Prefrontal allopregnanolone synergizes with D_1 receptor activation to disrupt sensorimotor gating in male Sprague-Dawley rats

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Abstract

Rationale. The prepulse inhibition (PPI) of the startle reflex is the best-established index of sensorimotor gating. We documented that the neurosteroid allopregnanolone (AP) is necessary to reduce PPI in response to D1 dopamine receptor agonists. Since Sprague-Dawley (SD) rats are poorly sensitive to the PPI-disrupting effects of these drugs, we hypothesized that AP might increase this susceptibility.

Objectives. We tested whether AP is sufficient to increase the vulnerability of SD rats to PPI deficits in response to the D1 receptor full agonist SKF82958. Methods SD rats were tested for PPI after treatment with SKF82958 (0.05-0.3 mg/kg, SC) in combination with either intraperitoneal (1-10 mg/kg) or intracerebral (0.5 µg/µl/side) AP administration into the medial prefrontal cortex (mPFC) or nucleus accumbens shell. To rule out potential confounds, we measured whether SKF82958 affected the endogenous mPFC levels of AP.

Results. SD rats exhibited marked PPI deficits in response to the combination of systemic and intra-mPFC AP with SKF82958 but not with the D2 receptor agonist quinpirole (0.3-0.6 mg/kg, SC). SKF82958 did not elevate mPFC levels of AP but enhanced the content of its precursor progesterone. The PPI deficits caused by SKF82958 in combination with AP were opposed by the AP antagonist isoallopregnanolone (10 mg/kg, IP) and the glutamate NMDA receptor positive modulator CIQ (5 mg/kg, IP).

Conclusion. The results suggest that AP enables the detrimental effects of D1 receptor activation on sensorimotor gating. AP antagonism or glutamatergic modulation counters these effects and may have therapeutic potential for neuropsychiatric disorders characterized by gating deficits.

Keywords: prepulse inhibition of the startle, sensorimotor gating, dopamine, D1 receptors, allopregnanolone, isoallopregnanolone, neuroactive steroids

Introduction

Sensorimotor gating is the information-processing domain whereby a neural system filters out extraneous external or internal information from higher-order processing to prevent information overload and enable the formation of saliency maps (Butler et al. 1990). Gating deficits are thought to reflect poor inhibitory functioning and disrupt the integrity of cognitive functioning. The relevance of assessing sensory gating abnormalities in the clinical setting lies in the presence of multiple neuropsychiatric disorders, encompassing schizophrenia, obsessive-compulsive disorder (OCD), and Tourette syndrome (TS) (Geyer 2006). One of the best operational measures to assess the integrity of sensorimotor gating is afforded by the prepulse inhibition (PPI) of the acoustic startle reflex. In this cross-species endophenotype, the startle response elicited by a sudden loud noise is robustly blunted by a weak, nonstartling prestimulus (Hoffman and Ison 1980).

Dopamine plays a critical role in shaping sensorimotor gating. In animal models, these effects have been primarily ascribed to the involvement of the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) through the activation of both D1 and D2 receptors; however, the specific sensitivity to these two classes of receptors varies, depending on the genetic background (Swerdlow et al. 2001). In rats, all the main strains available for experimental use display PPI deficits in response to D2 receptor agonists. Conversely, while D1 receptor agonists reduce PPI in Long-Evans rats (Mosher et al. 2016), they impair this parameter only at relatively high doses (Wan et al. 1996; Ralph and Caine, 2005; Mosher et al. 2016) in Sprague- Dawley (SD) rats, possibly due to the activation of other dopamine receptors (Wan et al. 1996; Mosher et al. 2016). In most mouse strains, D1-like receptor agonists disrupt PPI in an antipsychotic-sensitive fashion (Ralph-Williams et al. 2003); however, selective D2 receptor agonists do not elicit PPI deficits in this species, except for a few strains (Ralph and Caine 2005).

While the mechanisms of this strain- and species-dependent differential sensitivity remain elusive, we previously documented that the sensitivity of mice and Long-Evans rats to D1 receptor agonists is opposed by the inhibition of 5α-reductase (5αR) (Bortolato et al. 2008; Frau et al. 2013; Frau et al. 2016). This family of steroidogenic enzymes catalyzes the saturation of the 4,5-double bond of the A ring of progesterone, converting it into dihydroprogesterone (DHP). In turn, this compound is metabolized into 5α-pregnan-3αol-20-one (allopregnanolone; AP) by 3α-hydroxysteroid oxidoreductase (3α HSOR) (Paba et al. 2011).

Building on this evidence, we recently showed that genetic deficiency of 5αR type 1 rendered C57BL/6 mice insensitive to the PPI-disrupting properties of D1 receptor agonists (Mosher et al. 2019). Most importantly, this sensitivity was restored by acute injection of AP, suggesting that this neurosteroid is necessary to enable the regulation of sensorimotor gating by D1 receptors (Mosher et al. 2019). We also showed that, in both

C57BL/6 mice and Long-Evans rats, but not SD rats, AP in the mPFC elicits PPI deficits (Cadeddu et al. 2022). These data align with previous evidence that 5αR inhibition reversed the PPI deficits induced by dopaminergic agonists by acting in the mPFC and the NAc shell (NAcS) (Devoto et al. 2012). Interestingly, the PPI-disruptive effects of AP were reversed by isoallopregnanolone (isoAP), the endogenous 3β-epimer of AP that acts as

an antagonist to the AP-binding site within GABA-A receptors (Johansson et al. 2016).

These premises suggest that the lack of sensitivity to the PPI deficits induced by D1 receptor agonists and AP may be underpinned by common mechanisms. Specifically, three key questions remain open: 1) Is AP administration sufficient to confer sensitivity to D1 receptor activation in spontaneously refractory subjects? 2) What brain regions and 3) What receptors are implicated in these effects? To answer these questions, we first tested whether AP administration may render SD rats sensitive to the PPI-disruptive effects of the potent D1 receptor full agonist SKF82958. Next, to assess what receptors mediate AP's effects, we tested their reversal by isoAP. Finally, since we previously showed that low doses of NMDA glutamate blockers rendered SD rats susceptible to the PPI-disrupting effects of D1 receptor agonists (Bortolato et al. 2005), we verified whether this receptor is also involved in the synergism of D1 receptor agonists and AP.

Materials and methods

Animals

Adult male SD (Envigo, Bresso, Italy) rats weighing 250- 300 g were used for this study. Animals were group-housed in cages (n=4) with ad libitum access to food and water. The room was maintained at 22±0.2°C on a 12/12-h dark/light cycle (with lights off at 07:00 AM). Experiments occurred between 9:00 AM and 12:00 PM to minimize potential circadian bias in the effects of dopaminergic agonists on PPI and startle parameters. Each rat was tested only once throughout the study, except for experiments 3.4-3.5, where animals were tested in counterbalanced order. Every effort was made to minimize animal pain, suffering, and distress. All the experimental procedures were carried out per the European Union directive (EU Directive 2010/63) and Italian legislation (DLgs. 2014/26).

Drugs

The following drugs were used: AP, SKF82958 hydrobromide, (−)-quinpirole hydrochloride, dizocilpine maleate (Sigma-Aldrich, St Louis, MO), CIQ (Tocris Bioscience, Bristol, UK), and isoAP (donated by Asarina Pharma Ab, Solna, Sweden). SKF82958 was dissolved in distilled water. Quinpirole and dizocilpine were dissolved in a sterile 0.9% saline solution. IsoAP and AP were suspended in 3% and 10% hydroxypropyl β-cyclodextrin, respectively. Finally, CIQ was suspended in 2.5% DMSO and 2.5% Tween 80.

SKF82958, quinpirole, and dizocilpine were administered subcutaneously (SC), while AP, isoAP, and CIQ intraperitoneally (IP) in an injection volume of 1 and 2 ml/ kg body weight,

respectively. For intracerebral infusions, AP and dizocilpine were dissolved in a cyclodextrin/Ringer solution (final concentration, 1:5 v: v). Doses (indicated as mg/kg of salts) and injection times of the drugs used in these experiments were based on previous studies from our group and others (Wan et al. 1996; Geyer et al. 2001; Bortolato et al. 2005; Devoto et al. 2012; Suryavanshi et al. 2014; Frau et al. 2016; Mosher et al. 2016; Cadeddu et al. 2020).

Stereotaxic surgery and intracerebral infusion procedures

Rats underwent general anesthesia with a mixture (20:1) of fentanyl (Pfizer, New York, NY) plus the α2 adrenergic agonist medetomidine hydrochloride (Orion Pharma, Turku, Finland; 5ml/kg, IP). Afterward, rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA) with blunt ear bars to avoid damage to tympanic membranes. Under aseptic conditions, rats were shaved, and their scalp was retracted. Bilateral craniotomies were performed above the target sites, and stainless steel 22-G guide-cannulae (Plastics One, Roanoke, VA) were lowered slowly into place and implanted using dental cement and two skull screws. The lengths of the cannulae were selected to end 1 mm above the targeted areas, with the corresponding injector projecting 1 mm beyond the guide tip. Cannulae were plugged with wire stylets, and wounds were closed with surgical staples. Stereotaxic surgeries targeted two regions: mPFC (coordinates from the skull surface: AP = +3.0 mm, ML = ± 0.5 mm; DV = −3 mm) and NAcS (coordinates from the skull surface: (AP = +1.7 mm, ML = ± 0.8 mm; DV = −7.4 mm). Coordinates were based on the brain atlas by Paxinos and Watson (1998).

Rats were given enrofloxacin for five days (Bayer HealthCare, Shawnee Mission, KS) and allowed to recover in their home cages for 7-10 days before testing. On the day of the test, AP was administered into the mPFC or NAcS through 33-gauge internal cannulae (Plastics One) connected to a 50 µl syringe (Hamilton, Reno, NV, USA) by PE tubing (Intramedic, New York, NY). The infusion rate (0.5 µl/min) was controlled by microinjection pumps (CMA Microdialysis, Stockholm, Sweden). Injections were confirmed by monitoring the movement of liquid in the tubing via a small air bubble. The injectors were left in place for 2 min after infusion to allow fluid diffusion. PPI testing took place immediately upon completion of AP infusion. Rats were sacrificed after testing, and the locations of cannula tips were histologically verified by trained operators blind to behavioral results. Four animals were excluded from the analysis due to incorrect cannula placement.

Startle reflex and PPI

Startle reflex and PPI were measured as previously described (Bortolato et al. 2004). The apparatus used to detect startle reflex (Med Associates, St Albans, VT) consisted of 4 standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 9 cm diameter mounted on a piezoelectric accelerometric platform connected to an analogdigital converter. Two separate speakers conveyed background noise and acoustic bursts, each adequately placed to produce a sound variation within 1 dB across the startle cage. Both speakers and startle cages were connected to the main PC, which detected and analyzed all chamber variables with

specific software. Before each testing session, acoustic stimuli and mechanical responses were calibrated via specific devices supplied by Med Associates. On the testing day, each rat was placed in the cage for a 5-min acclimatization period consisting of 70-dB white noise background, which continued for the remainder of the session. Each session consisted of three consecutive sequences of trials (blocks).

During the first and third blocks, rats were presented with only five pulse-alone trials of 115 dB. The second block consisted of a pseudorandom sequence of fifty trials, including twelve pulse-alone trials; thirty trials with a pulse preceded by 74-, 78-, or 82-dB prepulses (10 for each level of prepulse loudness); and eight no-stimulus trials, where only the background noise was delivered. Pulse and prepulse durations were set at 40 and 20 ms, respectively. Inter-trial intervals were selected randomly between 10 and 15 s, while the inter-stimulus intervals were set at 100 milliseconds. The startle response was based on the first positive wave that meets the minimum wave criteria and determined as the mean startle amplitude of the pulse-alone trials relative to the second block. Startle amplitude was measured by converting the analog voltage signal (recorded by the startle sensor and ranging from −10 to +10 V) to arbitrary digital units between –2048 and +2048. The % PPI was calculated only on the values relative to the second block using the following formula: [(mean startle amplitude for pulse alone trials - mean startle amplitude for prepulse + pulse trials)/mean startle amplitude for pulse alone trials] × 100. Although our protocol was specifically designed to measure PPI, both startle amplitude and PPI values were consistently described, as changes in startle reflex may lead to computational artifacts in the parallel calculation of PPI.

Steroid quantification

Rats were treated with SKF82958 (0.3 mg/kg, SC) and, after 15 min, they were sacrificed, and the PFCs were collected and snap-frozen. Samples were extracted and purified as previously described (Gaikwad 2013). Following extraction with 0.7 ml chloroform, samples were centrifuged, and the chloroform fraction was transferred to a 2 ml tube and dried. The resulting residue was again extracted with 0.5 ml methanol. The methanol fraction was then added to the above chloroform fraction; the mixture was dried and resuspended in 130 µl methanol, filtered, and filtrates were transferred to vials for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC- MS/MS) analysis. UPLC-MS/MS analyses were performed using a Waters Acquity UPLC system connected with the high-performance triple quadrupole (TQD) mass spectrometer. Analytical separations were conducted as described by Bosse et al. (2021). The elution from the UPLC column was introduced to the TQD mass spectrometer. All MS experiments were performed by using electrospray ionization (ESI) in positive ion (PI) mode, with an ESI-MS capillary voltage of 4.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 500 V. Desolvation gas was kept at 500 l/h, with the temperature at 350 °C and source temperature at 150 °C. Pure standards of all targeted steroids were used to optimize the UPLC- MS/MS conditions before analysis and making calibration curves. Reference standards were purchased from Steraloids (Newport, RI). Stock steroid standards were prepared by mixing 5 µl of 1 mg/ml solution of each steroid and adjusting the final volume to 1 ml using methanol. All the stock solutions were stored at - 80 °C.

Reference standards were run before the first sample, in the middle of the runs, and after the last sample to prevent errors due to the matrix effect and day-to-day instrument variations. In addition, spiked samples were run before the first sample and after the last sample to calibrate for the drift in the retention time due to the matrix effect. After standard and spiked sample runs, several blanks were injected to wash the injector and avoid carry-over effects. The resulting data were processed using Waters Target Lynx 4.1 software (Bosse et al. 2021). The analyte concentrations were normalized per gram of tissue before extraction.

Data analysis

Normality and homoscedasticity of data distribution were verified by the Kolmogorov-Smirnov and Bartlett's tests. Analyses were performed by multiple-way ANOVAs, as appropriate, followed by Tukey's test (with Spjøtvoll-Stoline correction for unequal N whenever required) for post

hoc comparisons of the means. For % PPI analyses, data relative to different prepulse levels were collapsed since no interactions were found between prepulse levels and other factors throughout the study. Significance was set at $P<0.05$.

Results

AP renders SD rats sensitive to the PPI-disrupting effects of SKF82958

The first series of experiments aimed to verify whether AP may modify the effects of the D1 receptor agonist SKF82958 (Fig. 1). We first measured the combined systemic effects of AP (10 mg/kg, IP, 15 min before testing) and SKF82958 (0.05-0.3 mg/kg, SC, 10 min before testing) (n=8-9/group) (Fig. 1A-B). While SKF82958 dosedependently increased startle amplitude (Fig. 1A), these effects were not modified by AP. Conversely, the combination of AP and SKF82958 significantly reduced PPI (Fig. 1B).

We then tested whether the same synergistic effects could be observed following intra-mPFC or intra-NAcS administration of AP. In preliminary experiments, we found that stereotaxic surgery in the mPFC also rendered rats sensitive to high SKF82958 doses, likely in relation to post-surgical alterations. Thus, we tested the effects of the lowest SKF82958 dose (0.05 mg/kg, SC, 10 min before testing) with intra-mPFC injections of AP (0.5 µg/µL/ side; 5 min before testing) (n=10-14/group). SKF82958 increased startle amplitude, but this effect was not modified by AP (Fig. 1C). The combination of intra-mPFC AP and SKF82958 significantly reduced PPI (Fig. 1D). Notably, the association of systemic SKF82958 and intra-NAcS AP failed to modify startle amplitude (Fig. 1E) and PPI (Fig. 1F). SKF82958 increases the levels of progesterone and some of its metabolites, but not AP, in the PFC.

To rule out any potential direct effect of SKF82958 on endogenous AP synthesis, we also assessed the steroidomic effects of this D1 receptor agonist in the PFC. We found that SKF82958 (0.3 mg/kg, IP, 15 min before euthanasia) increased the content of progesterone and its metabolites 17- and 21-hydroxyprogesterone (deoxycorticosterone)

and reduced DHEA, but had no direct effect on pregnenolone, 17-hydroxypregnenolone, androstenedione, DHP, or AP (Fig. 2). Similarly, SKF82958 treatment failed to change the levels of all the other tested neuroactive steroids (Supplementary Table 1).

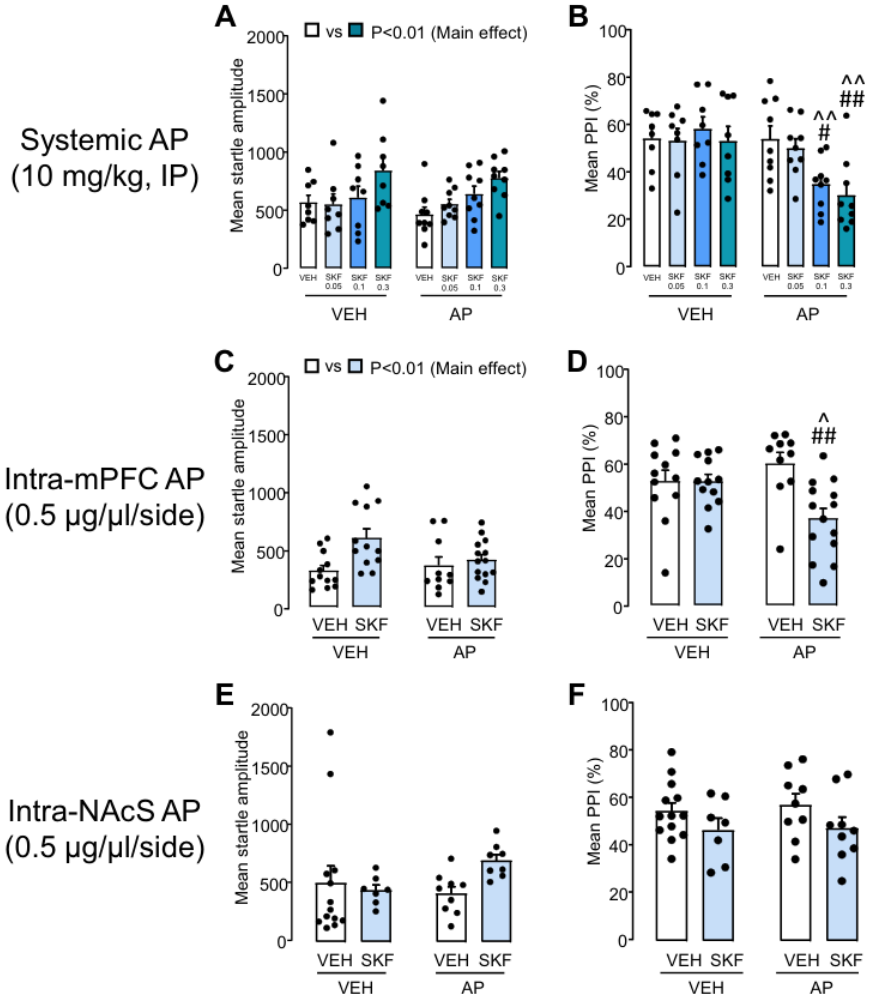


Fig. 1 The synergism of allopregnanolone (AP) and SKF82958 (SKF) disrupts the prepulse inhibition (PPI) of the startle reflex in male Sprague-Dawley rats. The effects of AP were tested after A-B) systemic (10 mg/kg, IP),C-D) intra-medial prefrontal cortex (mPFC) (0.5 µg/µl/side), and E-F) intra-Nucleus Accumbens Shell (NAcS) (0.5 µg/µl/side) administration. A) SKF (0.05-0.3 mg/kg, SC) dose-dependently increased startle amplitude [Main SKF effect: $F(3,60)=5.93$, $P<0.01$, $\eta2=0.22$; $P<0.01$ for comparisons between SKF 0.3 and its vehicle (VEH)]; conversely, systemic AP had no direct impact on startle [Main AP effect: $F(1,60)=0.40$, $P=0.53$, $\eta2=0.005$] and did not modify the elevation of startle caused by SKF [SKF × AP Interaction: $F(3,60)=0.32$, $P=0.81$, $\eta2=0.01$]. B) In the same experiment, the combination of systemic AP and SKF significantly reduced PPI [SKF × AP interaction: (3,60) 3.16, $P<0.05$, $\eta2=0.11$]. C) SKF (0.05 mg/kg, SC) increased startle amplitude [Main SKF effect: $F(1,44)=7.48$, $P<0.01$, $\eta2=0.13$]. Intra-mPFC infusions of AP did not intrinsically affect startle amplitude [Main AP effect: $F(1,44)=1.43$, $P=0.24$, $\eta2=0.03$] and did not modify the effects of SKF82958

[SKF × AP interaction: F(1,44)=3.77, P=0.06, η2=0.07]. D) However, while intra-mPFC AP failed to affect PPI [Main AP effect: F(1,44)=0.96, P=0.33, η2=0.02], its combination with SKF significantly reduced PPI [SKF × AP interaction: F(1,44)=7.55,

P<0.01, η2=0.13]. E) Neither systemic SKF (0.05 mg/kg, SC) nor intra-NAcS AP infusion altered startle amplitude [Main SKF effect: F(1,33)=0.90, P=0.35, η2=0.02; Main AP effect: F(1,33)=0.50, P=0.48, η2=0.01]. ANOVA also failed to detect any significant interaction between these treatments [SKF × AP Interaction: F(1,33)=2.31, P=0.14, η2=0.06]. F) These treatments also failed to modify PPI values, either per se [Main SKF effect: F(1,34)=3.95, P=0.06, η2=0.10; Main AP effect: F(1,34)=0.14, P=0.71, η2=0.004] or in combination [SKF × AP Interaction: F(1,34)=0.04, P=0.84, η2=0.001]. All data were analyzed by two-way ANOVA designs followed by Tukey's test. PPI values are indicated as the mean of the three values corresponding to each prepulse loudness. Main effects of SKF (followed by post-hoc comparisons where appropriate) are indicated as comparisons between white columns (VEH) vs. colored columns (SKF). SKF doses are given in mg/kg and indicated under the colored bars. #, P<0.05; ##, P<0.01 for post-hoc comparisons between AP-treated rats, injected with either SKF or its vehicle. ^, P<0.05; ^^, P<0.01 for post-hoc comparisons between AP-treated rats injected with SKF and VEH-treated rats treated with the same dose of SKF. All data are shown as means ± SEM. A-B) n=8-9/group; C-D- E-F) n=10-14/group. For further details, see text.

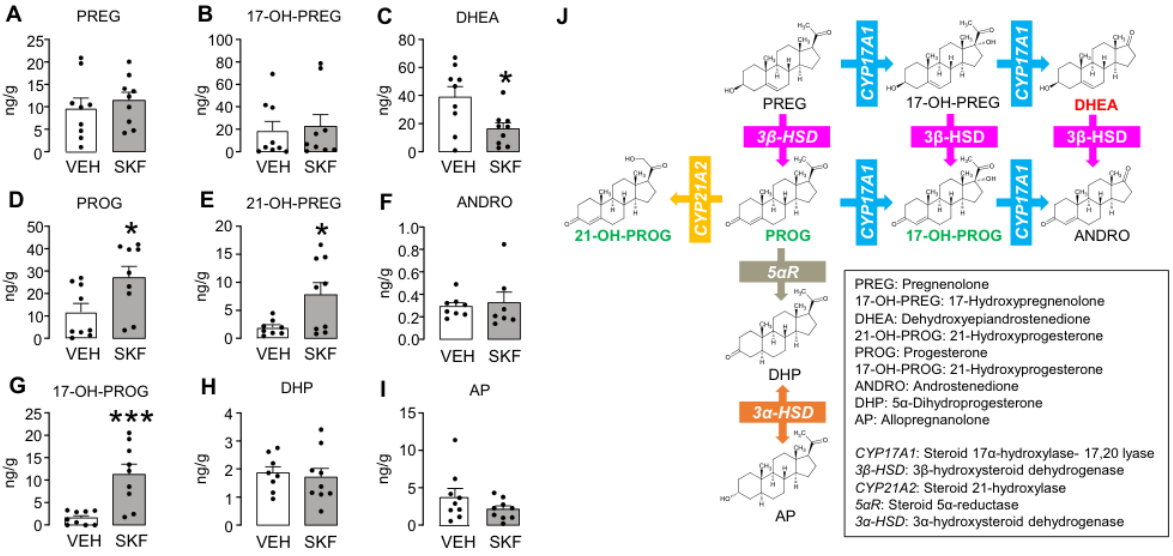


Fig. 2 Effects of SKF82958 (SKF: 0.3 mg/kg, IP) on the steroidomic profile of the medial prefrontal cortex of male Sprague- Dawley rats. SKF failed to increase the mPFC of either A) pregnenolone [F(1,16)=1.60, P=0.56, η2=0.02] or B) 17-OH-pregnenolone [F(1,16)=1.55, P= 0.78, η2=0.005]. Conversely, this treatment significantly reduced C) dehydroepiandrosterone (DHEA) levels [F (1,16) =6.81, P<0.05, η2=0.30] and increased the levels of D) progesterone [F (1,16) =5.81, P<0.05, η2=0.27] and its two metabolites E) 21-hydroxyprogesterone (deoxycorticosterone) [F (1,16) =6.20, P<0.05, η2=0.29] and G) 17-OH progesterone [F (1,16) =17.14, P<0.001, η2=0.52]. Finally, SKF elicited no

effects on the levels of F) androstenedione [F (1,13) =0.11, P=0.74, η2=0.009]. H) dihydroprogesterone (DHP) [F(1,15)=0.12, P= 0.73, η2=0.008], or I) allopregnanolone (AP) [F (1,16) =1.96, P=0.18, η2=0.11]. Values represent mean ± SEM for each steroid level in mPFC. *P<0.05, ***, P<0.001 for comparisons between SKF and its vehicle (VEH). For further details, see text. J) Schematic representation of the effects of SKF on steroid metabolic pathways in the medial prefrontal cortex. All abbreviations are explained in the rectangular box. The name of those steroids whose levels were significantly decreased or increased after SKF treatment are indicated in bold red and green fonts, respectively.

AP fails to modify the effects of quinpirole on startle and PPI

We next tested whether AP (10 mg/kg, IP, 15 min before testing) may alter the impact of quinpirole (0.3-0.6 mg/ kg, SC, 10 min before testing) on startle and PPI (n=8/ group). While neither drug affected startle amplitude values (Fig. 3A), the highest dose of QUIN impaired PPI (Fig. 3B), in line with previous results from our group and others (Swerdlow et al. 2000; Mosher et al. 2016). However, this effect was not significantly impacted by AP (Fig. 3B). Similar effects were observed in relation to the impact of intra-mPFC AP (0.5 µg/µl/side) on the PPI deficits caused by quinpirole (0.6 mg/kg, SC, 10 min before AP) (n=9-13/group). Again, no significant effect was observed on startle (Fig. 3C) and AP failed to modify the PPI reduction induced by quinpirole (Fig. 3D). Afterward, we explored whether the local infusion of AP in the NAcS might change the effects mediated by the systemic injection of quinpirole. While this drug reduced the mean startle amplitude (Fig. 3E) and disrupted PPI (Fig. 3F), neither effect was modified by NAcS infusions of AP.

IsoAP opposes the PPI deficits caused by the combination of AP and SKF82958

We then evaluated whether isoAP, the endogenous selective antagonist of AP, may oppose the PPI disruption induced by the combination of SKF82958 and AP. Rats (n=9-10/group) were treated with isoAP (10 mg/kg, IP, 20 min before testing) or its vehicle, followed by AP (10 mg/kg, IP, 15 min before testing) and SKF82958 (0.3 mg/ kg, SC, 10 min before testing) or their vehicles. These studies showed that isoAP significantly increased startle amplitudes, but this effect was not altered by any other treatment (Fig. 4A). The analysis of PPI confirmed that, while the combination of AP and SKF82958 significantly reduced PPI, isoAP rescued this effect (Fig. 4B).

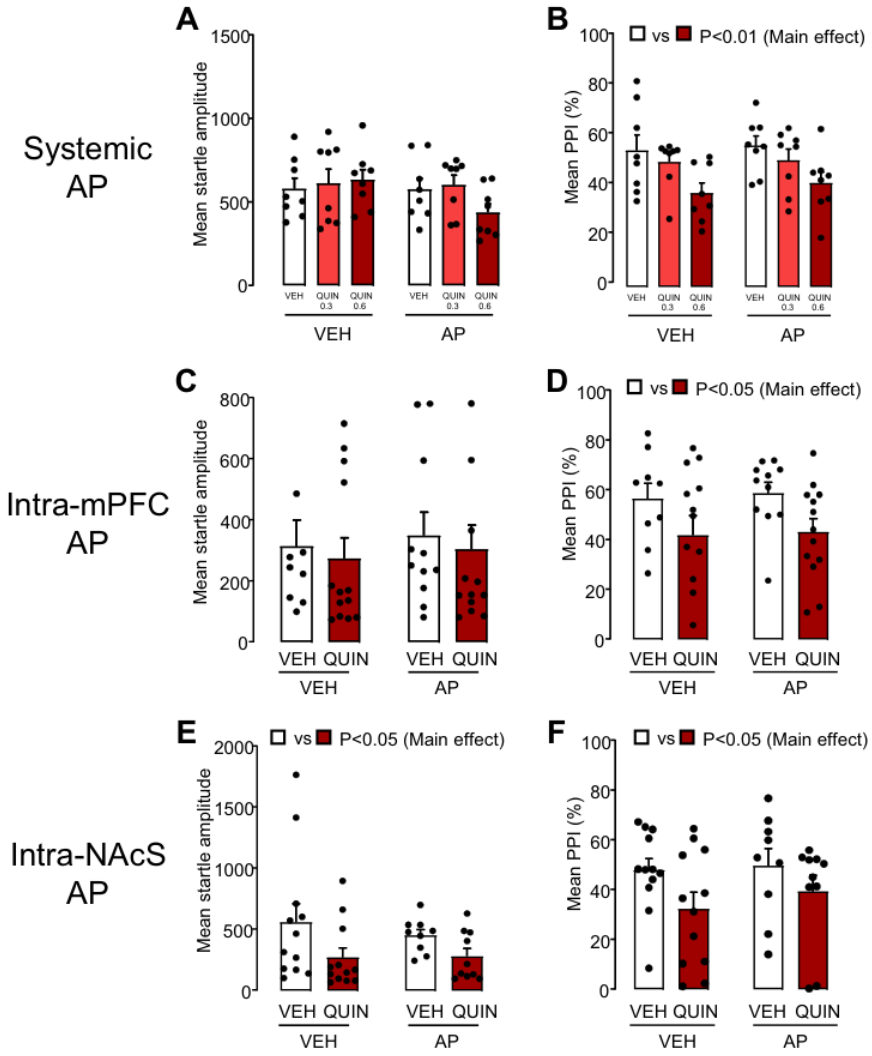


Fig. 3 Allopregnanolone (AP) does not modify the effects of quinpirole (QUIN) on the prepulse inhibition (PPI) of the startle reflex in male Sprague-Dawley rats. The effects of AP were tested after A-B) systemic (10 mg/kg, IP),C-D) intra-medial prefrontal cortex (mPFC) (0.5 µg/µl/side), and E-F) intra-Nucleus Accumbens Shell (NAcS) (0.5 µg/µl/side) administration. A) Startle amplitude was not modified by systemic QUIN (0.3-0.6 mg/kg, SC) [Main QUIN effect: $F(2,42)=0.62$, $P=0.54$, $\eta2=0.03$], systemic AP [Main AP effect: $F(1,42)=1.67$, $P=0.20$, $\eta2=0.04$] or their interaction [$F(2,42)=1.37$, $P=0.26$, $\eta2=0.06$]. B) While QUIN significantly reduced PPI [Main QUIN effect: $F(2,42)=6.51$, $P<0.01$, $\eta2=0.23$], AP did not affect this index [Main AP effect: $F(1,42)=0.34$, $P=0.56$, $\eta2=0.006$] and failed to modify the PPI-reducing effects of QUIN [$F(2,42)=0.07$, $P=0.94$, $\eta2=0.002$]. C) Startle amplitude was not affected by either QUIN (0.6 mg/kg, SC) [Main QUIN effect: $F(1,42)=0.30$, $P=0.59$, $\eta2=0.007$], intra-mPFC AP [main AP effect: $F(1,42)=0.18$, $P=0.68$, $\eta2=0.004$] or their combination [$F(1,42)=0.0009$, $P=0.98$, $\eta2=0.0006$] induced changes of startle amplitude. D) In the same experiment, while QUIN reduced PPI [$F(1,42)=5.71$, $P<0.05$, $\eta2=0.12$], AP did not

[Main AP effect: F(1,42)=0.08, P=0.78, η2=0.002], and no significant interaction between QUIN and AP was found [F(1,42)=0.005, P=0.95, η2=0.0001]. E) Systemic QUIN (0.6 mg/kg, SC) reduced startle amplitude per se [Main QUIN effect: F(1,39)=5.01, P<0.05, η2=0.11]; conversely, intra-NAcS infusions of AP did not alter the startle reflex [Main AP effect: F(1,39)=0.22, P=0.64, η2=0.005] and did not modify the effects of QUIN [QUIN × AP Interaction: F(1,39)=0.32, P=0.57, η2=0.007]. F) While QUIN significantly disrupted PPI [Main QUIN effect: F(1,39)=4.23, P<0.05, η2=0.10], this effect was not modified by AP [QUIN × AP Interaction: F(1,39)=0.17, P=0.68, η2=0.004]. AP had no intrinsic effect on PPI [F(1,39)=0.48, P=0.49, η2=0.01]. All data were analyzed by two-way ANOVA designs followed by Tukey's test. PPI values are indicated as the mean of the three values corresponding to each prepulse loudness. Main effects of QUIN are indicated as comparisons between white columns (vehicle; VEH) vs-colored columns (QUIN). QUIN doses are given in mg/kg and indicated under colored bars. All data are shown as means ± SEM. A-B) n=8/group; C-D-E-F) n=9- 13/group. For further details, see text.

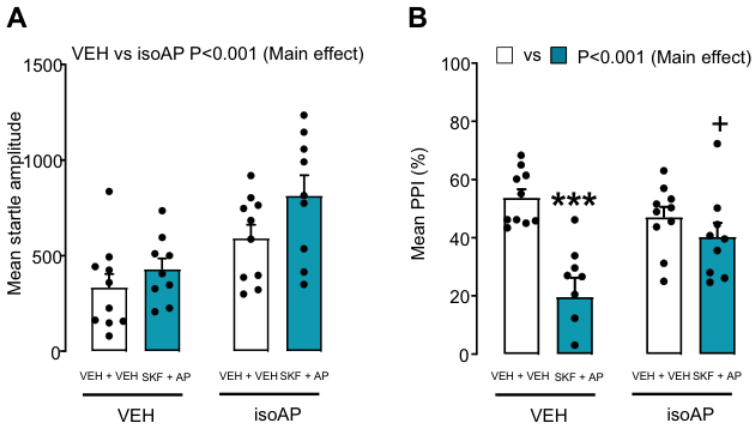


Fig. 4 Isoallopregnanolone (isoAP, 10 mg/kg, IP) reverses the prepulse inhibition (PPI) deficits induced by the combination of SKF82958 (SKF; 0.3 mg/kg, SC) and allopregnanolone (AP, 10 mg/kg, IP). A) While isoAP significantly increased startle amplitude [Main effect: F(1,34)=16.69, P<0.001, η2=0.30], this parameter was not modified by either the combination of AP (10 mg/kg, IP) and SKF (0.3 mg/kg, SC) [F(1,34)=4.06, P=0.05, η2=0.07] or their interaction with isoAP [F(1,34)=0.65, P=0.42, η2=0.01]. B) The significant PPI disruption elicited by SKF in combination with

AP was rescued by isoAP treatment [AP+SKF × isoAP interaction: F(1,34)=8.60, P<0.01, η2=0.13]. All data were analyzed by two-way ANOVA designs followed by Tukey's test. PPI values are indicated as the mean of the three values corresponding to each prepulse loudness. ***, P<0.001 for post-hoc comparisons between groups treated with SKF and AP, and the veh (VEH) of isoAP and those treated with their three VEH. +, P<0.05 for post-hoc comparisons between VEH+SKF+AP and isoAP+SKF+AP. All data are shown as means ± SEM. n=9-10/groups. For further details, see text.

CIQ opposes the PPI deficits caused by the combination of AP and SKF82958

We previously showed that SD rats were made susceptible to the PPI-disrupting effects of D1 receptor agonists (Bortolato et al. 2005) by low doses of dizocilpine, a potent NMDA glutamate receptor blocker. This evidence led us to hypothesize that NMDA receptors may also be involved in the observed synergism of AP and SKF82958. Since our previous studies had been conducted using a partial D1 receptor agonist, SKF38393 (Bortolato et al. 2005), we first tested whether the combination of SKF82958 (0.3 mg/kg, SC, 10 min before testing) with low, ineffective doses of dizocilpine (0.05 mg/kg, SC, 5 min before testing) may also disrupt PPI (n=9-10/group) (Fig. S1). While neither drug modified startle amplitude (Fig. S1A), their concomitant administration elicited significant PPI deficits (Fig. S1B).

Our next step was to verify whether intra-mPFC administration of dizocilpine (1.5-4.5 µg/µL/side; 5 min after SKF82958) might also render rats sensitive to SKF82958induced PPI deficits (Fig. S1C-D). Neither dizocilpine (at both doses tested) nor SK82958 altered startle amplitude significantly (Fig. S1C). However, the combination of the two drugs produced a significant deficit of PPI (Fig. S1D).

Given that the PPI-disruptive effects of dizocilpine are reversed by CIQ, the positive allosteric modulator of GluN2C/D-containing NMDA receptors (Suryavanshi et al. 2014), we tested whether this drug might also counteract the PPI deficits induced by the association of SKF82958 with either low dizocilpine doses or AP. The last group of rats (N=4-9 group) received pretreatment with CIQ (5 mg/kg, IP, 20 min before testing); 10 minutes

later, rats were administered SKF82958 and dizocilpine at the doses that efficiently disrupted PPI in the previous experiment (SKF82958: 0.3 mg/kg, SC. 10 min before testing; dizocilpine: 0.05 mg/kg, SC, 5 min before testing). Our results showed that CIQ opposed the PPI deficits, albeit not selectively, induced by the combination of dizocilpine and SKF82958 (Fig. 5B), although none of these drugs affected startle amplitude (Fig. 5A).

Finally, using a similar design, rats (n=8/group) were treated with CIQ followed by AP (10 mg/kg, SC) and SKF82958 (0.3 mg/kg, IP). Again, while none of these treatments modified startle amplitude (Fig. 5C), CIQ completely reversed the PPI disruption caused by the association of AP and SKF82958 (Fig. 5D).

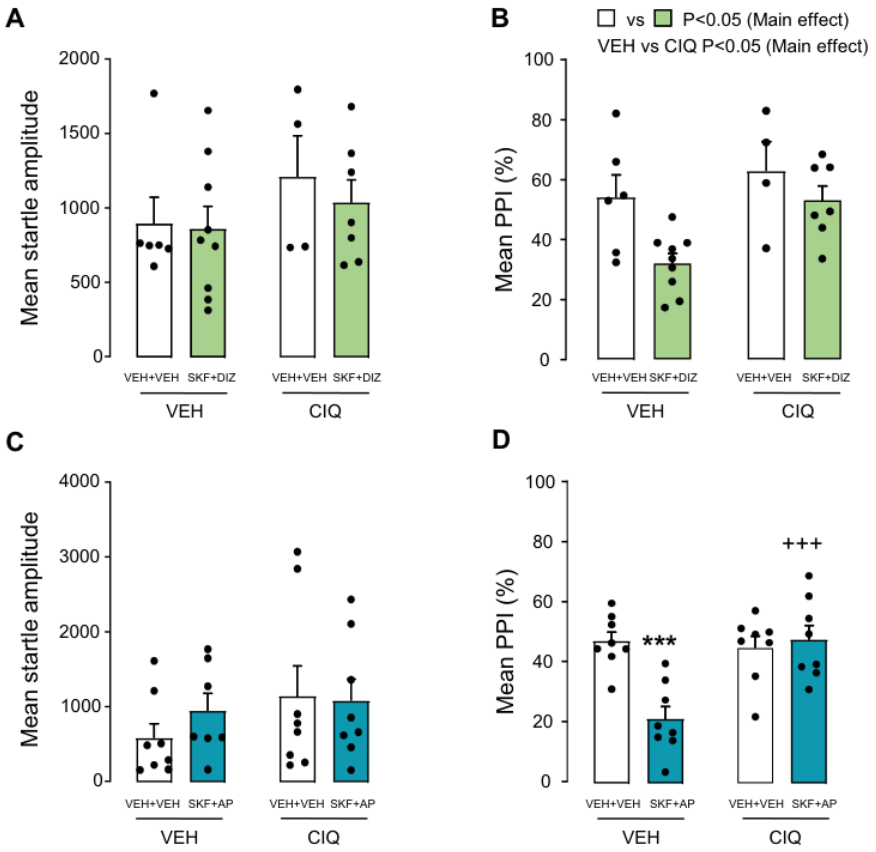


Fig. 5 CIQ (5 mg/kg, IP) opposes the prepulse inhibition (PPI) deficits induced by the synergism of SKF82958 (SKF; 0.3 mg/kg, SC) with either A-B) low-dose dizocilpine (DIZ; 0.05 mg/kg, SC) or C-D) allopregnanolone (AP; 10 mg/kg, IP). A) Startle amplitude was not affected by CIQ [Main CIQ effect: $F(1,22)=1.76$], SKF and low-dose DIZ [Main DIZ+SKF effect: $F(1,22)=0.32$, $P=0.58$, $\eta2=0.01$;, $P=0.20$, $\eta2=0.07$] or their interaction [$F(1,22)=0.13$, $P=0.72$, $\eta2=0.006$]. B) The combination of dizocilpine and SKF reduced PPI [$F(1,22)=7.02$, $P<0.05$, $\eta2=0.17$], while CIQ increased it [Main effect: $F(1,22)=6.25$, $P<0.05$, $\eta2=0.17$]. However, no interaction between these treatments was found [$F(1,22)=1.02$, $P=0.32$, $\eta2=0.02$]. C) Startle amplitude was not affected by CIQ [Main CIQ effect: $F(1,27)=1.37$, $P=0.25$, $\eta2=0.05$], SKF and AP [Main SKF+AP effect: $F(1,27)=0.27$, $P=0.61$, $\eta2=0.009$] or their interaction [$F(1,27)=0.13$, $P=0.50$, $\eta2=0.02$]. D) While SKF and AP reduced PPI [Main SKF+AP effect: $F(1,28)=8.26$, $P<0.01$, $\eta2=0.14$] CIQ increased it [Main CIQ effect: $F(1,28)=9.02$, $P<0.01$, $\eta2=0.15$] and reversed the effects of SKF+AP [CIQ x SKF+AP interaction: $F(1, 28)=12.50$, $P<0.01$, $\eta2=0.21$]. All data were analyzed with twoway ANOVAs followed by Tukey's test for post-hoc comparisons. PPI values are indicated as the mean of the three values corresponding to each prepulse loudness. The main effect for SKF+DIZ is indicated as a comparison between green and white bars. ***, $P<0.001$ for post-hoc comparisons between groups treated with SKF, AP and the vehicle (VEH) of CIQ and those treated with the three VEHs. +++, $P<0.001$ for post-hoc comparisons

between CIQ+SKF+AP and VEH+SKF+AP. All data are shown as means ± SEM. A-B) n=4-9/ group; C-D) n=8/group. For further details, see text.

Discussion

As mentioned in the Introduction, previous evidence showed that SD rats are relatively resistant to the PPI deficits induced by D1 receptor agonists. Indeed, this strain was shown to impair PPI in response to high doses of D1 receptor agonists (Wan et al. 1996; Swerdlow et al. 2001; Ralph and Caine, 2005), even though these effects may not entirely reflect D1 receptor activation, as they were countered by D2, but not D1 receptor antagonists (Wan et al. 1996; Mosher et al. 2016). Capitalizing on these premises, the main results of this study documented that systemic and intra-mPFC injections of the neurosteroid AP, while intrinsically unable to affect PPI, rendered SD rats susceptible to PPI deficits in response to the potent D1 receptor agonist SKF82958. Conversely, AP did not alter the effects of the D2 receptor agonist quinpirole. Complementary to these results, we previously documented that genetic inactivation of 5αR type 1 countered the PPI-disrupting effects of D1 receptor agonists in C57BL/6 mice, and this effect was reversed by AP treatment (Mosher et al.

2019). Together with the present findings, these data indicate that AP is necessary and sufficient to confer sensitivity to the PPI-disruptive effects of D1 receptor agonists. Several other results from our groups further support this conclusion: first, we documented that AP disrupts PPI in the same rodents that exhibit sensitivity to the gating impairments produced by D1 receptor activation, such as C57BL/6 mice and Long-Evans rats (Ralph-Williams et al. 2003; Mosher et al. 2016; Cadeddu et al. 2022); second, we showed that 5αR inhibitors, such as finasteride, reverse

the PPI disruption produced by D1, but not D2 receptor agonists (Frau et al. 2013, 2016). Furthermore, it is worth noting that, in preliminary studies, we found that low doses of D1 receptor agonists significantly worsen the PPI deficits induced by sleep deprivation (Frau et al. 2008) - a manipulation that enhances AP synthesis in the PFC (Frau et al. 2016).

Our steroidomic analyses showed that, in SD rats, D1 receptor activation did not lead to any significant change in AP, ruling out that the synergism between AP and SKF82958 may be supported by any indirect effect of the latter drug on AP synthesis. However, we found that SKF82958 increased the levels of its precursor progesterone and its metabolites 17- and 21-hydroxyprogesterone; furthermore, it significantly reduced the levels of DHEA. These findings suggest that activating D1 receptors in the PFC redirects the metabolic fate of pregnenolone from its conversion into DHEA (via CYP17A1) to progesterone (via 3β-hydroxysteroid dehydrogenase). Interestingly, previous studies have shown that D1 receptor stimulation increases progesterone in rat ovarian cells by enhancing the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) (Mori et al. 1994). Furthermore, the activation of D1 receptors has been shown to augment 3β-HSD expression (Matsuo et al. 2017). Irrespective of these specific mechanisms, our results suggest that, in animal models exhibiting PPI deficits in response to D1 receptor

stimulation, an increase in progesterone may lead to greater AP concentrations, possibly due to greater 5αR expression and/or activity.

Our data imply that different animal models may exhibit variable responsiveness to the effects of D1 on information processing based on their levels of AP in this region. Several rat studies have shown interstrain differences, both in relation to baseline levels and sensitivity to dopaminergic drugs (Swerdlow et al. 2001; Swerdlow et al. 2004; Swerdlow et al. 2006; Swerdlow et al. 2007; Mosher et al. 2016). SD rats are an albino strain that was first developed in 1925 by crossing a Wistar female and a male hooded rat. In rats, albinism is due to a genetic defect in tyrosinase (Searle, 1990). This enzyme degrades tyrosine, the primary precursor of dopamine, and thus influences the synthesis and release of this neurotransmitter. For example, SD rats show lower dopamine turnover than hooded rats (Swerdlow et al. 2005), which likely affects D1 receptor responsiveness. Indeed, previous results suggest that alterations in the dopaminergic system may be a byproduct of the domestication process of experimental rats (Nikulina et al. 1992). In addition to these changes in dopaminergic regulation and behavioral responsiveness, it is possible that SD rats may feature specific alterations in neurosteroids, contributing to the observed reduction in sensitivity to D1 receptor stimulation. Further studies will be needed to evaluate whether divergences in neurosteroidogenic enzyme levels and prefrontal

neurosteroid content may account for strain differences in the effects of D1 receptor agonists in sensorimotor gating and other behavioral domains.

The best-characterized mechanism of action of AP is the activation of GABA-A receptors (Majewska et al. 1986; Paul and Purdy 1992; Rupprecht 2003) through two different sites, respectively localized in the α subunit and at the interface between α and β subunits of this receptor (Hosie et al. 2006, 2009). Our studies showed that the disruption of PPI induced by the combination of AP and SKF82958 was opposed by isoAP, the 3β-epimer of AP that acts as an endogenous antagonist for AP sites within GABA-A receptors. These data further support the idea that the mechanisms of AP are at least partially mediated by GABA-A receptors; these data agree with previous findings from our group showing that this compound reduces the adverse effects of AP on PPI (Cadeddu et al. 2020, 2022).

In addition to the involvement of GABA-A receptors, our data indicate that the deficits induced by AP are also countered by CIQ, the activator of NMDA receptors containing NR2C and NR2D subunits (Mullasseril et al. 2010), two subunits primarily expressed in cortical interneurons (Monyer et al. 1994). These effects parallel those observed against low doses of the NMDA receptor antagonist dizocilpine, which also renders SD rats sensitive to PPI deficits induced by D1 receptor agonists (Bortolato et al. 2005). These data suggest that the PPI deficits caused by the synergism of AP and D1 receptor agonists are reversed by the activation of NR2C/NR2D-containing NMDA receptors. The antagonism of NMDA receptors in the interneurons is regarded as a central mechanism for PPI disruption (Linderholm et al. 2010); indeed, previous evidence has shown that the PPI deficits induced by higher doses of dizocilpine are reversed by CIQ (Suryavanshi et al. 2014).

Several interpretations can help explain the implication of interneuronal NMDA receptors in the synergism of AP and SKF82958. One possibility may be a direct action of AP on NMDA receptors. Although the role of AP on NMDA is not fully well-understood, several studies suggested that AP may negatively affect NMDA currents. For example, Wang et al. (2008) found that AP significantly reduces NMDA currents in the central amygdala. At the same time, other research has shown no significant effect of AP on NMDA receptors (Hu et al. 2006). Other evidence indicates that AP may exert positive, rather than negative, modulation of NMDA receptors (Giuliani et al. 2011). Briz and colleagues showed that, by activating GABA-A function, AP might prevent NMDA receptor internalization induced by dieldrin (Briz et al. 2012). An intriguing alternative may be that AP mediates its adverse effects through conversion into one of its metabolites. For example, AP sulfate has been shown to exert a subtle negative modulation of these receptors (Weaver et al. 2000). Finally, PPI deficits may also result from imbalances

between the activity of pyramidal neurons and interneurons; thus, the effects of CIQ may reverse the synergism between D1 receptors and AP simply by promoting the activation of interneurons, which in turn may be critical to reverse these mechanisms. Future studies are needed to understand the specific mechanisms underlying the role of D1 receptors in regulating sensorimotor gating.

NMDA receptor antagonism in the PFC likely impairs PPI by inactivating interneurons, which results in a lower stimulation threshold for pyramidal neurons. Thus, the activation of D1 receptors on these cells may further contribute to these effects. Recent data have shown that AP modulates oscillatory activity generated by interneurons in the amygdala (Antonoudiou et al. 2022). Future studies are needed to understand whether similar mechanisms may also be responsible for the effects of AP in the PFC. Irrespective of these observations, our data suggest that transient functional deficits of the PFC (including the activation of GABA-A receptors or the partial inhibition of NMDA receptors) may increase the predisposition to PPI deficits in response to D1 receptor activation. In line with this idea, our preliminary observations also showed that the sensitivity to the D1-induced PPI deficits is increased after stereotaxic surgery.

The role of AP as a factor in the effects of D1 receptor activation may be highly relevant in some of the psychopathological conditions associated with gating deficits. For example, our studies have highlighted the relevance of AP as a pathogenic factor in TS (Bortolato et al. 2022). Recent studies have shown that D1 receptor antagonists reduce tics (Gilbert et al. 2018). A potential problem of D1 receptor antagonists, mainly in the PFC, is the potentially damaging impact of these compounds on attention (Wulaer et al. 2021), given the key role of these targets in attention regulation and associative learning (Sawaguchi and Goldman- Rakic 1994; Seamans et al. 1998; Granon et al. 2000; Puig and Miller 2012). From this perspective, our data may suggest that interfering with AP synthesis or signaling may be a preferable alternative to D1 receptor antagonism for the therapy of tics or TS-related deficits, leading to therapeutic effects without additional adverse effects on attention. As mentioned above, PPI deficits are typical of several neuropsychiatric disorders, including TS and OCD; notably, the ability of drugs to restore PPI in rodents treated with dopaminergic agonists has been shown to predict antipsychotic potency

(Swerdlow et al. 1994) or validate therapeutic efficacy for TS (Godar et al. 2014). In line with this idea, it is worth noting that finasteride may have therapeutic effects on TS (Bortolato et al. 2007; Muroni et al. 2011) and schizophrenia (Koethe et al. 2008). Our results support the possibility that selective antagonists of AP, such as isoAP, could have therapeutic effects for these conditions without some of the adverse effects of finasteride, including low libido

and depression (Traish et al. 2015). In line with this idea, we have recently shown that isoAP reduces tic-like manifestations in animal models of TS (Cadeddu et al. 2020).

Several limitations to this study should be acknowledged. First, we did not test female rats. Prior testing in rats showed that the antidopaminergic effects of finasteride were preserved in both males and females, as well as castrated males (Paba et al. 2011; Devoto et al. 2012). While these premises suggests that females may display similar effects to those observed in males, rich evidence has documented sex differences in dopaminergic regulation (Godar and Bortolato, 2014; Becker, 1999) and PPI (Lehmann et al. 1999). Indeed, PPI varies across the menstrual cycle in female rats, with the lowest levels in the proestrous phase (Koch et al. 1998; but see Adams et al. 2008 for contrasting results). Given that the circulating levels of AP and its precursor progesterone rise in the luteal phase (Bäckström et al. 2014), future studies are needed to assess how these fluctuations may affect the behavioral effects of D1 agonists throughout the estrous cycle. Second, while our analyses were limited to AP, other neuroactive steroids may participate in some observed effects. Research has indicated that neuroactive steroids extensively interact with dopamine signaling to regulate various physiological functions (Sanchez et al. 2010). For example, both progesterone and testosterone alter dopamine release and turnover in rodents (Sanchez et al. 2010). Further studies are warranted to address how these interactions may contribute to the pathophysiology of gating disorders. Third, our previous studies have shown that several types of acute stressors reduce PPI through the enhancement of AP in the PFC (Cadeddu et al. 2022); future research will be needed to understand whether acute stress can also change the sensitivity of PPI to D1 receptor agonists. These limitations notwithstanding, the results of these studies strongly support a role for AP in the mediation of dopamine-induced PPI deficits and raise important questions for future research on the balance between D1 and D2 receptors in the regulation of sensorimotor gating mechanisms.

Conclusion

Several studies report that neurosteroids can modulate dopamine system and transmission. Allopregnanolone has been shown to affect dopamine levels both in the nucleus accumbens and prefrontal cortex, two key regions of sensorimotor gating. To our knowledge, this is the first study that demonstrates a region-specific/synergistic effect between the neurosteroid AP and a potent D1 receptor agonist. This synergy enables the detrimental effects of D1 receptor activation on information processing, through functional deficits of the PFC. As such, this finding unveils the critical role of cortical AP in dopamine-related disorders characterized by impairments of sensorimotor gating

function, especially those psychiatric conditions that involve deregulations of D1 receptor signaling, such as TS.

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Declarations

Conflict of interest: PN is CEO and CMO for Asarina Pharma and has a 1.2% equity stake in Asarina Pharma. MB consults for Asarina Pharmaceuticals and receives research funding from Asarina and Lundbeck Pharmaceuticals. All the other authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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