Prefrontal 5α-reductase 2 mediates male-specific acute stress response

Roberto Cadeddu [1*], Giulia Braccagni [1*], Gabriele Floris [1], Caterina Branca [1],

Eleonora Corridori [3], Sara Salviati [3], Pilar Sánchez [4], Luca Spiro Santovito [5],

Jesus M Torres [4], Esperanza Ortega [4], Graziano Pinna [5,6,7], Philip J. Moos [1],

Simona Scheggi [3*], Marco Bortolato [1,2*]

1. Department of Pharmacology and Toxicology, College of Pharmacy, University of Utah, Salt Lake City, UT, USA
2. Department of Pharmacology and Toxicology, School of Pharmacy. University of Kansas, Lawrence, KS, USA
3. Department of Molecular and Developmental Medicine, University of Siena, Italy
4. Department of Biochemistry, Molecular Biology 3 and Immunology, Faculty of Medicine, University of Granada, Spain
5. The Psychiatric Institute, Department of Psychiatry, College of Medicine, University of Illinois at Chicago, IL, USA
6. UI Center on Depression and Resilience (UICDR), Department of Psychiatry, College of Medicine, University of Illinois at Chicago, IL, USA
7. Center for Alcohol Research in Epigenetics (CARE), Department of Psychiatry, College of Medicine, University of Illinois at Chicago, IL, USA

* These authors contributed equally to the manuscript

Corresponding author:

Marco Bortolato, MD PhD

Dept. of Pharmacology and Toxicology
College of Pharmacy
University of Utah
L.S. Skaggs Hall, Room 3916 30 S 2000 E
Salt Lake City, UT 84112 USA
e-mail: marco.bortolato@utah.edu

Abstract

Acute stress triggers rapid brain responses, including increased allopregnanolone (AP) synthesis via 5α-reductase (5αR). However, the involvement of this enzyme in stress response remains elusive. Here, we investigated the roles of the two major 5αR isoenzymes in acute stress. Acute stress increased mRNA and protein levels of 5αR2, but not 5αR1, in the medial prefrontal cortex (mPFC) of male, but not female, rats. Targeted mPFC downregulation of 5αR2, but not 5αR1, markedly reduced the responsiveness of males, but not females, to both stressful and arousing stimuli. Similar sex differences were observed in 5αR2 knockout rats. While 5αR1 regulated AP synthesis under baseline conditions, 5αR2 enabled this process in response to acute stress. Single-nucleus transcriptomic analyses revealed that 5αR2 enabled stress-induced protein synthesis in pyramidal neurons and glia of the mPFC. These findings underscore the pivotal role of 5αR2 in shaping sex-related differences in acute stress reactivity.

INTRODUCTION

The acute stress response is a transient physiological and psychological reaction elicited by an immediate perceived threat or demand. It manifests as a heightened activation of the sympatho-adrenomedullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis, aimed at the mobilization of organism's energy resources to cope with immediate situational demands (Russell & Lightman, 2019). Despite its short duration, acute stress can have significant physiological and psychological impacts, insofar as it influences cognitive function and emotional regulation (McEwen et al., 2015; McEwen & Sapolsky, 1995). Notably, while stress-induced signals are initially adaptive, they can exacerbate health risks and contribute to the development of stress-related disorders when excessively activated or dysregulated.

Glucocorticoids, catecholamines, and neurotrophins have long been recognized for their pivotal roles in orchestrating the physiological and behavioral aspects of adaptation to acute stress; additionally, emerging research has shed light on the significance of allopregnanolone (3α-hydroxy-5α-pregnan-20-one; AP), a neurosteroid derivative of progesterone, which exhibits potent anxiolytic and neuroprotective properties (Gunn et al., 2015). In male rodents, exposure to acute stressors increases AP levels in the prefrontal cortex (PFC) (Barbaccia et al., 1996; Cadeddu et al., 2022; Purdy et al., 1991). Given the primary role of this brain region in coping and resilience (Maier & Watkins, 2010), this phenomenon has been extensively interpreted as an adaptive mechanism to reinstate homeostasis by mitigating the excessive activation of other systems implicated in the acute stress response, such as the HPA axis (Gunn et al., 2015). Indeed, AP acts as a positive allosteric modulation of GABA-A channels (Legesse et al., 2023), the most prominent inhibitory neurotransmitter receptors in the central nervous system.

Several details remain elusive with respect to the mechanisms that enable AP biosynthesis in response to acute stress. The conversion of progesterone to AP occurs through a two-step process. Initially, progesterone is transformed into dihydroprogesterone (DHP) by the enzyme 5α reductase (5αR), followed by the conversion of DHP into AP by the enzyme 3α hydroxysteroid oxidoreductase (3αHSOR) (Diviccaro et al., 2022). Unlike 3αHSOR, 5αR catalyzes a unidirectional reaction, regarded as the key rate-limiting step in AP synthesis (Paba et al., 2011). In line with this evidence, 5αR inhibitors produce a profound reduction in AP synthesis (Concas et al., 1998). Notably, recent evidence indicates that finasteride, a prototypical 5αR inhibitor, hinders the capacity to mount an effective adaptive acute response to both stressful and incentive stimuli (Godar et al., 2019), suggesting that AP may be relevant in promoting coping and shaping the broader behavioral response to acute

challenges. The two most extensively characterized 5αR isoenzymes, 5αR1 and 5αR2, share similar structures and genetic ancestry (Paba et al., 2011). Despite this, their substrate affinity and distribution differences throughout the body suggest distinct physiological roles. For instance, 5αR2 exhibits a markedly higher affinity for testosterone and progesterone than 5αR1 (Paba et al., 2011).

Accordingly, 5αR2 is implicated in the peripheral conversion of testosterone into its more active metabolite, 5α-androstan-17β-ol-3-one (dihydrotestosterone; DHT), a potent androgen hormone responsible for developing numerous secondary sexual characteristics in males. While both isoenzymes are expressed in the brain (Castelli et al., 2013), 5αR2 shows significantly lower levels in the PFC of male and female rats, along with a sexually dimorphic regulation exerted by androgens (Torres & Ortega, 2003, 2006). Collectively, the functional role of this enzyme remains less comprehensively understood. Our study exposed male and female rats to various acute stressors to elucidate the specific roles of these 5αR isozymes in stress-induced AP synthesis and behavioral reactivity. Additionally, we specifically suppressed the expression of each isoenzyme in the medial PFC and nucleus accumbens (NAc), two crucial regions in the regulation of reward, motivation and mood (Nestler & Carlezon, 2006; Russo & Nestler, 2013). Then, we assessed the impact of this manipulation on behavior, AP synthesis, and cell-specific transcriptomic alterations.

In contrast to previous assumptions, our results identify 5αR2, rather than 5αR1, as the primary contributor to the acute synthesis of AP in the mPFC of male rats. Specifically, our observations reveal that acute stress rapidly upregulates 5αR2 expression in the mPFC, and this increase is instrumental for the production of AP and the enactment of several behavioral responses to salient stimuli. This distinctive response is accompanied by marked transcriptomic alterations in PFC pyramidal neurons, with 5αR2 deficiency resulting in a generalized reduction of the acute stress response across most cell types. These effects were observed exclusively in males under stressful conditions, indicating a significant sexual dimorphism in the role of 5αR2 in behavioral regulation. This distinction provides valuable insights into the potential contribution of this enzyme to sex-specific stress susceptibility and may also yield valuable insights into the mechanisms underlying sex differences in the vulnerability to psychiatric disorders characterized by aberrant stress responses.

RESULTS

Acute stress upregulates 5αR2 in males.

To ascertain the specific reactivity of each 5αR isoenzyme to acute stress, we first measured the expression levels of 5αR1 and 5αR2 across various brain regions and at different time intervals following two well-established experimental protocols that induce inescapable acute stress: forced swim and foot-shock. Both procedures have been extensively validated for their capacity to elicit consistent and measurable stress responses across various behavioral, physiological, and neurobiological parameters, including the elevation of AP in the medial prefrontal cortex (mPFC). Strikingly, these protocols elicited distinct signature panels with respect to brain-regional 5αR isoenzyme expression.

Specifically, male rats exposed to the forced swim showed a significant upregulation of 5αR1 in the nucleus accumbens (NAc) and hypothalamus (Fig. 1A), while the foot-shock reduced the levels of 5αR1 in the NAc and hippocampus (Fig. 1B). Interestingly, male rats exposed to forced swim rapidly increased 5αR2 protein levels in the mPFC and hypothalamus, coupled with a modest yet significant reduction in amygdala (Fig. 1C). Notably, foot-shock led to a similar elevation in 5αR2 expression in the mPFC (Fig. 1D). In contrast with males, females showed no alterations in the 5αR isoenzyme expression levels in response to the two stressors (Fig. 1E-H). Taken together, these data indicate that both forced swim and foot-shock led to the upregulation of 5αR2 in the mPFC of males, both at protein and mRNA level (Fig. S1), as well as other specific brain-regional changes in 5αR levels.

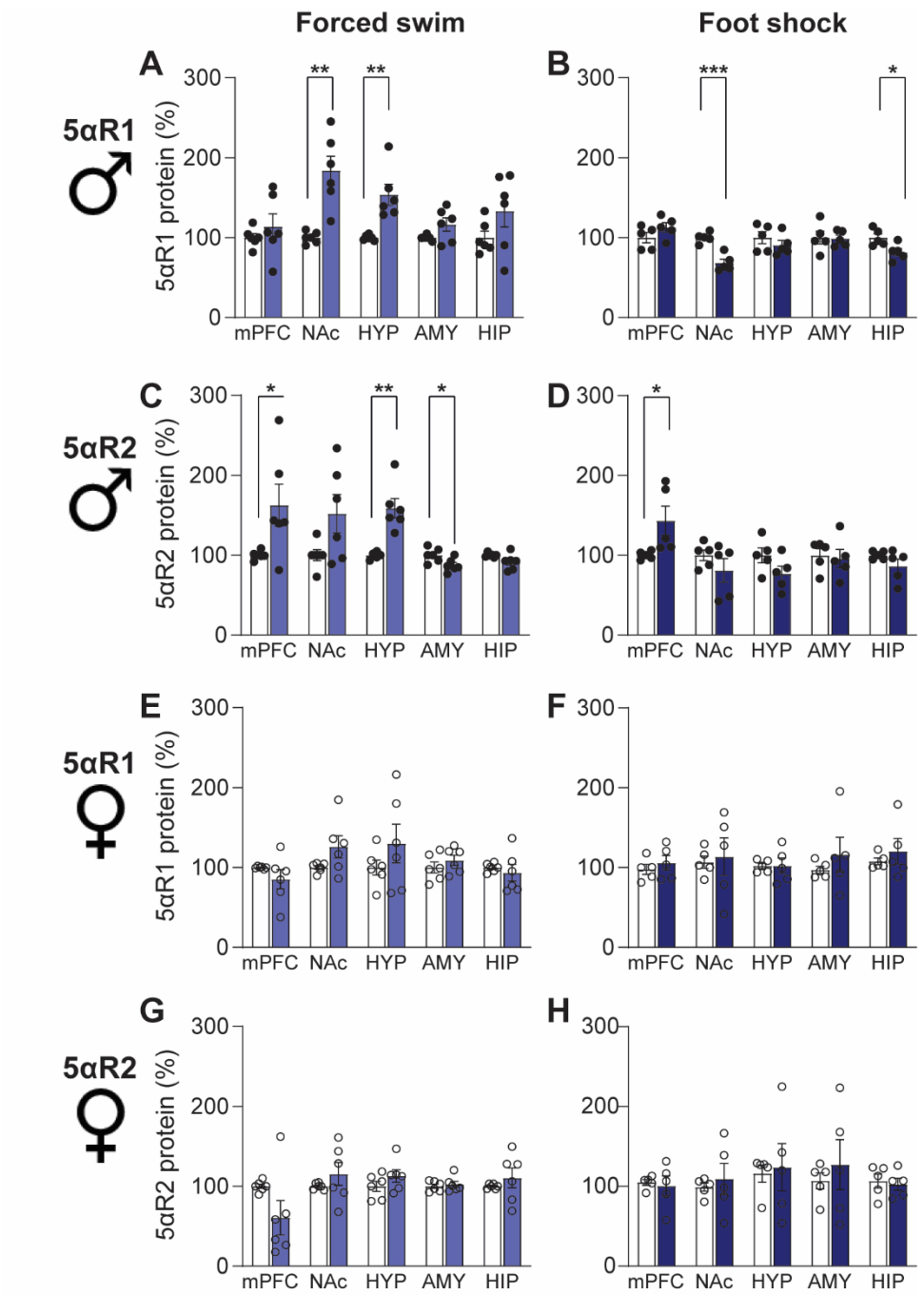

Figure 1: Stress upregulates 5αR2 in males. Stressed rats (colored columns) were exposed either to the forced swim test (left panels) or the foot-shock (right panels) and were sacrificed 30 minutes after the task. 5αR1 and 5αR2 protein levels were evaluated in: medial prefrontal cortex (mPFC), nucleus accumbens (NAc), hypothalamus (HYP), amygdala (AMY), and hippocampus (HIP). Control rats (white columns) were left undisturbed in their home-cage. (A) 30 minutes after forced swim exposure, 5αR1 was significantly upregulated by stress in NAc (one-way ANOVA, F(1,5.258)=20.709, p=0.005, partial η^2=0.674 with Brown-Forsythe correction) and HYP (one-way ANOVA, F(1,5.114)=16.769, p=0.009, partial η^2=0.626 with Brown-Forsythe correction) of male rats. (B) In males, 5αR1 was significantly downregulated by foot-shock in NAc (one-way ANOVA, F(1,8)=33.272, p<0.001, partial η^2=0.806) and HIP (one-way ANOVA, F(1,8)=7.612, p=0.025, partial η^2=0.488), (C) 5αR2 was upregulated by forced swim in PFC (one-way ANOVA, F(1,5.070)=5.633, p=0.039, partial η^2=0.360 with Brown-Forsythe correction) and HYP (one-way ANOVA, F(1,5.206)=23.531, p=0.004, partial η^2=0.702 with Brown-Forsythe correction), while it was downregulated in AMY (one-way ANOVA, F(1,10)=6.439, p=0.029,

partial η^2=0.392) of male rats. (D) In males, 5αR2 was upregulated by foot-shock in mPFC (one-way ANOVA, F(1,4.123)=5.469, p=0.048, partial η^2=0.406 with Brown-Forsythe correction). (E-H) Neither forced swim nor foot-shock significantly altered 5αR1 or 5αR2 protein levels in female rats. For further details, see Supplementary Results. Data are presented as mean ± SEM. Light blue, forced swim. Dark blue, foot-shock. Full dots, males. Empty dots, females. *p<0.05, **p<0.01, and ***p<0.001.

5aR2 is necessary to mount a response to stress in males.

Leveraging these findings, we characterized the specific effects of 5αR1 or 5αR2 in the mPFC and NAc, given that these two brain regions exhibited the most prominent changes in the expression of these enzymes in response to acute stress. To this end, we knocked down the expression of either isoenzyme by subjecting male and female rats to stereotaxic bilateral injections of adeno-associated serotype 5 viral vectors (AAV5) carrying shRNA constructs directed against 5αR1 or 5αR2, along with the GFP reporter gene. Scrambled RNA was used as control. This procedure effectively and selectively reduced the protein levels of the enzyme of interest within the designated brain region, as validated by the expression of the GFP reporter used for monitoring virus distribution (Fig. S2).

Two weeks after surgery, we conducted a comprehensive behavioral assessment in these animals. Examination of the phenotypes induced by 5αR1 knockdown in male rats showed that this manipulation yielded minimal impact (Fig. 2), except for an increase in locomotor activity following mPFC targeting (Fig. 2A) and decreased social interaction following targeting of either the mPFC (Fig. 2E) or the NAc (Fig. 2K). Conversely, 5αR2 knockdown in the mPFC of male rats compromised their ability to appropriately respond to acute exposure to arousing or stressful stimuli. Notably, these animals did not exhibit noticeable alterations in locomotion (Fig. 3A), yet they demonstrated prolonged immobility during the forced swim test (Fig. 3B) and a reduced tendency to exit the dark chamber in the defensive withdrawal paradigm (Fig. 3C). Additionally, they displayed diminished responsiveness to novel olfactory stimuli, as evidenced by decreased exploratory rearing towards a new odor (Fig. 3D), and reduced interaction time with unfamiliar counterparts (Fig. 3E). Furthermore, male rats did not show sucrose preference over time (Fig. 3F). Interestingly, most of these phenotypes were specific to males with 5αR2 knock-down in the PFC; targeting the NAc with the same RNA interference construct did not elicit this spectrum of responses (Fig. 3G-3L), with the exception of reduced social interaction (Fig. 3K) and sucrose preference (Fig. 3L). Finally, analyses of the behavior in female rats showed that the same manipulation in females elicited no significant differences (Fig. 4), with the only exception of the significant increase in locomotor activity following 5αR1 knock-down in the mPFC (Fig. 4A).

To confirm the contribution of 5αR2 to the regulation of environmental reactivity, we generated 5αR2 full-body knock-out rats (Fig. 5A-B). Interestingly, male knock-out rats showed a significant increase in immobility time during the forced swim test (Fig. 5C) and a decrease in time spent outside the chamber in the defensive withdrawal paradigm (Fig. 5D), as well as sucrose preference (Fig. 5E). Again, these phenotypes were only detected in males (Fig. 5F-H).

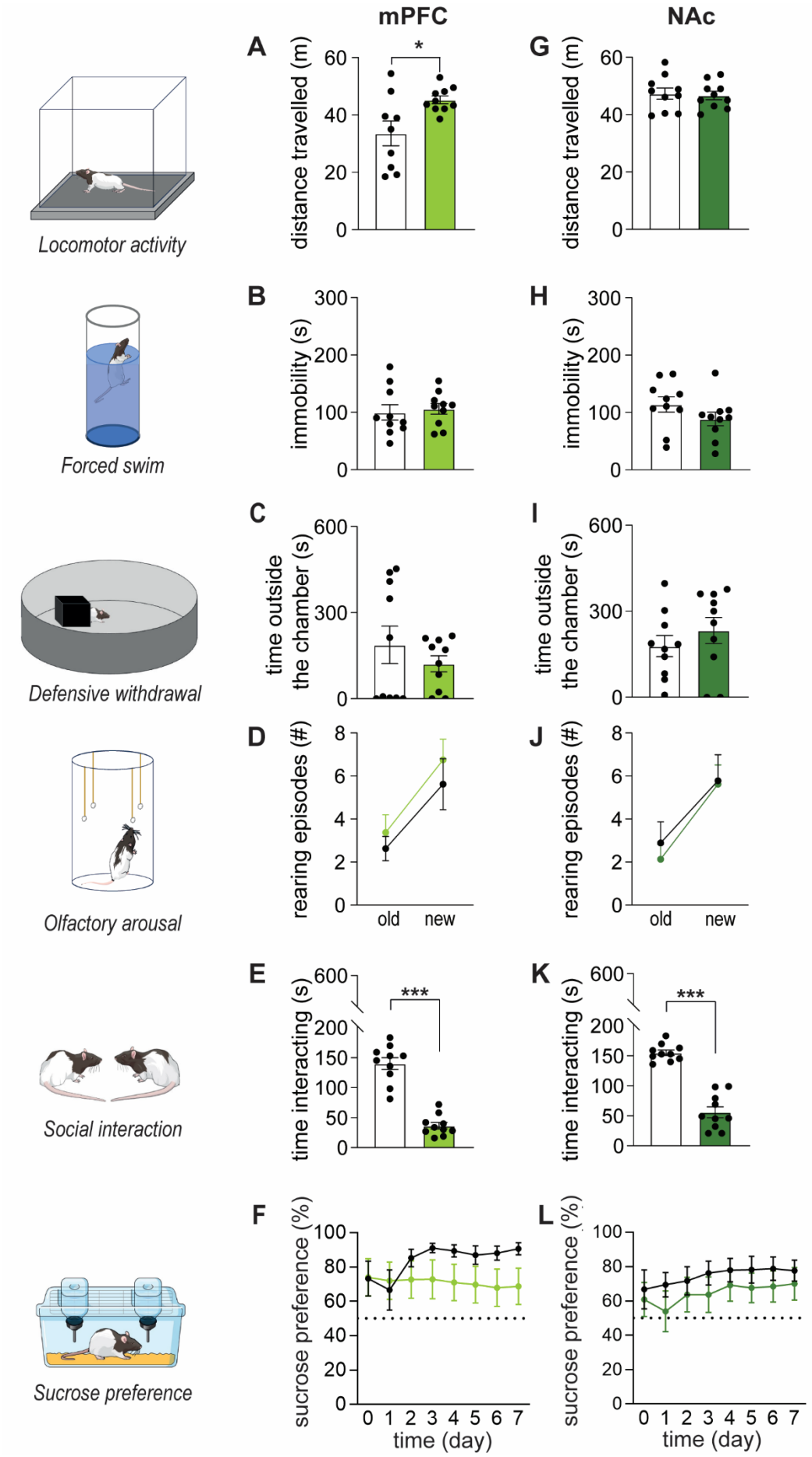
mPFC
NAc
A
G
distance travelled (m)
Locomotor activity
B
H
immobility (s)
Forced swim
C
I
time outside the chamber (s)
Defensive withdrawal
D
J
rearing episodes (#)
old
new
Olfactory arousal
E
K
time interacting (s)
Social interaction
F
L
sucrose preference (%)
time (day)
Sucrose preference

Figure 2: Behavioral characterization of male animals with downregulation of 5αR1. Male rats were injected stereotaxically into the prefrontal cortex (A-F, PFC, light green) or the nucleus accumbens (G-L, NAc, dark green) with construct carrying scrambled-shRNA or 5αR1-targeting shRNA and the behavior was evaluated 2 weeks after surgery. (A) Locomotory activity was significantly increased in knock-down (KD) rats for 5αR1 in the PFC (one-way ANOVA, $F(1,9.548)=6.716$, $p=0.028$, partial $\eta^2=0.302$ with Brown-Forsythe correction). No differences were highlighted in the forced swim test (B) and in the defensive withdrawal paradigm (C). (D) New odor exposure elicited similar arousal between groups (repeated measure two-way ANOVA, main effect of odor, $F(1,14)=16.75$, $p=0.0011$, partial $\eta^2=0.545$). (E) 5αR1 KD rats interacted significantly less with foreigner counterparts than control animals (one-way ANOVA, $F(1,18)=82.224$, $p<0.001$, partial $\eta^2=0.820$). (F) Sucrose preference test showed no difference. (G-I) 5αR1 downregulation in NAc did not elicit any differences for locomotory activity (G), forced swim test (H) and defensive withdrawal (I). (J) New odor exposure elicited similar arousal between groups (repeated measure two-way ANOVA, main effect of odor $F(1,15)=9.644$, $p=0.0072$, partial $\eta^2=0.391$). (K) 5αR1 KD rats interacted significantly less with foreigner counterparts than control animals (one-way ANOVA, $F(1,13.071)=93.961$, $p<0.001$, partial $\eta^2=0.839$ with Brown-Forsythe correction). (L) Sucrose preference test showed no difference. For further details, see Supplementary Results. Data are presented as mean ± SEM. *, $p<0.05$ and ***, $p<0.001$.

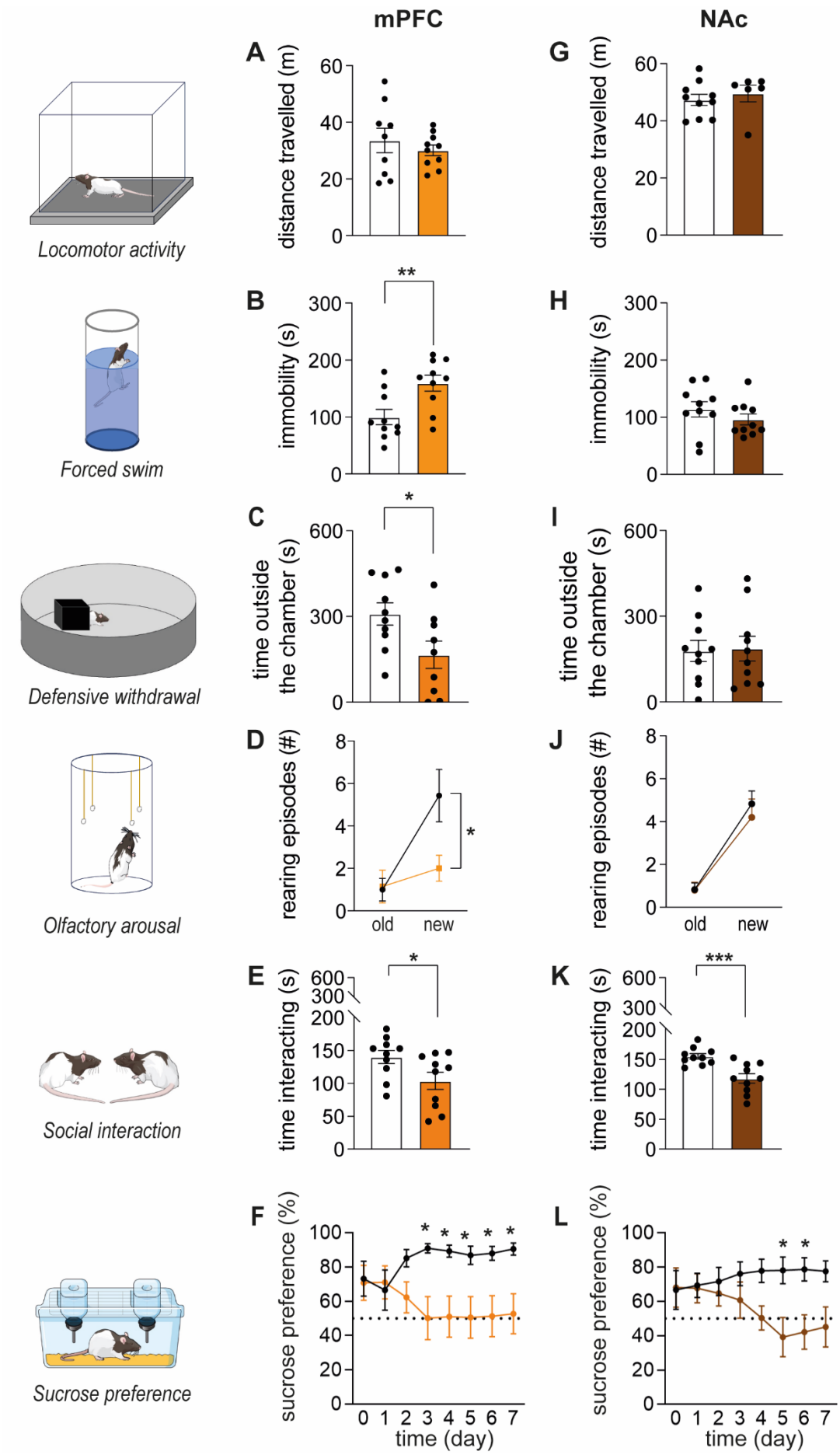
mPFC
NAc
A
G
distance travelled (m)
Locomotor activity
B
H
immobility (s)
**
Forced swim
C
I
time outside the chamber (s)
*
Defensive withdrawal
D
J
rearing episodes (#)
old
new
Olfactory arousal
E
K
time interacting (s)
***
Social interaction
F
L
sucrose preference (%)
time (day)
Sucrose preference

Figure 3: Behavioral characterization of male animals with downregulation of 5αR2. Rats were injected into the prefrontal cortex (A-F, PFC, light orange) or the nucleus accumbens (G-L, NAc, dark orange) with construct carrying scrambled-shRNA or 5αR2-targeting shRNA and the behavior was evaluated 2 weeks after surgery. (A) Locomotor activity did not reveal any differences. (B) 5αR2 knock-down (KD) rats floated significantly more than scrambled animals in the forced swim test (one-way ANOVA, $F(1,18)=9.515$, $p=0.006$, partial $\eta^2=0.346$). (C) 5αR2 KD rats spent significantly less time outside the chamber in the defensive withdrawal paradigm (one-way ANOVA, $F(1,17)=5.454$, $p=0.032$, partial $\eta^2=0.243$). (D) 5αR2 KD rats exposed to new odor showed a reduced arousal (repeated measure two-way ANOVA, main effect of odor, $F(1,12)=24.159$, $p=0.0004$, partial $\eta^2=0.668$; interaction, $F(1,12)=11.029$, $p=0.006$, partial $\eta^2=0.479$). In the panel is reported the p value from post-hoc analyses with Holm´s correction. (E) The 5αR2 KD animals interacted less with foreign counterparts than scrambled animals (one-way ANOVA, $F(1,18)=4.876$, $p=0.040$, partial $\eta^2=0.213$). (F) To test for anhedonia, rats were exposed to the sucrose preference test. 5αR2 KD rats over time showed no preference for sucrose. Data were analyzed by repeated measure two-way ANOVA (main effect of condition, $F(1,18)=6.374$, $p=0.0183$, partial $\eta^2=0.272$; interaction, $F(7,126)=4.123$, $p=0.0004$, partial $\eta^2=0.186$). In the panel are reported p values (*, $p<0.05$) from post-hoc analyses with Sidak´s correction. (G-L) Downregulation of 5αR2 in NAc did not elicit any differences in locomotor activity (G), forced swim test (H), and the defensive withdrawal paradigm (I). (J) New odor exposure elicited similar arousal between groups (repeated measure two-way ANOVA, main effect of odor $F(1,9)=101.8$, $p<0.0001$, partial $\eta^2=0.919$). (K) 5αR2 KD rats interacted significantly less than scramble with a foreigner counterpart (one-way ANOVA, $F(1,18)=16.380$, $p<0.001$, partial $\eta^2=0.476$). (L) In the sucrose preference test, 5αR2 KD rats did not show preference for sucrose at day 5 and 6 (repeated measure two-way ANOVA, repeated measure two-way ANOVA, main effect of condition, $F(1,18)=5.357$, $p=0.0327$, partial $\eta^2=0.229$; interaction, $F(7,126)=2.638$, $p=0.0141$, partial $\eta^2=0.128$). In the panel are reported p values (*, $p<0.05$) from post-hoc analyses with Sidak´s correction. For further details, see Supplementary Results. Data are reported as mean ± SEM. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$.

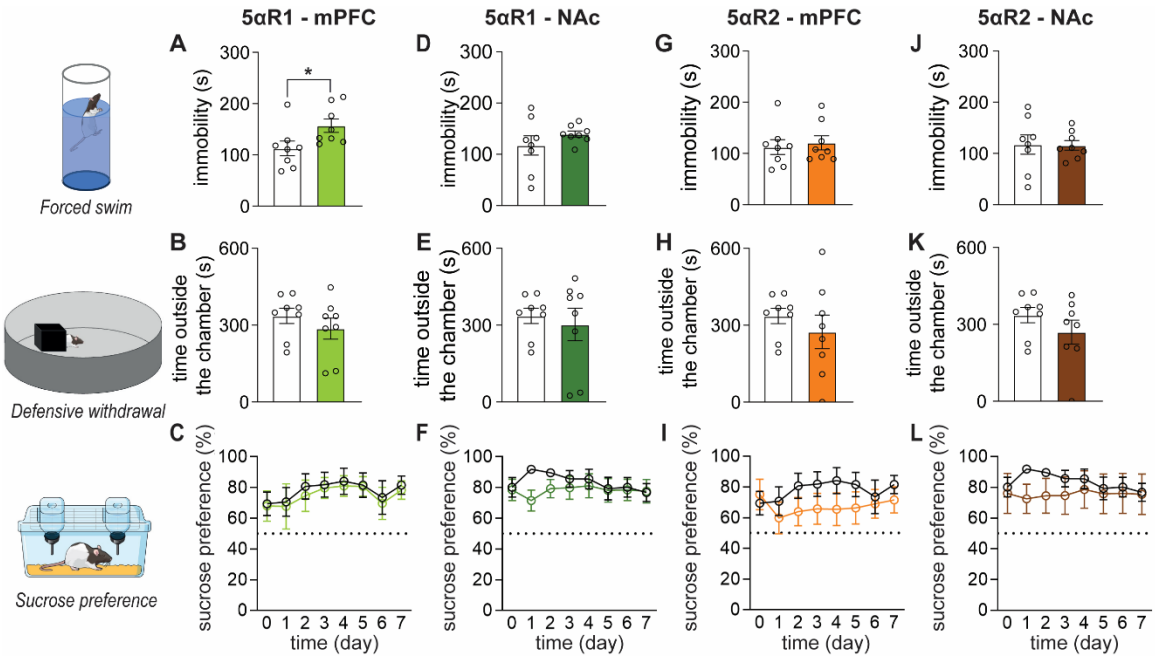

Figure 4: Behavioral characterization of female rats. Female rats were injected stereotaxically into the prefrontal cortex (PFC) or the nucleus accumbens (NAc) with construct carrying scrambled-shRNA, 5αR1- or 5αR2-targeting shRNA and the behavior was evaluated 2 weeks after surgery. (A-C) Knock-down (KD) of 5αR1 in the PFC increased the immobility time in the forced swim (A, one-way ANOVA, $F(1,14)=5.235$, $p=0.038$, partial $\eta^2=0.272$), while elicited no effects in the defensive withdrawal paradigm (B) and in the sucrose preference test (C). (D-F) Knock-down of 5αR1 in the NAc did not generate any significant shift in the tested behaviors. (G-L) Knock-down of 5αR2 in female rats did not induce any significant differences irrespective of the targeted area (PFC, G-I, or NAc, J-L). For further details, see Supplementary Results. Data are presented as mean ± SEM. *,$p<0.05$.

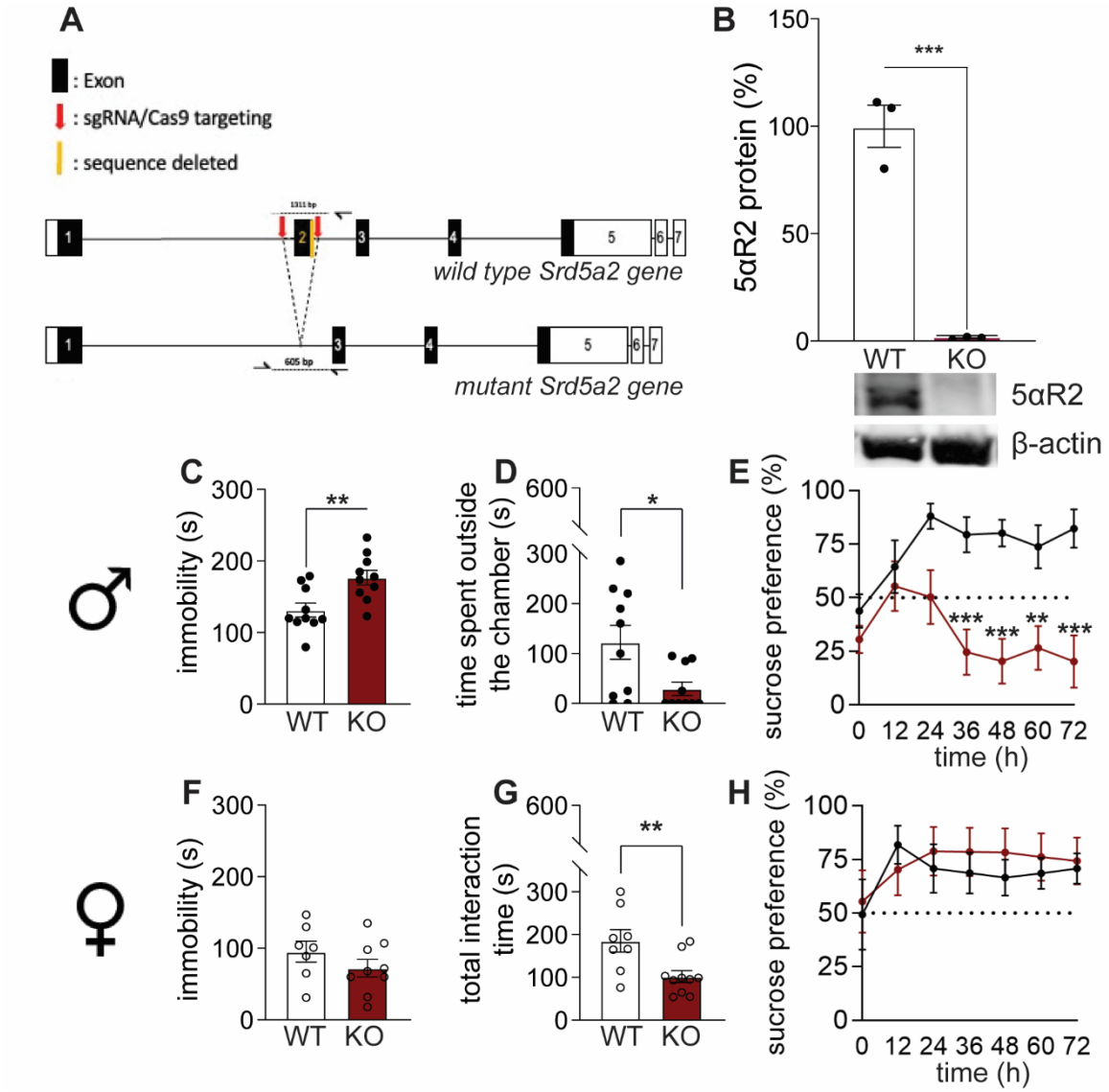

Figure 5: Generation and behavioral characterization of 5αR2 knock-out (KO) rats. (A-B) Representation of the targeted mutations generated using Crispr/Cas9-based genome editing strategies to remove exon 2 from the 5αR2 gene. To validate the generated knock-out (KO) model, 5αR2 levels were evaluated in the prostate tissue by Western blot (representative image reported in the bottom of panel B). Crispr/CAS9 approach efficiently deleted 5αR2 and abolished 5αR2 expression (one-way ANOVA, $F(1,4)=99.560$, $p<0.001$, partial $\eta^2=0.961$). (C-E) KO male rats showed an increase in the floating time during the forced swim test (C, one-way ANOVA, $F(1,18)=10.416$, $p=0.005$, partial $\eta^2=0.367$), a decrease in the time spent outside the chamber in the defensive withdrawal paradigm (D, one-way ANOVA, $F(1,11.724)=6.418$, $p=0.027$, partial $\eta^2=0.263$ with Brown-Forsythe correction), and a decrease in the sucrose preference test (E, repeated measure two-way ANOVA, main effect of time, $F(6,108)=3.437$, $p=0.004$, partial $\eta^2=0.160$; main effect of genotype, $F(1,18)=17.290$, $p=0.0006$, partial $\eta^2=0.490$; interaction, $F(6,108)=4.179$, $p=0.007$, partial $\eta^2=0.188$. In the panel are reported significance from post-hoc analysis with Sidak's correction). (F-H) KO female rats showed a dimmed phenotype, with no differences in the forced swim (F) and in the sucrose preference (H) tests, and a significant decrease in the time spent interacting with a foreigner counterpart (G, one-way ANOVA, $F(1,16)=8.796$, $p=0.009$, partial $\eta^2=0.355$). For further details, see

Supplementary Results. Data are presented as mean ± SEM. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$.

Stress increases AP levels with a 5aR2-dependent mechanism.

Building on these premises, we measured the levels of progesterone and AP (as well as their relative ratio) in the PFC in basal condition and after stress exposure. Male rats knock-down for either 5αR1 or 5αR2 were sacrificed 30 minutes after exposure to forced-swim stress, in comparison with no-stress conditions. At baseline, knocking-down 5αR1 increased progesterone levels and decreased AP levels, while knocking-down 5αR2 decreased both progesterone and AP levels (Fig. 6A-B). However, the AP/progesterone ratio was significantly affected only by the knock-down of 5αR1 (Fig. 6C). After stress exposure, we detected a significant upregulation of progesterone and a significant downregulation of AP levels in 5αR2 knock-down animals (Fig. 6D-E), leading to a significant downregulation of the ratio AP/progesterone (Fig. 6F). Taken together these data suggest that while 5αR1 is crucial for maintaining the proper levels of progesterone/AP at baseline (tonic phase), 5αR2 presence is instrumental to increase AP during stress exposure (inducible phase).

These data led us to the hypothesis that the inability to synthesize AP in response to acute stress may account for the impairment in acute stress response in male rats with 5αR2 knock-down of the mPFC. To test this idea, we reevaluated the behavioral responses of these animals following AP treatment (6 mg/kg, IP, 15 minutes before testing). Notably, AP treatment ameliorated the behavioral abnormalities observed in these animals. Indeed, male rats showed a decrease in the latency to exit the dark chamber in the defensive withdrawal paradigm (Fig. 6G), as well as increases in the latency to immobility in the forced swim test (Fig. 6H), in social interaction (Fig. 6I, evaluated as the duration of anogenital sniffing), and new odor arousal (Fig. 6J).

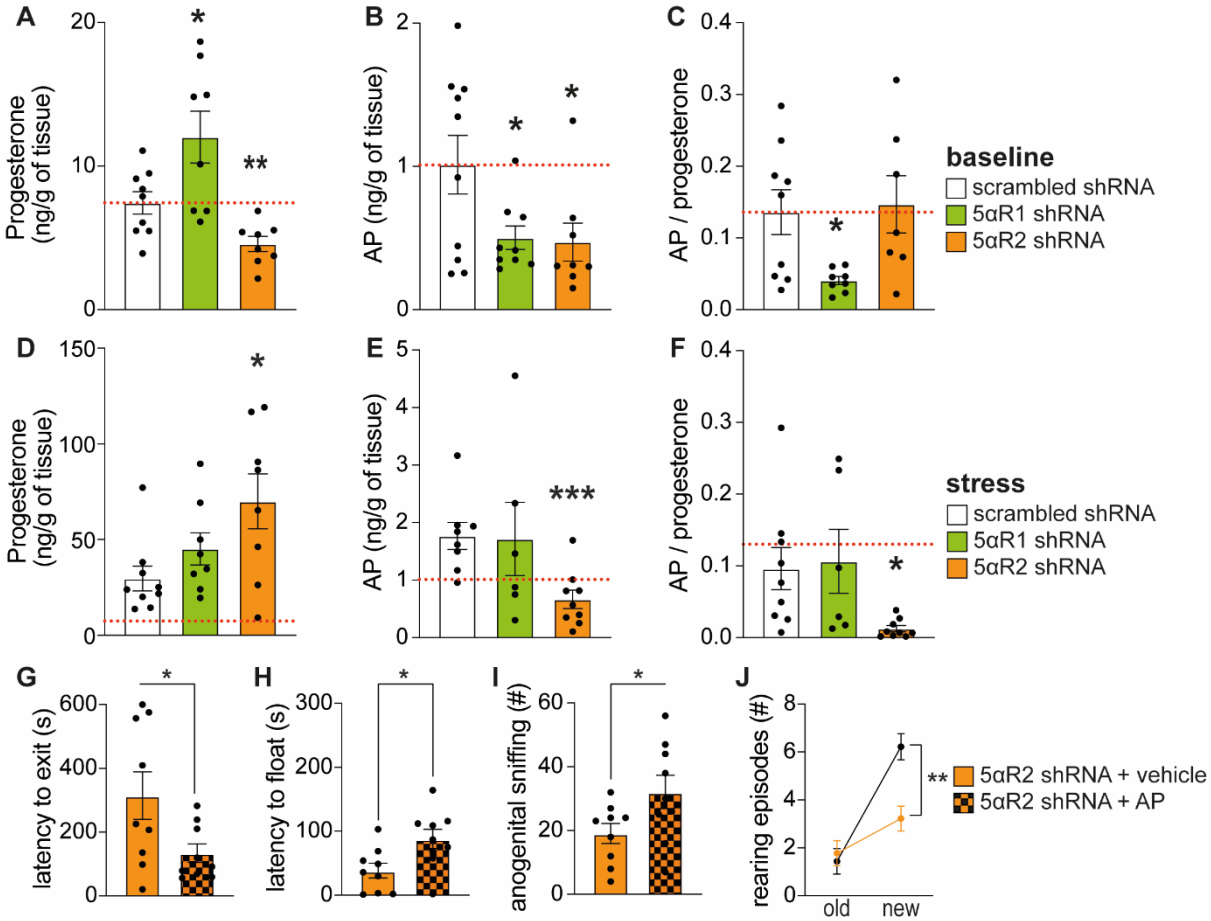

Figure 6: Levels of neuroactive steroids in rat prefrontal cortex and effects of systemic administration of allopregnanolone in 5αR2 deficient rats. (A-F) Analysis of progesterone and allopregnanolone (AP) in prefrontal cortices from experimental animals (scrambled, shRNA for 5αR1, shRNA for 5αR2) in basal condition (A-C) or 30 minutes after stress (forced swim) exposure (D-F). (A-C) At baseline, while progesterone levels were significantly increased by shRNA targeting 5αR1, they were significantly decreased by shRNA targeting 5αR2 (A, one-way ANOVA, $F(1,9.524)=5.407$, $p=0.044$, partial $\eta^2=0.281$ with Brown-Forsythe correction for 5αR1 and $F(1,15)=8.870$, $p=0.009$, partial $\eta^2=0.372$ for 5αR2). AP was significantly decreased by the knock-down of either 5αR1 or 5αR2 (B, one-way ANOVA, $F(1,11.772)=5.366$, $p=0.039$, partial $\eta^2=0.225$ with Brown-Forsythe correction for 5αR1 and $F(1,14.807)=4.896$, $p=0.043$, partial $\eta^2=0.214$ with Brown-Forsythe correction for 5αR2). However, only the downregulation of 5αR1 decreased the ratio between AP and progesterone (C, one-way ANOVA, $F(1,8.531)=8.953$, $p=0.016$, partial $\eta^2=0.347$ with Brown-Forsythe correction). (D-F) As expected, stress increased progesterone and AP levels, while the respective ratio did not change magnitude (in the graphs, the dashed red lines represent the average of the scrambled group at baseline). Stress exposure in animals with a downregulation of 5αR2 significantly increased progesterone levels (D, one-way ANOVA, $F(1,9.804)=6.582$, $p=0.029$, partial $\eta^2=0.322$ with Brown-Forsythe correction), while reduced AP levels (E, one-way ANOVA, $F(1,15)=15.377$, $p=0.001$, partial $\eta^2=0.506$). In line with these results, also the ratio was significantly affected by the condition (F, one-way ANOVA, $F(1,8.341)=7.923$, $p=0.022$, partial $\eta^2=0.331$ with Brown-Forsythe correction). No significant effects were detected in animals with downregulation of 5αR1. (G-J) Given the effects driven by 5αR2 downregulation, we tested whether AP treatment was able to rescue the phenotype of these rats. Indeed, treatment with AP (IP, 6mg/kg, 15 minutes before testing) was able to

decrease the latency to exit in the defensive withdrawal paradigm (G, one-way ANOVA, $F(1,10.335)=5.073$, $p=0.047$, partial $\eta^2=0.241$ with Brown-Forsythe correction), and to increase the latency to immobility in the forced swim test (H, one-way ANOVA, $F(1,16)=6.790$, $p=0.0169$, partial $\eta^2=0.298$). Furthermore, AP treatment increased the social interaction, evaluated as the duration of anogenital sniffing (I, one-way ANOVA, $F(1,16)=4.599$, $p=0.048$, partial $\eta^2=0.223$), and odor arousal (J, repeated measure two-way ANOVA, main effect of treatment, $F(1,16)=4.655$, $p=0.0171$, partial $\eta^2=0.225$; main effect of odor, $F(1,16)=53.837$, $p<0.0001$, partial $\eta^2=0.771$; $F(1,16)=15.451$, interaction, $p=0.001$, partial $\eta^2=0.491$). In the panel is reported significance from post-hoc analysis with Holm's correction. For further details, see Supplementary Results. Data are presented as mean ±SEM. *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$.

5αR2 enables RNA translation processes in the mPFC in response to acute stress.

To understand the cellular mechanisms underlying the regulation of stress reactivity by 5αR2 in the mPFC, we conducted snRNA-seq of this region in male rats subjected to either scrambled-RNA or 5αR2-shRNA administration. Specifically, to elucidate the selective impact of this enzyme on molecular changes induced by acute stress, different groups of animals were either exposed to forced swim stress or maintained under control, no-stress conditions. Gamma-Poisson modeling identified 13 distinct clusters of cells, which were recognized via cell markers as: pyramidal neurons (PN) from various cortical layers; interneurons (VIP positive and VIP negative); oligodendrocyte progenitor cells (OPCs); oligodendrocytes; astrocytes; microglia; stromal cells; and endothelial cells (Fig. 7A-B, S3). To elucidate the effects of 5αR2 downregulation in the PFC under both baseline conditions and in the context of acute stress response, we compared snRNA-seq data between unstressed 5αR2 knockdown and control rats. Notably, no discernible differences in gene expression were observed in the interneuron, stromal, and endothelial cell clusters, as well as most PN clusters (Supplementary Tables 1-3). In contrast, layer-V PNs, microglia, astrocytes, OPCs, and oligodendrocytes exhibited substantial transcriptomic variations between groups, suggesting their heightened susceptibility to the effects of 5αR2 deficiency.

Within the oligodendrocyte cluster, we identified 164 differentially expressed genes (DEGs). Gene Ontology (GO) analyses for biological processes revealed significant differences in various modules, including "Cytoplasmic Translation", "Macromolecule Biosynthetic Process", and "Peptide Biosynthetic Process." Similar findings were observed in astrocytes (48 DEGs) and OPCs (22 DEGs). In another cluster exhibiting characteristics of microglia, we detected 231 DEGs, with prominent variations associated with the "Regulation Of Peptidyl-Tyrosine Phosphorylation" pathway. Analyses within layer V PNs unveiled 76 DEGs, with notable downregulation observed in pathways related to "Cell-Cell Adhesion Via Plasma-Membrane Adhesion Molecules" and "Homophilic Cell Adhesion Via Plasma Membrane Adhesion Molecules" (Fig. 7C). Similar effects were identified in GO cellular component and molecular function analyses (Fig. S4).

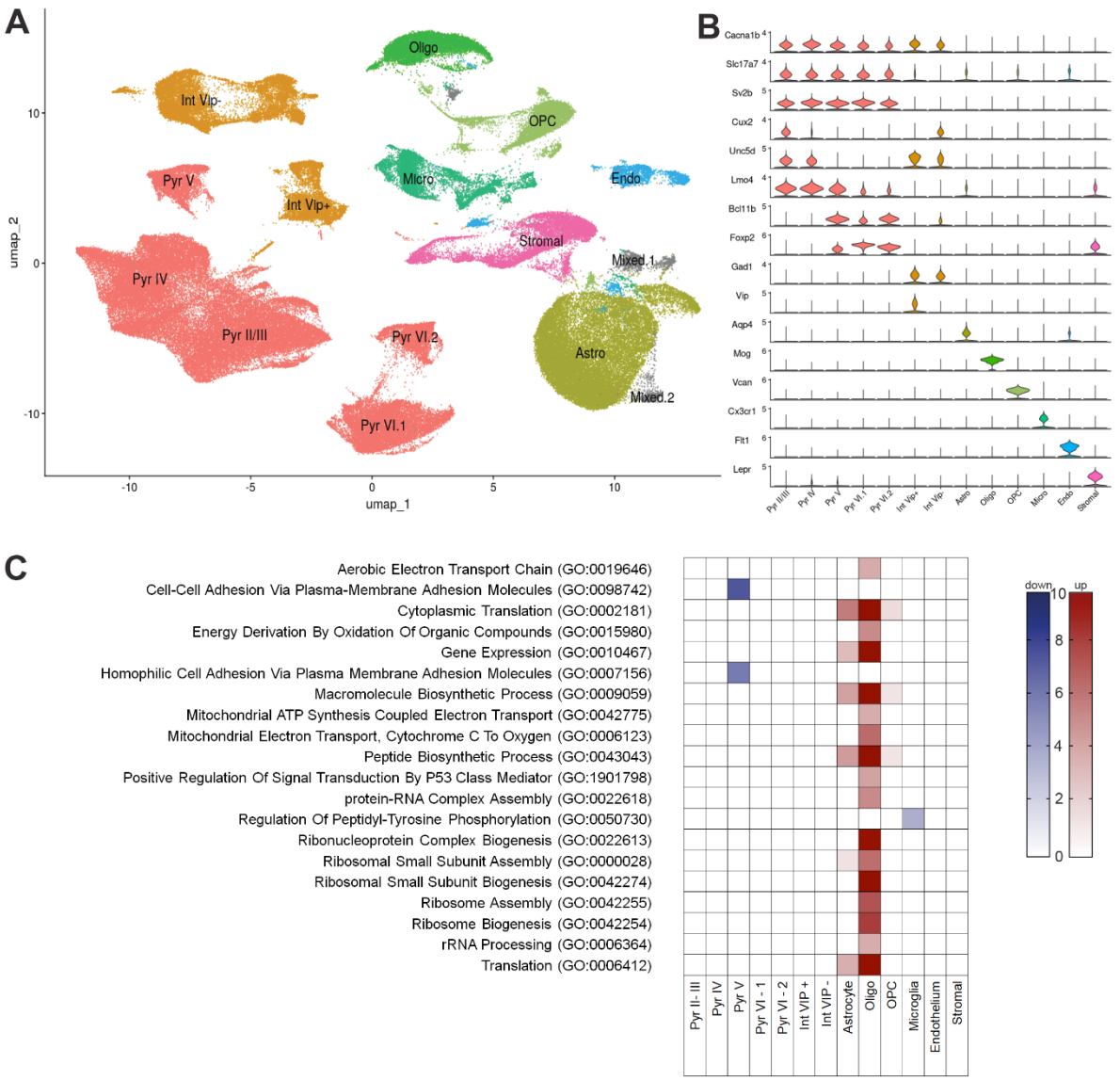

Figure 7: Single-nucleus transcriptomic analyses of cortical tissues of 5aR2-shRNA and scrambled-shRNA rats. (A) Uniform Manifold Approximation and Projection (UMAP) following SCTransformation with the generalized linear model, Gamma Poisson method, annotated by major cell types, including astrocytes, oligodendrocytes, microglia, pyramidal neurons, interneurons, oligodendrocyte progenitor cells (OPC), and others. (B) Violin plots of marker genes used to distinguish the clusters differentiating known cell types. (C) Gene ontology analysis for biological processes highlights the most differential ontologies among these cell clusters. Abbreviations: Pyr, pyramidal neurons; Int, interneurons; Astro, astrocytes; Oligo, oligodendrocytes; OPC, oligodendrocyte precursor cell; Micro, microglia; Endo, endothelial cells.

Given that our analyses revealed that most effects of 5αR2 are best captured in relation to the response to acute stress, we conducted a detailed analysis of snRNA-seq data with respect to the effects of forced swim stress in rats previously subjected to mPFC 5αR2 knockdown or scrambled RNA injections. To highlight the transcriptomic effects of stress exposure, we ranked the overall GO biological processes in the scrambled RNA groups (baseline and after stress exposure). Subsequently, we examined the same modules in the 5αR2-shRNA groups. Remarkably, while stress exposure led to a significant upregulation of several processes related to translation and gene expression across neurons and glial cells (Fig. 8A, left panel), we observed a striking downregulation of these pathways in tissues with 5αR2 knockdown (Fig. 8A, right panel). Specifically, the effects in the mPFC neuron clusters were driven by 262 DEGs in the control group (Supplementary Tables 4-6) and 1191 DEGs in the 5αR2-shRNA group (Supplementary Tables 7-9). Moreover, the specific downregulation of pathways such as "Axon Guidance", "Chemical Synaptic Transmission", and "Nervous System Development" in interneurons was completely abolished by 5αR2 deficiency. Similar trends were observed in the GO cellular component analysis (Fig. 8B) and the GO molecular function analysis (Fig. S5).

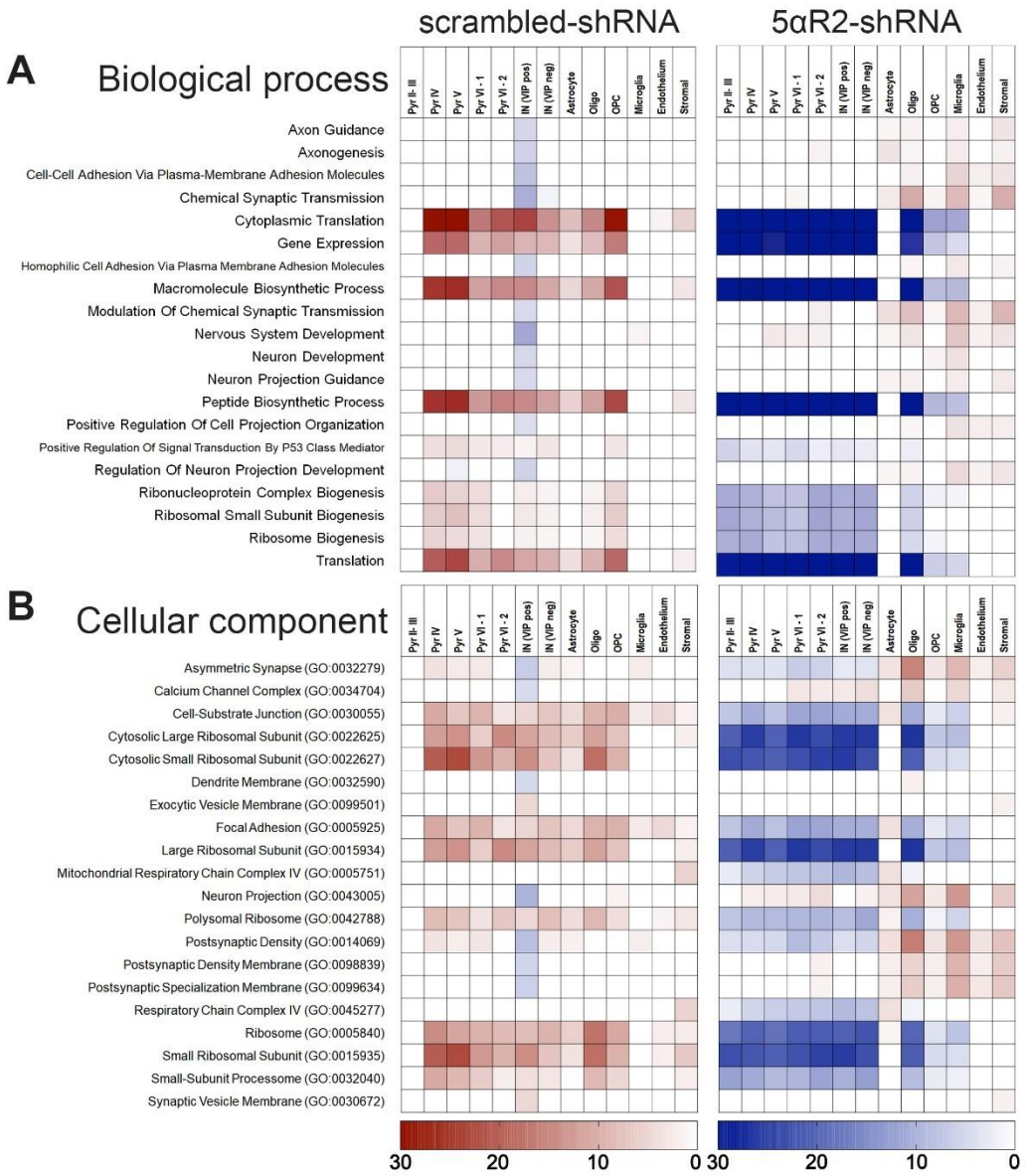

Figure 8: Single-nucleus transcriptomic analyses of cortical tissues of 5aR2-shRNA and scrambled-shRNA rats exposed to acute stress. (A-B) Gene ontology analysis for biological process (A) and cellular component (B) highlighting the most differential ontologies among these cell clusters. Two comparisons are shown side by side, on the left differences induced by stress exposure in prefrontal cortex (PFC) of control rats, on the right the same behavioral paradigm applied to rats knocked-down for 5αR2 in the PFC. Abbreviations: Pyr, pyramidal neurons; Int, interneurons; Oligo, oligodendrocytes; OPC, oligodendrocyte precursor cell.

DISCUSSION

The results of our study point to a paramount, previously unrecognized role of prefrontal 5αR2, rather than 5αR1, in enabling acute stress response exclusively in male subjects. This conclusion is supported by several key findings: first, 5αR2 expression was rapidly upregulated in the mPFC in response to two distinct, well-established experimental protocols producing inescapable acute stress; however, this response was exclusively observed in males, in sharp contrast with the lack of changes in females; second, in contrast with 5αR1**,** targeted genetic downregulation of 5αR2 expression in this region led to a profound reduction in the ability of male, but not female, rats to mount an adaptive behavioral response to both stressful and arousing stimuli; third, a selective reduction of 5αR2, but not 5αR1, abolished stress-induced elevation of AP levels in males, identifying this enzyme as a pivotal factor for the biosynthesis of this neurosteroid in response to acute stress. In support of this idea, AP administration reversed the behavioral deficits observed in male rats subjected to 5αR2 knock-down in the mPFC. Finally, 5αR2 suppression in this region led to a complete reversal of the cellular response to stress in males, as evidenced by single-nucleus transcriptomic analyses. Overall, these results underscore 5αR2's central role in regulating behavioral reactivity in males, but not in females, and point to the implication of this enzyme in sexually dimorphic regulation of the response to acute stress. These findings are in line with our prior research indicating a diminished reactivity to environmental stimuli following finasteride administration (Godar et al., 2019) by pointing to the role of 5αR2, rather than 5αR1, inhibition, as critical to blunt the ability to react to salient stimuli. Most importantly, these results challenge established beliefs regarding the functions of 5αR1 and 5αR2 in behavioral regulation.

As previously delineated, AP biosynthesis proceeds from progesterone through a two-step enzymatic pathway, initially involving the transformation of progesterone into DHP via the action of 5αR, followed by the subsequent conversion of DHP into AP by 3αHSOR. Taken together these data highlighted a distinction between the dynamics of 5αR1 and 5αR2 regarding AP production. Our findings indicate that, in rats subjected to knock-down of 5αR1 in the mPFC, the basal concentrations of progesterone were significantly elevated, while AP levels were reduced. However, no behavioral differences were observed between these rats and their counterparts treated with scrambled shRNA. These data suggest that 5αR1 functions as a constitutive enzyme responsible for the continuous conversion of progesterone to DHP and the sustained production of basal levels of AP. In contrast, 5αR2 knock-down in the mPFC actively abolished stress-induced augmentation of AP levels. These findings collectively suggest that 5αR1 and 5αR2 exhibit distinct functional roles in regulating AP synthesis. These

data indicate that 5αR2 orchestrates a transient, stress-adaptive response by enabling the rapid synthesis of AP in response to swift environmental variations. The characteristics of 5αR2, including its synthesis within 30 minutes from acute stress exposure, align with those of an adaptive or inducible enzyme responsive to sudden environmental changes. Consistent with this distinction, 5αR2 exhibits a markedly higher affinity for progesterone and other ketosteroids than 5αR1. Furthermore, the lower and more localized distribution of 5αR2 within the brain aligns with the specific expression pattern characteristic of inducible enzymes. Steroidogenic cells do not store preformed hormones but synthesize steroids *de novo* in response to physiological demands; thus, the functional bifurcation between 5αR1 and 5αR2 appears perfectly tailored to enable either slow and sustained or rapid and transient adaptive responses to environmental contingencies.

One of the most striking results of this study was that the role of 5αR2 in acute stress response was only observed in males, pointing to a marked sexual dimorphism in the involvement of 5αR2 in behavioral regulation. In line with these data, previous studies have shown that acute stress does not lead to an elevation of AP levels in the brains of female rodents (Pisu et al., 2016). By identifying 5αR2 as a critical player in the acute stress response in male rats, this study highlights the specific role of this isoform in mediating acute stress response and suggests potential therapeutic implications for modulating AP levels in stress-related disorders. These findings provide a potential mechanistic explanation for sex differences in stress vulnerability and resilience. In addition, they deepen our understanding of the neurochemical mechanisms underlying stress adaptation but also offer potential targets for therapeutic intervention in stress-related neuropsychiatric disorders. The mechanisms underlying the divergent responses in males and females with respect to 5αR2 remain unknown; however, this divergence may be contributed by the well-defined role of this enzyme in converting testosterone into the even more potent androgen agonist DHT. Irrespective of the underlying mechanisms, it is worth noting that the distinction between males and females with respect to the regulation of 5αR2 may yield valuable insights into the potential contribution of this enzyme to sex-specific susceptibility in psychopathological conditions characterized by aberrant stress responses, such as anxiety and mood disorders. Thus, previous report shows that environmental stress exposure significantly increased 5αR2 in male and female rats, with an interesting sexual dimorphism in 5αR1 (Sánchez et al., 2009), Furthermore, our experiments showed that 5αR2 down-regulation in the mPFC led to a blunted behavioral response to salient stimuli, paralleled by reduced stress-induced AP synthesis. Notably, some of these results are in alignment with the behavioral aberrations commonly associated with chronic stress and depression-related responses, such as reduced preference for palatable stimuli and reduced social interaction and response in the forced swim test. For this reason,

future studies are warranted to verify the changes in 5αR2 related to chronic stress. Understanding the molecular underpinnings of these sex-specific responses could inform the development of sex-tailored therapeutic interventions for stress-related disorders, such as depression.

Acute environmental stress triggers survival responses, including alterations in gene expression and physiological functions, collectively constituting a defensive reaction. Single-nucleus transcriptomic analyses of mPFC tissues with a 5αR2 knockdown revealed marginal baseline changes, mostly limited to oligodendrocytes and deep-layer pyramidal neurons; however, in striking contrast with these results, the downregulation of this isoenzyme led to dramatic differences with respect to the acute stress response. We found that acute stress induced widespread transcriptional changes in most neuronal cells and glia, characterized by the enhancement in translational activities and activation of the endoplasmic reticulum across these cell populations. Strikingly, acute stress exerted the opposite effects in the absence of 5αR2, with a dramatic reduction in these biological processes. These results suggest that 5αR2 enables neuroprotective actions aimed at buffering the detrimental impact of acute stress in the PFC, likely due to the production of AP or other 5α-reduced neurosteroids. Insufficient 5αR2 levels, coupled with overwhelming stress exposure, result in a substantial decrease in translation and energy production. Consistent with this notion, AP exerts neurogenic and neuroprotective effects, which likely mitigates the adverse impacts of acute stress and supports translation maintenance.

Several limitations in this study should be acknowledged. Firstly, while our investigation provides valuable insights into the roles of 5αR1 and 5αR2 within the mPFC and NAc, it is essential to recognize that our analysis was confined to these specific brain regions and isoenzymes. We did not comprehensively explore the involvement of other critical brain regions implicated in the acute stress response, such as the hippocampus, amygdala, and hypothalamus, nor did we assess the potential contributions of other 5αR isoenzymes, such as 5αR3, or other neurosteroidogenic enzymes. Future research should investigate the involvement of these additional factors in relation to the acute stress response to provide a more comprehensive understanding of the neurobiological mechanisms underlying stress adaptation.

Secondly, while our study primarily focuses on AP, it is crucial to acknowledge that other 5α-reduced neurosteroids, such as tetrahydrodeoxycorticosterone (THDOC), may also be synthesized concurrently. These neurosteroids exert diverse effects on receptors, including but not limited to GABA-A, NMDA, and progesterone membrane receptors. Given the interconnected nature of neurosteroid signaling pathways, it is plausible that changes in 5αR2

synthesis may impact the activation of these receptors. Therefore, future studies are warranted to investigate whether alterations in 5αR2 expression are accompanied by corresponding changes in the signaling of these neurosteroid-interacting receptors, thereby providing further insight into the intricate mechanisms underlying stress response and neurological function.

Thirdly, the behavioral profile of rats subjected to 5αR2 knockdown in the mPFC aligns with several characteristics associated with chronic stress and depression-related responses, including a low preference for palatable stimuli, reductions in exploratory activity and social interaction, and blunted response to acute stressors. In line with this observation, it is important to note that AP plays a key role in the pathophysiology of several neuropsychiatric disorders, including anxiety and depression (Schüle et al., 2014). In particular, AP exerts potent, rapid antidepressant properties, which have led to its recent approval as a new therapy for post-partum depression (Pinna, 2020). This profile raises key questions regarding the potential functions of 5αR2 in the context of chronic stress and depression. In line with this idea, previous research has demonstrated a reduction in 5αR2 levels in rats subjected to isolation rearing (Bortolato et al., 2011), suggesting a potential vulnerability of this enzyme to chronic stress. Understanding how changes in 5αR2 may be implicated in depression and other disorders associated with chronic stress may inform the development of novel therapeutic strategies for these conditions.

Even with these limitations, our findings have revealed that 5αR2 is a key contributor to the intricate cascade of molecular events governing the adaptive stress response, shedding light on its potential importance in neurological and psychiatric contexts. Possibly due to its low baseline expression in the brain, this enzyme has received limited attention in neurobiological research. These findings pave the way for further investigations into the molecular mechanisms underlying stress resilience and vulnerability. Further investigation into the regulatory mechanisms and functional implications of 5αR2 in the brain is warranted to elucidate its precise role in stress adaptation and related neurological processes. Moreover, translational studies in humans are needed to validate the relevance of these findings in clinical settings. Understanding the interplay between 5αR isoenzymes, neurosteroid synthesis, and stress-related disorders could lead to the development of novel pharmacological interventions targeting these pathways.

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