suppressed to levels that were comparable to WT vehicle control.

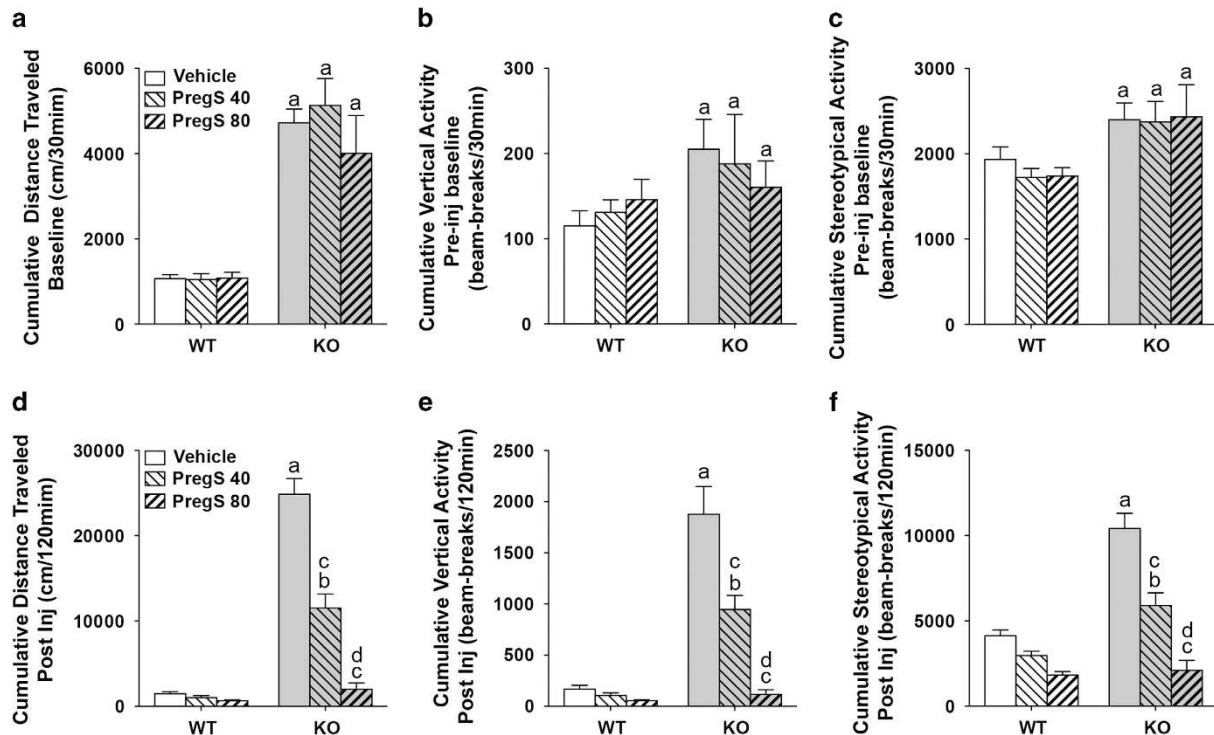


Figure 1. Pregnenolone sulfate (PregS) suppresses hyperlocomotor activities in DAT-KO mice. Baseline activities (pre-injection baseline) were measured for 30 min before mice were injected (intraperitoneally) with vehicle (Veh), or 40 or 60 mg kg⁻¹ PregS, and locomotor activities were monitored for a further 2 h (post-injection). Cumulative distance traveled (a), vertical activity (b) and stereotypical activity (c) at baseline are shown. Cumulative post-injection activities are shown for horizontal activity (d), rearing (e) and stereotypy (f). *N* = 10–16 mice per genotype per treatment condition; ^a*P* < 0.05, WT-Veh versus DAT-KO-Veh; ^b*P* < 0.05, within treatment groups WT versus DAT-KO; ^c*P* < 0.05, within groups versus Veh; ^d*P* < 0.05, within groups PregS40 versus PregS80. DAT-KO, dopamine transporter knockout; WT, wild type.

PregS rescues PPI deficits in DAT-KO mice

The mice received treatments of either 20 or 40 mg kg⁻¹ PregS to investigate the effects of PregS on PPI. There were no genotype differences for the percentage of null activity (WT: 1.32–10.31%; DAT-KO: 2.23–10.76%) and for the startle response. In addition, the treatment did not affect startle responses (Figure 2b). There were significant main effects of genotype, treatment and prepulse ($F_{1,204} = 82.2$, $F_{2,204} = 22.9$ and $F_{2,204} = 161$, respectively, $P_s < 0.001$), with significant treatment by genotype interaction ($F_{2,204} = 21.8$, $P < 0.001$). DAT-KO mice showed distinct PPI deficits compared with WT controls at all prepulse intensities ($P_s < 0.005$). WT mice were not affected by 20 or 40 mg kg⁻¹ PregS treatment. Relative to WT controls, PPI deficits of DAT-KO mice were partially rescued with 20 mg kg⁻¹ PregS and fully rescued with 40 mg kg⁻¹ PregS ($P_s < 0.05$, Figure 2a).

PregS alleviates the cognitive deficits in DAT-KO mice

To evaluate the effect of PregS on cognition, WT and DAT-KO mice were administered 40 mg kg⁻¹ PregS for 10 days, and then examined in the novel object recognition and social transmission of food preference tests. For the novel object recognition test, there were significant main effects of genotype, treatment and test session ($F_{1,176} = 21.5$, $F_{1,176} = 21.5$ and $F_{3,176} = 13.4$, respectively, $P_s < 0.001$), with significant genotype by treatment interaction ($F_{1,176} = 14.0$, $P_s < 0.001$). During training, no preference was shown for either identical object used by genotype or treatment group (Figure 3a). During the testing, vehicle-treated WT mice showed preference for the novel object for all the three test sessions ($t(>0.318) < 4.46$, $P_s < 0.01$). PregS treatment did not affect the preference scores of WT mice, with them showing preference for the novel object ($t(>0.170) < 7.15$, $P_s < 0.001$). In contrast, vehicle-treated DAT-KO mice showed no preference for

either the novel or familiar object during each of the three test sessions. For all the three test sessions, PregS-treated DAT-KO mice show increased preference for the novel object relative to vehicle-treated DAT-KO mice ($P_s < 0.001$), to levels similar to vehicle- or PregS-treated WT mice (Figure 3a). For short-term, long-term and remote memory, a one-sample *t*-test showed that the preference scores of PregS-treated DAT-KO mice were significantly higher than 0 ($t(>4.14) < 6.17$, $P_s < 0.001$). For the total duration of object exploration, the only significant main effect was that of test session ($F_{3,96} = 5.72$, $P < 0.01$), with no other significant main effects or interactions (Figure 3b). Thus, the differences in preference scores were not due to genotypic differences in the total duration of object exploration. As such, the reduced preference for the novel object by the DAT-KO mice is not due to genotype differences in durations of object explorations.

For the social transmission of food preference test, there were significant main effects of genotype, treatment and test session ($F_{1,105} = 16.9$ and 17.7, respectively, $P_s < 0.001$), and significant genotype by treatment interaction ($F_{1,105} = 20.4$, $P_s < 0.001$). Vehicle-treated WT mice showed positive preference, indicating that they preferred the familiar diet over the novel diet across all the three test sessions ($t(>6.23) < 6.56$, $P_s < 0.001$). Preference scores of WT mice were not affected by PregS treatment ($t(>4.53) < 7.45$, $P_s < 0.001$, Figure 3c). Vehicle-treated DAT-KO mice showed no preference for either diet across the three sessions, whereas those treated with 40 mg kg⁻¹ PregS preferred the familiar diet for all the three test sessions ($t(>5.6) < 9.01$, $P_s < 0.001$), with preference scores that were significantly higher than that of their DAT-KO controls ($P_s < 0.05$) and that were similar to vehicle- or PregS-treated WT mice (Figure 3c). To ensure that the preference scores were not biased by genotype differences in motivation to consume food, the total amount of

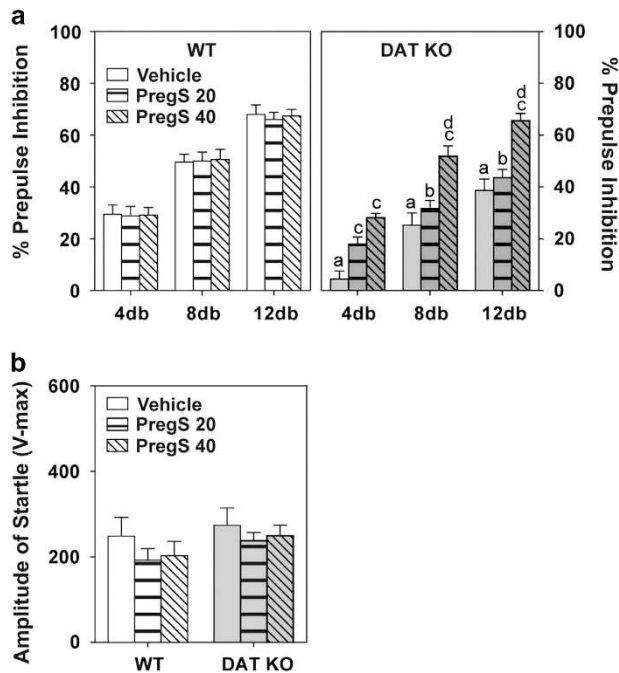


Figure 2. Pregnenolone sulfate (PregS) rescues PPI deficits in DAT-KO mice. WT and DAT-KO mice were injected (intraperitoneally) with vehicle (Veh), or 20 or 40 mg kg⁻¹ PregS before being tested. **(a)** PPI levels of WT (left) and DAT-KO (right) mice. **(b)** Amplitude of startle responses of WT and DAT-KO mice. *N* = 9–17 mice per genotype per treatment condition; ^a*P* < 0.05, WT-Veh versus DAT-KO-Veh; ^b*P* < 0.05, within treatment groups WT versus DAT-KO; ^c*P* < 0.05, within groups versus Veh; ^d*P* < 0.05, within groups PregS40 versus PregS80. DAT-KO, dopamine transporter knockout; PPI, prepulse inhibition; WT, wild type.

food consumed by each group of mice for each test session were calculated. The only significant main effect detected was that of test session ($F_{2,105} = 9.37$, $P < 0.01$; Figure 3d). Therefore, the differences in preference scores between the test groups are not due to differing motivations for consuming the different diets.

PregS antagonizes MK801-induced behavioral deficits in WT mice
As PregS is a known positive allosteric modulator of NMDA receptors, WT mice were administered MK801 to induce behavioral deficits, and then treated with PregS or its precursor, Preg (Figure 4). Both locomotor activity and PPI paradigms were used to determine the efficacy of PregS versus Preg. Cumulative post-injection distance traveled showed a significant main effect of treatment for distance traveled in the open field test ($F_{5,53} = 54.1$, $P < 0.001$; Figures 4a and b). WT mice treated with 0.1 mg kg⁻¹ MK801 had significantly increased locomotion relative to vehicle-treated mice ($P < 0.001$). MK801-induced hyperlocomotion was fully suppressed by 80 mg kg⁻¹ PregS ($P < 0.001$), but was partially suppressed by 60 mg kg⁻¹ Preg ($P < 0.001$) to levels that were still higher than that of vehicle-treated WT mice ($P_s < 0.05$).

In PPI, the main effects of treatment and prepulse intensity were significant ($F_{5,222} = 36.0$ and $F_{2,222} = 214$, respectively, $P_s < 0.001$), but the treatment and prepulse intensity interaction were not. MK801 treatment suppressed PPI relative to vehicle-treated WT for all the three prepulse intensities ($P_s < 0.001$; Figure 4c). At 40 mg kg⁻¹, PregS restored PPI of MK801-treated WT mice ($P_s < 0.001$) to levels of the vehicle-treated WT mice at all the three prepulse intensities, whereas 60 mg kg⁻¹ Preg only partially rescued the MK801-induced PPI deficits at the 4 and 8 db prepulse intensities ($P_s < 0.01$). At the 12 db prepulse intensity, 60 mg kg⁻¹

Preg could not fully rescue PPI deficits of MK801-treated WT mice ($P_s < 0.05$).

Acute treatment of PregS, but not Preg, activates AKT/GSK signaling pathway

As PregS could antagonize MK801-induced hyperlocomotion, we tested whether acute treatment with PregS affected NMDA receptor-related downstream signaling pathways, such as the AKT/GSK3 β pathway, in a time-dependent manner. We found that there was significant main effect of time for phosphorylation levels of AKT and GSK3 β ($F_{3,13} = 10.7$; 12.4, respectively, $P_s < 0.001$; Figures 5a to c) in striatal tissues of PregS-treated WT mice. Maximal phosphorylation levels of AKT and GSK3 β occurred at 15 min after 80 mg kg⁻¹ PregS treatment. At 15 min, pAKT and pGSK3 β showed a 2.08 (± 0.185) and 1.87 (± 0.212) fold increase relative to vehicle ($n = 4$; $P_s < 0.01$).

For the DAT-KO treated groups, the phosphorylation levels of AKT and GSK3 β showed a significant main effect of time ($F_{1,21} = 77.2$; 29.2, respectively, $P_s < 0.001$, Figures 5d to f). At 15 and 30 min, pAKT and pGSK3 β levels were higher than that of baseline ($P_s < 0.005$). Maximal levels of pAKT and pGSK3 β occurred at 30 min after 80 mg kg⁻¹ PregS treatment, which were significantly higher than at 15 and 60 min ($P_s < 0.001$). Phosphorylated GSK3 β levels returned to baseline levels 60 min after PregS treatment, whereas pAKT levels still remained elevated ($P_s < 0.001$).

In contrast, Preg, the precursor of PregS, showed no significant effect on pAKT in DAT-KO mice (Supplementary Figures S1A and B). There was a significant main effect of time on the phosphorylation levels of GSK3 β ($F_{1,11} = 4.00$, $P_s = 0.0375$, Supplementary Figures S1A and C), with maximal levels of pGSK3 β occurring at 30 min after 60 mg kg⁻¹ Preg treatment ($P_s = 0.038$, relative to baseline). Phosphorylation levels of pGSK3 β at 15 min and 60 min were not significantly different from 0 min.

Long-term treatment of PregS, but not Preg, increases NMDA receptor NR1 subunit levels

As the 10-day treatment with PregS improved the performance of DAT-KO mice in learning and memory tests (Figure 3), we tested whether long-term PregS treatment changes NMDA receptor levels in the hippocampus of WT and DAT-KO mice (Figure 5g). In WT mice, there was no significant main effect of treatment on the expression of the NR1 subunit of the NMDA receptor (Figure 5h), whereas in DAT-KO mice, long-term PregS treatment significantly increased NR1 levels in the hippocampus relative to vehicle treatment ($F_{1,7} = 37.62$, $P < 0.001$; Figure 5i). In contrast, although long-term Preg treatment has also been shown to improve cognitive performance in DAT-KO mice,²⁸ hippocampal NR1 expression levels did not increase with treatment in both WT and DAT-KO mice (Supplementary Figure S1D to F).

DISCUSSION

In this study, we characterized the efficacy of PregS on alleviating the schizophrenia-like behaviors in DAT-KO mice. DAT-KO mice show an elevated dopaminergic tone and as a result, exhibit certain schizophrenia-like endophenotypes, including hyperlocomotion, stereotypy,²⁹ deficits in prepulse inhibition^{28,31} and impaired spatial memory.²⁸ Previous studies have also shown that dysregulation in the AKT/GSK3 β pathway is the underlying cause of hyperlocomotion in DAT-KO mice.¹³

Here, we showed that acute administration of PregS in DAT-KO mice dose-dependently suppressed their hyperlocomotion and stereotypic activity (Figure 1), and rescued prepulse inhibition deficits (Figure 2). Long-term administration of 40 mg kg⁻¹ PregS alleviated the cognitive deficits of DAT-KO mice in the novel object recognition and social transmission of food preference paradigms (Figure 3). Therefore, increasing the circulating levels of

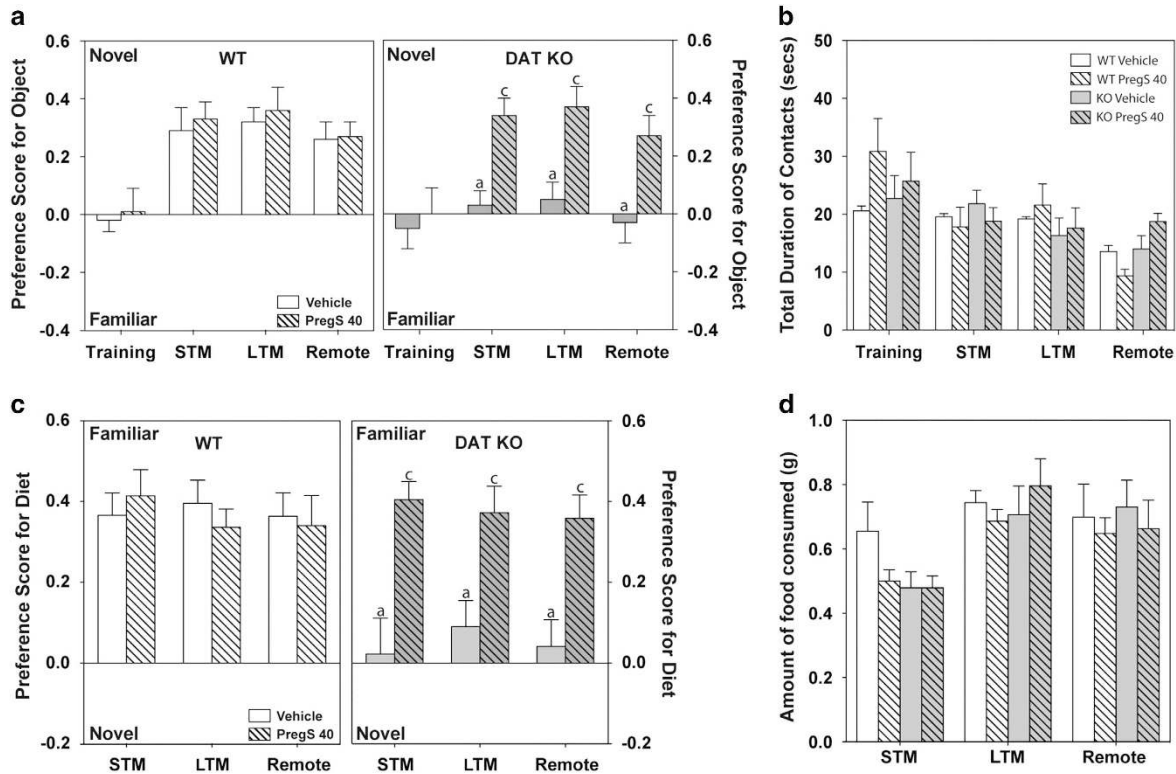


Figure 3. Pregnenolone sulfate (PregS) alleviates cognitive deficits in DAT-KO mice. WT and DAT-KO mice were treated with vehicle (Veh) or 40 mg kg⁻¹ PregS for 10 consecutive days before being tested in the paradigms of novel object recognition (a) and social transmission of food preference (c) for short-term (STM), long-term (LTM) and remote memory. Total duration of contacts with both objects (b) in the novel object recognition test and the total amount of food consumed (d) in the social transmission of food preference test were also calculated. *N* = 9–12 mice per genotype per treatment condition for each test. ^a*P* < 0.05, WT-Veh versus DAT-KO-Veh; ^c*P* < 0.05, within groups versus Veh. DAT-KO, dopamine transporter knockout; WT, wild type.

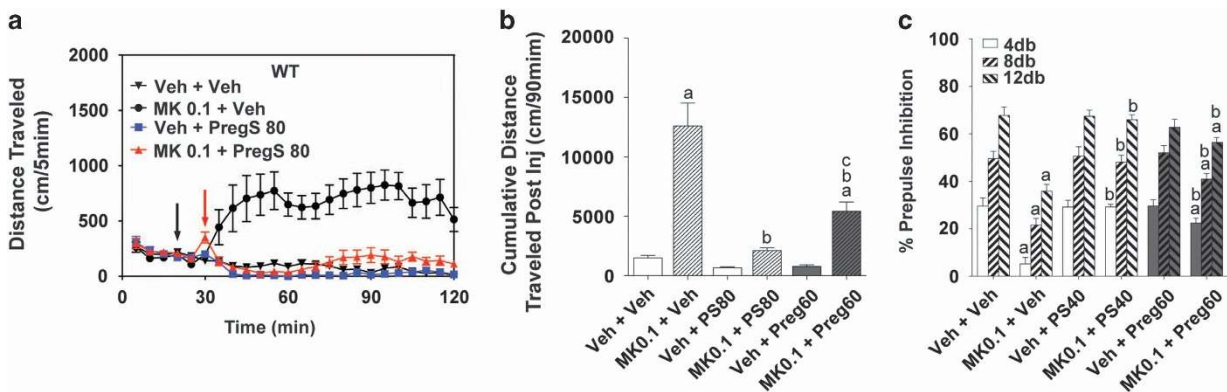


Figure 4. Pregnenolone sulfate (PregS) rescues MK801-induced hyperlocomotion and PPI deficit. (a) WT mice were treated with vehicle or MK801, followed by an injection of vehicle (Veh) or PregS 80 mg kg⁻¹ before being monitored for horizontal activity for 90 min. (b) PregS 80 mg kg⁻¹ fully suppressed MK801-induced hyperactivity, whereas 60 mg kg⁻¹ Preg showed partial suppression. (c) WT mice were treated with vehicle or MK801, followed by vehicle, PregS 40 mg kg⁻¹ or Preg 60 mg kg⁻¹, and subsequently tested in PPI. PregS 40 mg kg⁻¹ fully rescued MK801-induced PPI deficits, whereas 60 mg kg⁻¹ Preg showed partial effects (c). *N* = 9–14 mice per treatment condition for each test. ^a*P* < 0.05, versus WT Veh-Veh; ^b*P* < 0.05, versus WT MK801-Veh; ^c*P* < 0.05, MK801-PregS-treated groups versus MK801-Preg treated groups. PPI, prepulse inhibition; WT, wild type.

PregS alleviates the positive and negative schizophrenia-like endophenotypes in DAT-KO mice without adversely affecting WT mice.

PregS rescues the aberrant behaviors of DAT-KO mice by acting on the central nervous system

PregS is a neurosteroid that is converted from Preg by a sulfotransferase.^{37–41} However, the level of PregS in human brain

is controversial due to technical limitations, as the level of PregS is below the detection limit of 0.3 ng per gram tissue.³⁴ Even then, it is likely that the brain tissue levels of PregS are likely to be lower than its precursor, Preg.³⁷

When systemically administered, PregS was able to cross the blood–brain barrier to increase brain levels of PregS.⁴² Here, we show that systemic administration of PregS changes phosphorylation levels of signaling molecules in brain tissues (Figure 5). This confirms that the observed behavioral effects following acute

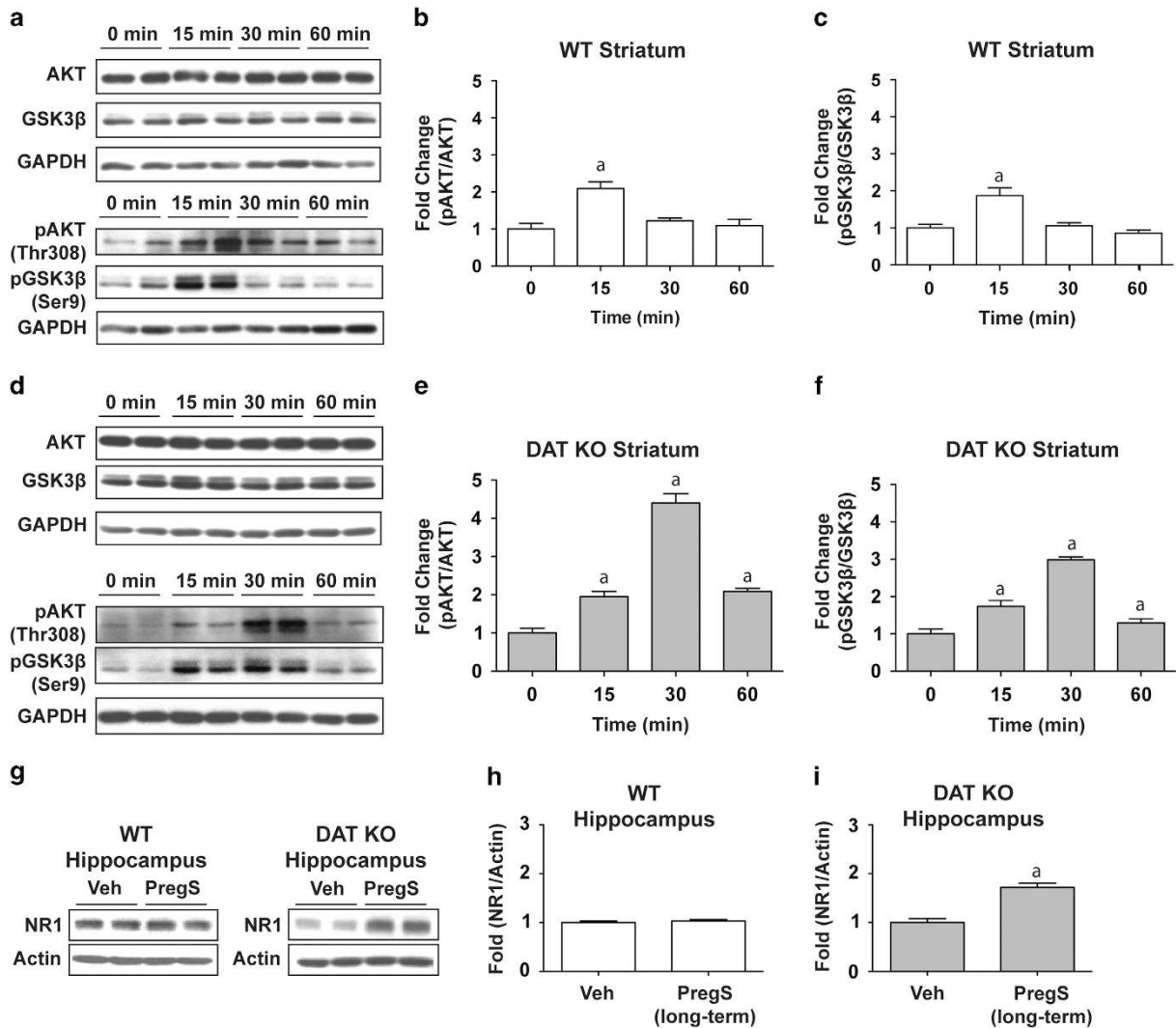


Figure 5. Modulatory effects of PregS on NMDA signaling. Effects of acute treatment of 80 mg kg⁻¹ PregS on AKT/GSK3β signaling in striatum were analyzed in WT (a–c) and DAT-KO (d–f) mice by antibodies against phospho-AKT (Thr308), phospho-GSK3β (Ser9) and their respective non-phosphorylated forms. (g–i) Long-term effects of chronic treatment of 40 mg kg⁻¹ PregS on NR1 subunit of NMDA receptor in hippocampus were characterized in WT and DAT-KO (g–i) mice. GAPDH (a,d) and actin (g) were used as loading controls for densitometry measures. Quantified ratios of phosphorylated/non-phosphorylated forms were normalized to 0 min. ^a*P* < 0.05, versus 0 min or vehicle (Veh) group. DAT-KO, dopamine transporter knockout; PregS, pregnenolone sulfate; WT, wild type.

PregS administration are due to the actions of PregS on the central nervous system.

PregS modulates NMDA signaling to rescue cognitive deficits
PregS has important regulatory effects on several neurotransmission signaling systems, such as those regulated by sigma-1 (ref. 43) and GABA_A⁴⁴ receptors. More importantly, PregS is a positive allosteric modulator of NMDA neurotransmission, possibly through its interaction with NR1/NR2B subunits.³⁵ Here, we showed that acute PregS treatment had antagonistic effects to a NMDA receptor antagonist, MK801, on locomotor activity and acoustic startle reflex behavior (Figure 4).

In addition, the concentration of PregS is known to be higher in the hippocampus, an area primarily associated with memory, than in the cortex,⁴⁵ which may give rise to the role of PregS in learning and memory. Previous studies have shown that PregS is an effective memory enhancer for rodent models.^{22,46,47} For example, the infusion of PregS into the hippocampus restored the memory deficits of aged rats in the Y maze.⁴⁸ PregS has also been shown to antagonize the memory deficits induced by NMDA receptor

antagonists.^{49,50} Here, we showed that long-term treatment of DAT-KO mice with PregS rescued their impaired episodic memory and poor discriminative abilities (Figure 3), and increased the expression of the NR1 subunit of the NMDA receptor in the hippocampus (Figure 5i). As NMDA receptor signaling is important for long-term potentiation,^{51,52} we postulate that the increased NR1 expression may be the underlying mechanism of the rescued cognitive deficits of DAT-KO mice. These data are also in agreement with recent findings that PregS can stimulate NMDA receptor surface expression.⁵³

Acute PregS treatment has modulatory effects on the AKT/GSK3β signaling pathway

It has been proposed that DAT-KO mice have dysregulated AKT/GSK3β signaling due to overstimulation of dopamine D2 receptor, which results in increased striatal GSK3β activation, that is, decreased GSK3β phosphorylation.¹³ Therefore, inhibition of GSK3β activity, (i.e. increased GSK3β phosphorylation), by a GSK3β inhibitor, such as 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione, lithium or dopamine D2 receptor antagonists, reduces

hyperlocomotion in DAT-KO mice.¹³ Interestingly, hyperdopamine-induced behavioral abnormalities of DAT-KO mice were further enhanced by MK801, but were attenuated by positive modulators of AMPA receptors, suggesting dysregulation of glutamatergic function in DAT-KO mice.⁵⁴ As NMDA receptor antagonists also affect AKT/GSK3 β signaling,^{55–57} the antagonistic action of PregS on MK801 effects has led us to hypothesize that PregS may modulate the NMDA receptor signaling through the phosphatidylinositol 3-kinase/AKT signaling.⁵⁸ To investigate this possibility, we treated WT and DAT-KO mice with 80 mg kg⁻¹ PregS for 15, 30 and 60 min, and measured the non-phosphorylated and phosphorylated forms of AKT and GSK3 β in the striatum (Figures 5a to f). In WT mice, we found that PregS treatment caused an increase in the phosphorylation levels of AKT and GSK3 β 15 min after treatment, and the phosphorylation levels returned to baseline after 30 min. In PregS-treated DAT-KO mice, there was a delayed activation of the AKT/GSK3 β phosphorylation levels, and AKT phosphorylation was persistent even at 60 min, concomitant with the attenuation of hyperlocomotion and stereotypic activity. Although PregS treatment increases phosphorylation levels of AKT and GSK3 β in both WT and DAT-KO mice, it is observed that there was a temporal difference in their responses. We propose that this temporal difference may be due to the dysregulated AKT/GSK3 β signaling in DAT-KO mice, evident from the decreased levels of AKT and GSK3 β phosphorylation at baseline.¹³

It is also worthwhile to note that in comparison to vehicle-treated controls, DAT-KO mice treated long term with PregS still exhibited hyperlocomotion, and showed no changes in pAKT/AKT and pGSK3 β /GSK3 β levels when assayed the day after the last PregS injection (data not shown). These results suggest that the different treatment regimens of PregS, acute versus long term, bring about different biological responses. The effect of PregS on striatal-driven behaviors, such as locomotion, is fast-acting and short-lasting; whereas the effect of PregS on hippocampal-driven behaviors, such as memory, requires prolonged treatment and is more long-lasting.

PregS and Preg have differing mechanisms of action

PregS and Preg are inter-convertible.^{38–41,59} Therefore, given our results presented here and that of Wong *et al.*,²⁸ where Preg and PregS treatment rescued the aberrant behaviors of DAT-KO mice, we carried out further experiments to determine whether the observed behavioral rescue was due to inter-conversion between PregS and Preg, or distinct PregS and Preg mechanisms of actions that converged on similar behavioral results.

Previous studies have shown that acute PregS treatment increases levels of Preg, allopregnanolone and 5 α -dihydroprogesterone in the brain.^{42,50} It has also been shown that in the brain, the conversion rate of PregS to Preg is lower than that of Preg to PregS.^{42,60} However, most older studies obtained the levels of PregS through indirect measurements, which are rather inaccurate,³⁴ and direct assays have shown that the level of PregS in the brain is actually very low.⁶¹ Yet, we would like to highlight that the rate of inhibition of locomotor responses of DAT-KO mice is similar between acute PregS and Preg treatments, which should not be the case if the conversion of PregS to Preg is at a different rate from the conversion of Preg to PregS.^{42,60} As such, it is unlikely that the inhibition of locomotor activity following PregS treatment is due to the conversion to Preg.

A previous study has shown that unlike PregS, Preg does not directly affect NMDA receptor actions.⁶² In addition, PregS, but not Preg, could potentiate NMDA-induced toxicity *in vivo*.⁶³ These evidences suggest that PregS directly, rather than through the conversion to Preg, affected NMDA receptor actions. Our results are congruent with previous findings, where PregS, but not Preg,

fully rescued MK801-induced hyperactivity and prepulse inhibition deficits (Figure 4).

When we investigated the effects of Preg on downstream signaling molecules, we found that Preg treatment did not increase pAKT levels in DAT-KO mice and the relative increase in pGSK levels is smaller than that of PregS-treated mice (Supplementary Figures S1A and C). These data suggest that even though PregS and Preg are inter-convertible, the observed effects on the AKT/GSK3 β signaling pathway following the administration of PregS, is not due to the conversion of PregS to Preg. This also points to the presence of distinct mechanisms underlying the action of PregS and Preg in attenuating schizophrenia-like endophenotypes in DAT-KO mice. In addition, we showed that long-term treatment with PregS, but not Preg (Supplementary Figure S1) increased expression of hippocampal NR1 subunit of NMDA receptor in DAT-KO mice. Therefore, our biochemical and behavioral results suggest that PregS, but not Preg, acts through the NMDA receptor-modulated AKT/GSK3 β signaling pathway to normalize the behaviors of DAT-KO mice.¹³

CONCLUDING REMARKS

Dysregulation of dopaminergic and glutamatergic signaling are key players in the pathophysiology of schizophrenia.^{2,3,5,6} Both of these signaling systems converge on the AKT/GSK3 β pathway, where dysregulation in the AKT/GSK3 β signaling pathway has been implicated in schizophrenia.^{18,64} In mouse models, mice deficient in AKT1 (ref. 65) or overexpressing GSK3 β ⁶⁶ show defects in working memory. The DAT-KO mice are known to have dysregulated AKT/GSK3 β signaling,¹³ and recapitulate several schizophrenia-like endophenotypes. We have shown here that acute PregS treatment increases striatal GSK3 β phosphorylation as a potential effect in normalizing the positive schizophrenia-like symptoms of DAT-KO mice. We have shown evidence that the actions of PregS is somewhat distinct from Preg, in terms of biochemical and temporal signaling patterns. However, the conversion of PregS to other neurosteroid metabolites, such as dehydroisoandrosterone sulfate,⁶⁷ cannot be ruled out without further studies to determine the metabolism of systemically delivered PregS, which will involve direct assays to measure the levels of the downstream biosynthesized neurosteroids. We also showed that long-term administration of PregS ameliorates the cognitive deficits of DAT-KO mice, possibly through increasing hippocampal NR1 levels.

The results obtained with this study suggest that PregS may be able to directly address the NMDA receptor hypofunction in schizophrenic patients, which in turn leads to the amelioration of the associated negative symptoms and cognitive deficits.^{68,69} This study also strengthens the potential use of neurosteroids as therapeutics in the treatment of neuropsychiatric disorders. However, more studies are required to understand the enzymatic kinetics that regulate the metabolism of Preg, PregS and the other downstream neurosteroids.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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