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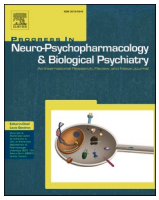

Emotional dysregulation following prenatal stress is associated with altered prefrontal cortex responsiveness to an acute challenge in adolescence

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ABSTRACT

Exposure to prenatal stress (PNS) has the potential to elicit multiple neurobiological alterations and increase the susceptibility to psychiatric disorders. Moreover, gestational stress may sensitize the brain toward an altered response to subsequent challenges. Here, we investigated the effects of PNS in rats and assessed whether these animals exhibit an altered brain responsiveness to an acute stress (AS) during adolescence. From gestational day 14 until delivery, Sprague Dawley dams were exposed to PNS or left undisturbed. During adolescence (PND38 to PND41), offspring were tested in the social interaction and splash test. At PND44 half of the animals were exposed to 5 min of forced swim stress. Males and Females exposed to PNS showed reduced sociability and increased anhedonic-like behavior. At the molecular level, exposure of adolescent rats to AS produced increased activation of the amygdala and ventral and dorsal hippocampus. Regarding the prefrontal cortex (PFC), we observed a pronounced activation in PNS males exposed to AS. Cell-type specific transcriptional analyses revealed a significant imbalance in the activation of PFC excitatory and inhibitory neurons in PNS males and females exposed to AS. Furthermore, stressed males exhibited disrupted HPA-axis function, while females showed impairments in the modulation of antioxidant genes. Our study shows that PNS induces emotional dysregulation and alters the responsiveness of the PFC to an acute stressor. Moreover, the disruption of excitatory and inhibitory balance during adolescence could influence the ability to respond to challenging events that may contribute to precipitate a full-blown pathologic condition.

1. Introduction

Gestation represents a pivotal phase in human development (Abbott et al., 2018). During this stage, the fetus is markedly susceptible to the conditions that the mother experiences. With this respect, there is a great deal of attention for the risk associated with the exposure to environmental adversities. Indeed, exposure to prenatal stress (PNS) has emerged as a significant event with the potential to elicit multiple neurobiological modifications in the fetus, thereby heightening the risk for the development of psychiatric disorders later in life (Glover et al., 2018; Buss et al., 2012). The long-term effects of PNS have been related to various factors including, but not limited to, sex, genetic predisposition, and stressor intensity (Abbott et al., 2018; Novais et al., 2017). Furthermore, it has been reported that not all individuals exhibit uniform responses to the exposure to stressors, since some individuals, classified as vulnerable, will eventually manifest phenotypic alterations, whereas others may be resilient (Creutzberg et al., 2023a; Franklin et al., 2012). Considering the multidimensional characteristics of stress-related alterations, it has become increasingly important to unravel the molecular underpinnings associated with the exposure to stressors early in life.

Several mechanisms have been implicated in the long-term consequences produced by PNS exposure, including a dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis, disruption in oxidant-antioxidant balance, heightened neuroinflammation, and enduring epigenetic alterations (Lopizzo et al., 2021; Cattaneo et al., 2018; Creutzberg et al., 2021; Fatima et al., 2019). These factors are considered pivotal for the susceptibility to developing psychiatric disorders later in life. Furthermore, we have recently shown that vulnerability or resilience to PNS is associated with significant changes in the basal

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activity state of different brain regions at adolescence, which represents a critical period for the manifestation of negative outcomes associated with stress exposure (Creutzberg et al., 2023a).

However, while it is well-established that PNS exposure may be associated with an enhanced risk to develop mental disorders, exposure to early life adversities may also prime the brain to subsequent challenges, which could precipitate a pathologic condition according to the classical 'two-hit' model (McEwen, 2003; Nederhof and Schmidt, 2012). Indeed, we have shown that adult rats previously exposed to PNS show significant changes in the responsiveness to an acute stress with brain region- and sex- selectivity (Luoni et al., 2016). However, adolescence is recognized as a developmental phase marked by vulnerability, which has the potential to further enhance brain reactivity to subsequent stressors (Andersen, 2003).

In the present study we aimed to identify mood-related alterations in animals previously exposed to PNS and to establish if such phenotype is associated with an altered ability to respond to an acute stressful experience. To this purpose, we investigated immediate-early genes (IEGs) as a proxy for brain activation and experience-induced cellular activity (Schuler et al., 2022), followed by cell-type specific transcriptional analysis in GABAergic and glutamatergic cells to investigate a possible imbalance of excitatory and inhibitory (E/I) mechanisms that have been linked to stress-related disorders (Caballero et al., 2021; McKlveen et al., 2019; Marchisella et al., 2021; Page and Coutellier, 2019).

2. Methods

2.1. Animals

Adult nulliparous male and female Sprague Dawley rats (*Rattus norvegicus*) were purchased from Charles River Laboratories (Italy). After two weeks of habituation, rats were bred with one male and one female for 48 h. After this period, two females were housed together until gestational day (GD) 14. At GD14 dams were single-housed and randomly allocated as control (CT) or prenatal stress (PNS). PNS dams were exposed to gestational restraint stress from GD14 until delivery, while CT dams were left undisturbed. A total of 6 litters for CT dams and 9 litters for PNS dams were utilized in this study. To reduce potential litter effects, 3 animals of each sex were utilized per litter. At birth the litters were culled to 10 pups (5 males and 5 females), and the day of birth was considered postnatal day (PND) 0. Pups were weaned at PND21 and housed with three same-sex animals per cage. Offspring behavioral testing was performed during adolescence (PND38 to PND41). The behavioral investigation was carried on a total of 80 animals: 16 CT males, 16 CT females, 24 PNS males, 24 PNS females. Behavioral data was video recorded and analyzed by two trained researchers with over 90 % of inter-rater reliability who were blind to the experimental groups. The reported results are the average of the individual observers.

After behavioral assessment (PND44), PNS animals with phenotypic alterations were identified using the cut-off method (Ardi et al., 2016). A behavioral score was calculated for each animal considering the total time and latency of the social interaction and splash test. To be considered affected, the score of the PNS animal must be higher than the average of the CT group plus one standard deviation. Next, half of CT and PNS animals that presented phenotypic alterations were randomly selected to be exposed to an acute stress (AS) before euthanasia. The experimental timeline can be seen in Fig. 1A.

All animals were housed in a facility with controlled temperature (21 ± 1 °C) and humidity (55 ± 5 %) under a 12 h/12 h light-dark cycle (lights on at 7 a.m.) with food and water ad libitum. Weekly cage cleaning was performed by the animal facility staff. All procedures included in the experiment were approved by the Italian Health Ministry under protocol n. 752/2020, in accordance with the Italian legislation on animal experimentation (Decreto Legislativo 116/92) and adherent to EU recommendation (EEC Council Directive 86/609). Furthermore, this experiment was conducted in accordance with the ARRIVE guidelines (Percie du Sert et al., 2020).

2.2. Prenatal stress protocol

The restrained stress protocol was performed during the last week of gestation (GD14 until delivery). PNS dams were exposed to three 45 min sessions of stress per day (randomized between 7 a.m. and 7 p.m.) under bright light (1500 lx) (Marchisella et al., 2021). The dams were placed into transparent Plexiglas cylinders (20 cm length x 9 cm diameter x 9 cm height) that could be regulated depending on the size of each animal. Body weight was checked to assess any effects of stress on weight gain during pregnancy.

2.3. Social interaction test

The social interaction test was performed at PND38. The test was performed in two phases, named sample and test. For the sample phase, the animal was allowed to explore for 3 min an arena (1 m × 1 m) that contained an empty grided enclosure. After that, the animal was removed from the apparatus and an unfamiliar animal (sex, age, and weight match) was introduced inside the grided enclosure. For the test phase, the animal was again free to explore the apparatus and the unfamiliar animal for 3 min. The latency for the first interaction and the total interaction time (sniffing, exploring, or touching) between animals was evaluated. The apparatus was cleaned with 70 % ethanol between animals.

2.4. Splash test

The splash test was performed at PND41. The test was performed by spraying a 10 % sucrose solution on the dorsal coat of the animals. The total time of grooming and latency to start grooming throughout a period of 3 min were evaluated.

2.5. Acute stress protocol

After behavioral testing (PND44) a subgroup of CT and PNS males and females were exposed to an acute swim stress (AS), while sham (not exposed to AS) animals were left undisturbed in their home cages. For the acute stressor, animals were placed into a transparent Plexiglas cylinder filled up with water (25 ± 2 °C) for 5 min. The water was changed, and the apparatus was cleaned between animals.

2.6. RNA extraction and transcriptional analysis

At PND44 sham animals were euthanized by decapitation, while on the same day, AS animals were euthanized 30 min after the AS protocol. The brain was removed, and one hemisphere was snap frozen in 2-methylbutane to be cut in a cryostat at 160 μm per section. Using the rat brain atlas as reference, the prefrontal cortex (PFC), dorsal hippocampus (dHIPP), ventral hippocampus (vHIPP), and amygdala (AMY) were micro-dissected utilizing punchers (Paxinos and Watson, 2006). The following bregma ranges were utilized for the microdissections: PFC (4.68 to 2.52 mm); dHIPP (−2.76 to −4.08 mm); vHIPP (−4.56 to 5.28 mm); AMY (−1.56 to −3.36 mm). Total RNA extraction from the selected brain regions was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Following quantification using NanoDrop spectrophotometer (Thermo Fisher), RNA samples were diluted at 10 ng/μl for quantitative real-time polymerase chain reaction (qRT-PCR) (CFX384 real-time system, Bio-Rad Laboratories). All samples were run in triplicates using β-Actin and GAPDH as internal controls. Primers ID's and sequences are reported in table S1. Amplification efficiencies for target and housekeeping genes were considered for data analysis (Pfaffl, 2001). Data is reported as fold

change % compared to the CT baseline group (set at 100 %).

2.7. RNAscope and immunofluorescence

For in situ hybridization analysis, the other hemisphere was post-fixed with 4 % paraformaldehyde for 24 h, dehydrated in 30 % sucrose for 48 h, and frozen with 2-methylbutane for further processing. The brain was cut in a cryostat at 20um, and 1 out of 6 PFC sections was used for the analysis in accordance with the rat brain atlas (Paxinos and Watson, 2006). The co-detection RNAscope™ Multiplex Fluorescent V2 Assay was performed following the manufacturer's instructions. Rat-Peptidylprolyl Isomerase B (Rn-Ppib – 313,921) was used as a positive control, Arc (Rn-Arc-C3 540,901) as the target gene, and DAPI was used to stain cell nuclei. In addition, the following primary and secondary antibodies were used: rabbit anti-parvalbumin (NB120–11427, Novus Bio – 1:1000), mouse anti-VGlut2 (MA5–27613, ThermoFisher Scientific – 1:100), Alexa Fluor 488 goat anti-rabbit (1:500), and Alexa Fluor 647 goat anti-mouse (1:500). Images were acquired in an LSM-900 confocal microscope (Carl Zeiss, Oberkochen, Germany) at 40×. Signal coverage area of the probe signal (Ppib or Arc) in PV+ and Vglut2+ cells were analyzed by an experimenter blind to the sample condition using ImageJ software (Schneider et al., 2012). The total Arc coverage was normalized using Ppib.

2.8. Corticosterone analysis

In the morning of the euthanasia, trunk blood from all groups was collected for corticosterone analysis under basal conditions or 30 min after the AS protocol (PND44 – between 10 a.m. and 1 p.m.). Total blood was collected in a tube containing ethylenediaminetetraacetic acid and centrifuged for 10 min at 1500*g* to separate the plasma. The analysis was performed using Corticosterone ELISA (Tecan – RE52211) assay, according to the manufacturer's instructions. Absorbance data was converted into concentration (ng/ml) based in a standard curve supplied by the assay.

2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v.29 and GraphPad Prism 9. Data is shown as group mean ± standard error of the mean (SEM). Statistically significant *p*-values indicated in the graphs are as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, while & symbol was utilized for significant interactions in the Two-way ANOVA test. Two-way ANOVA was utilized for behavior (PNS x sex) and molecular analysis (PNS x AS). RNAscope analysis was performed with two-way ANOVA (PNS X AS) considering 6 animals per group (20–30 cells per group). Tukey's post-hoc tests were conducted to identify the specific effects in pairwise comparison. The Shapiro-Wilk test was performed to verify the normality of data distribution. Sample size was based on previous experiments of the research group that investigated the behavioral and molecular effects of PNS and were calculated in accordance with estimates by power analysis using G*power software (Creutzberg et al., 2023a; Luoni et al., 2016; Marchisella et al., 2021). Outlier analysis was conducted for both behavioral and molecular data, with samples identified as outliers excluded from the analysis. Furthermore, detailed information of all statistical analysis can be found in the supplementary table 2.

The IEGs (*Arc*, *Npas4*, *cFos*, and *Zif268)* investigated were used to calculate a z-score for an integrated molecular overview. The following formula was utilized: z-score = $(x-\mu)/\sigma$; where x is the individual gene expression value of each animal, μ is the average gene expression of the control group, and σ is the standard deviation of the control group. The average of all IEGs corresponded to the overall "z-activation score", which was calculated when the expression data for at least three genes were available (Creutzberg et al., 2023b).

3. Results

3.1. Analysis of the emotional behaviors of affected adolescent rats exposed to prenatal stress

Since only a subgroup of animals exposed to prenatal stress shows a behavioral phenotype in adolescence (Creutzberg et al., 2023a), we first investigated the effects of PNS exposure on emotional behavior using the social interaction and splash test and we applied a cut-off method in order to identify animals vulnerable to PNS exposure (Ardi et al., 2016). Indeed, for these animals we found a significant impairment in sociability in both males and females, as shown by the increased latency to interact with the unfamiliar animal [$F(1,52) = 77.76$, $p < 0.001$; Fig. 1B] and the decreased interaction time [$F(1,52) = 54.66$, $p < 0.001$; Fig. 1C]. Regarding the splash test, we observed a significant increase in grooming latency [$F(1,49) = 7.948$, $p = 0.006$; Fig. 1D] and decrease in grooming time [$F(1,46) = 9.842$, $p = 0.016$; Fig. 1E] in males and females vulnerable to PNS. All the subsequent analyses of this study were performed on vulnerable animals, either under resting conditions or following the acute stress challenge.

3.2. Analysis of peripheral HPA responsiveness following prenatal stress and modulation by the acute challenge in adolescence

Considering that the aim of this work was to investigate if animals previously exposed to PNS would respond differently to an acute challenge during adolescence, we first measured plasmatic corticosterone levels as an index for the peripheral responsiveness of the HPA axis to the AS. While we did not observe any significant changes between CT and PNS rats under resting conditions, we found, as expected, a significant increase in corticosterone levels after exposure to AS in both males [$F(1,27) = 24.45$, $p < 0.001$; Fig. 2A] and females [$F(1,25) = 6.611$, $p = 0.016$; Fig. 2B]. Such effect was independent from the prenatal condition.

3.3. Analysis of brain activation in animals exposed to prenatal stress and modulation by the acute challenge in adolescence

Altered brain activation is one of the mechanisms through which PNS exposure may exert its priming effects to a secondary challenge. For this reason, we investigated the expression of four activity-regulated genes (*Arc, Npas4, cFos, and Zif268*) to calculate a z-activation score in order to investigate the activation state of different brain regions following PNS and AS during adolescence.

Within the PFC of males, we observed a significant PNS x AS interaction [$F(1,23) = 5.736$, $p = 0.025$; Fig. 3A]. *Post-hoc* analysis showed that PNS rats exposed to AS have a significant z-activation score increase, as compared to CT + AS animals as well as to their sham counterpart ($p = 0.028$ and $p < 0.001$, respectively). Furthermore, male animals exposed to AS display increased z-activation score of the dHIPP [$F(1,24) = 9.447$, $p = 0.005$; Fig. 3B], vHIPP [$F(1,24) = 13.17$, $p = 0.001$; Fig. 3C], and AMY [$F(1,21) = 17.62$, $p < 0.001$; Fig. 3D], regardless of the prenatal condition.

Considering the analysis of females, we found that animals exposed to AS show an increased activation of different regions as demonstrated by the significant AS effect in the PFC [$F(1,23) = 10.32$, $p = 0.003$; Fig. 3E], dHIPP [$F(1,24) = 5.425$, $p = 0.028$; Fig. 3F], and AMY [$F(1,21) = 8.334$, $p = 0.008$; Fig. 3H], suggesting a similar effect regardless of the prenatal condition. For the vHIPP, while we found a similar AS effect [$F(1,23) = 4.663$, $p = 0.042$; Fig. 3G], we also observed a trend for a PNS x AS interaction ($p = 0.077$). An exploratory *post-hoc* analysis revealed that CT animals exposed to AS show increased z-activation score ($p = 0.028$), an effect that was not observed in PNS rats.

Since the major differences between CT and PNS animals in the response to AS were observed in the PFC, we decided to focus on this brain region, which also exerts an important top-down role in

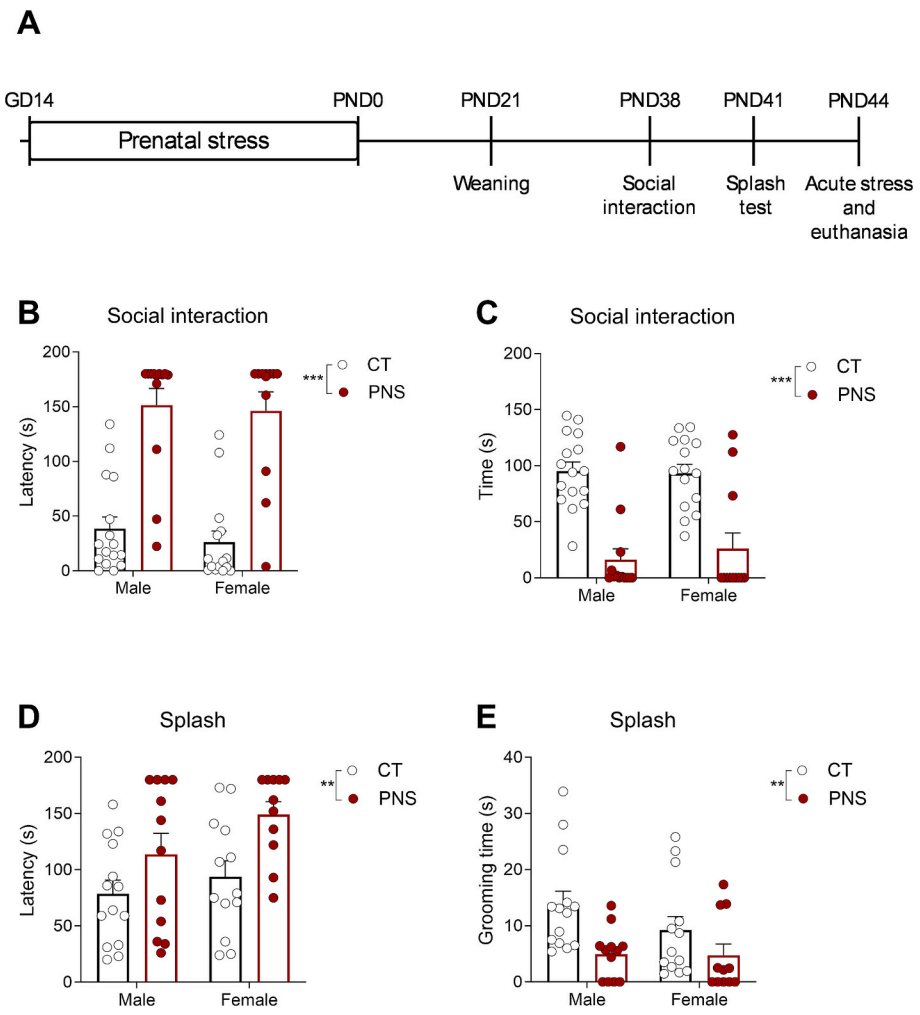

Fig. 1. Behavioral characterization of adolescent animals exposed to prenatal stress.
Pregnant dams were exposed to prenatal stress (PNS) or left undisturbed (CT). During adolescence, the latency and time of interaction in the social interaction test and latency and grooming time in the splash test were investigated in the offspring. A) Experimental design and timeline. Social interaction (B and C) and splash test (D and E) were performed on control and PNS animals that were subsequently randomized either to sham condition or to the acute stress exposure for the molecular analyses. Data are expressed as mean ± SEM of 11–16 animals per group. $^{**}p < 0.01$; $^{***}p < 0.001$ (Two-way ANOVA).

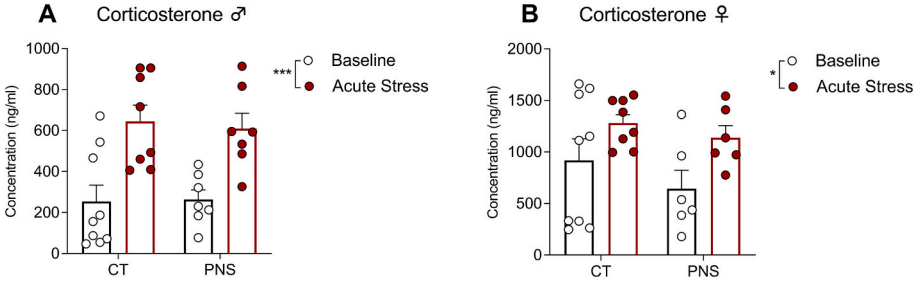

Fig. 2. Plasma corticosterone levels in animals exposed to prenatal stress: modulation by an acute challenge in adolescence.
Effect of a 5 min swim stress in adolescent male (panel A) and female (panel B) rats whose mothers were exposed to prenatal stress (PNS) or left undisturbed (CT). Values are the mean corticosterone concentrations ± SEM of 6–9 animals per group. $^{*}p < 0.05$; $^{***}p < 0.001$ (Two-way ANOVA).

controlling the functional activity of other brain structures.

Regarding the analysis of the individual genes within the PFC of male animals, we found a significant PNS x AS interaction for *Arc* mRNA levels [F (1,23) = 7.855, $p = 0.010$; Fig. 4A]. *Post-hoc* analysis revealed that PNS animals exposed to the acute challenge in adolescence showed higher mRNA levels compared to their sham counterpart ($p < 0.001$) as well as to CT animals exposed to the acute stressor ($p = 0.017$). Moreover, we found a significant AS effect for *Npas4* [F (1,23) = 13.36, $p =$ 0.001; Fig. 4B], *cFos* [F (1,23) = 51.80, $p < 0.001$; Fig. 4C], and *Zif268* [F (1,23) = 16.81, $p < 0.001$; Fig. 4D], with increased mRNA levels regardless of PNS exposure. When considering female rats, the pattern of modulation for the different IEGs was not homogeneous. Indeed, we found a significant PNS x AS interaction for *Npas4* mRNA levels [F (1,23) = 4.363, $p = 0.048$; Fig. 4F] as well as for *cFos* gene expression [F (1,23) = 5.226, $p = 0.031$; Fig. 4G]. Accordingly, *post-hoc* analysis revealed that exposure of CT females to the AS produced a significant

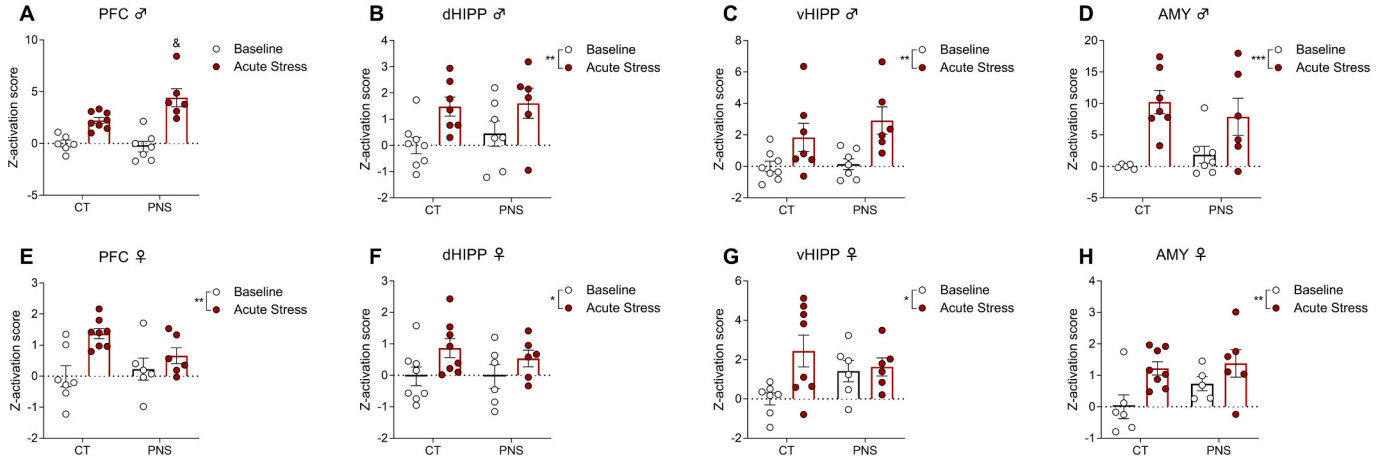

Fig. 3. Analysis of the 'activity state' in different brain regions of animals exposed to prenatal stress: modulation by an acute challenge in adolescence.
The *Z*-Activation score for control (CT) and prenatally stressed (PNS) animals exposed or not to the acute challenge was calculated based on the mRNA levels of four IEGs (Arc, Npas4, cFos, and Zif268) in the following brain areas: prefrontal cortex (PFC), dorsal Hippocampus (dHIPP), ventral hippocampus (vHIPP) and amygdala (AMY). The analyses were performed in male (A, B, C, D) and female (E, F, G, H) animals. Data are expressed as mean ± SEM of 5–8 animals per group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Panel A: $^{\&}$PNS x AS interaction ($p < 0.001$ PNS + AS vs. PNS; $p < 0.05$ PNS + AS vs. CT + AS). Two-way ANOVA with Tukey's post hoc test.

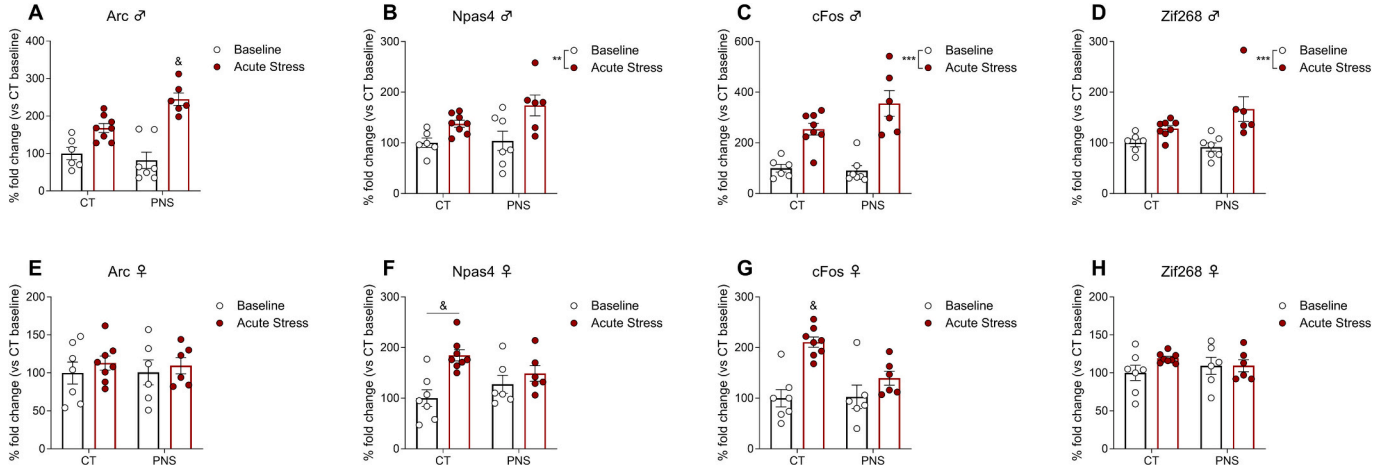

Fig. 4. Gene expression analysis of activity-dependent genes in the prefrontal cortex of animals exposed to prenatal stress: modulation by an acute challenge in adolescence.
The mRNA levels for Arc, Npas4, cFos, and Zif268 were measured in control (CT) and prenatally stressed (PNS) animals that were exposed or not to the acute challenge. The analyses were performed in male (A, B, C, D) and female (E, F, G, H) rats. Data are expressed as mean ± SEM of 6–8 animals per group. $**p < 0.01$; $***p < 0.001$. Panel A: $^{\&}$PNS x AS interaction effect ($p < 0.001$ PNS + AS vs. PNS; $p < 0.05$ PNS + AS vs. CT + AS); Panel F: $^{\&}$PNS x AS interaction ($p < 0.01$ CT + AS vs. CT); Panel G: $^{\&}$PNS x AS interaction effect ($p < 0.001$ CT + AS vs. CT; $p < 0.05$ CT + AS vs. PNS + AS). Two-way ANOVA with Tukey's post hoc test.

up-regulation of *Npas4* ($p = 0.001$) and *cFos* ($p < 0.001$) mRNA levels as compared to CT animals, whereas such increase was not found when PNS animals were exposed to the acute challenge. Conversely, we did not observe significant alterations regarding the mRNA levels of *Arc* (Fig. 4E) and *Zif268* (Fig. 4H).

The description of the changes for each individual activity-regulate gene in dHIPP, vHIPP, and AMY of males and females can be found in the supplementary material (see Fig. S1-S3, respectively).

3.4. Analysis of excitatory/inhibitory balance in the prefrontal cortex of animals exposed to prenatal stress and modulation by the acute challenge in adolescence

Considering the pattern of activation of the PFC following AS exposure as well as to the prenatal condition, we decided to deepen our analysis to identify possible differences in the responsiveness of excitatory and inhibitory neurons. With this respect, we used RNAscope to investigate the modulation of *Arc* expression within glutamatergic excitatory pyramidal neurons (Vglut2+) as compared to the sub-group of parvalbumin-positive/fast-spiking GABAergic interneurons (PV+).

When analyzing *Arc mRNA levels* in Vglut2+ cells of male rats we found a significant effect of PNS [$F(1,95) = 23.55$, $p < 0.001$; Fig. 5A] and of AS [$F(1,95) = 8.304$, $p = 0.004$; Fig. 5A]. Indeed, Arc expression in these cells was elevated in animals exposed to stress during gestation and was also elevated following exposure to the acute challenge. Conversely, when investigating its mRNA levels in PV+ neurons, we found a significant PNS x AS interaction [$F(1,95) = 7.632$, $p = 0.006$; Fig. 5B]. *Post-hoc* analysis revealed that AS increases the activation of PV neurons in CT animals ($p < 0.001$), an effect that was not observed in PNS rats. Based on these data, we also calculated the ratio of *Arc* expression between the two populations (Vglut2+/PV+) in order to establish possible changes in the activity dependent balance between excitatory and inhibitory (E/I) neurotransmission. We observed a significant PNS X AS interaction [$F(1,95) = 8.980$, $p = 0.003$; Fig. 5C] and

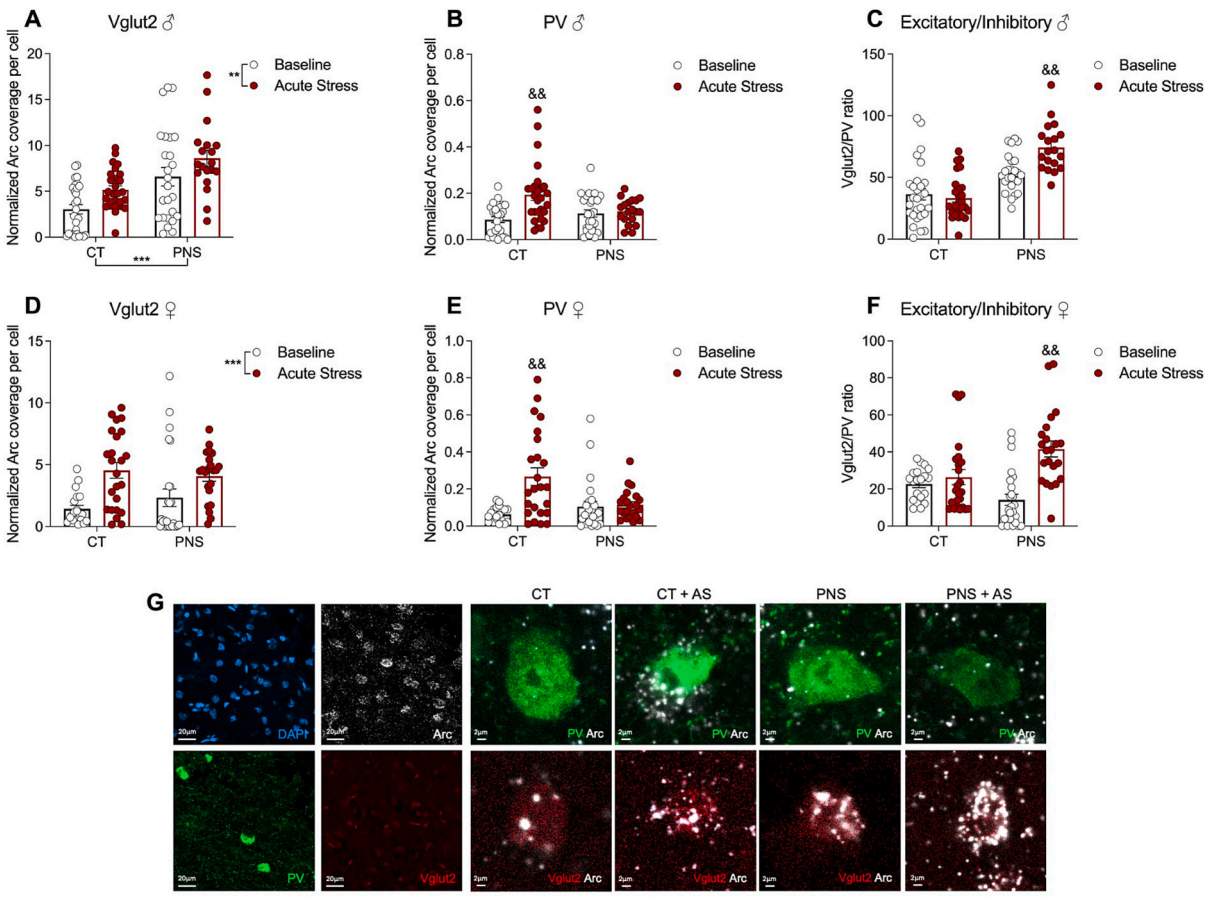

Fig. 5. Analysis of Arc mRNA expression in excitatory and inhibitory cells of the prefrontal cortex from animals exposed to prenatal stress: modulation by an acute challenge in adolescence.

Arc mRNA levels were measured by RNA scope analysis in Vglut2+ and PV+ cells of control (CT) and prenatally stressed (PNS) animals that were exposed or not to the acute challenge. Excitatory/inhibitory balance was calculated using a ratio of Vglut2+/PV+. The analyses were performed in male (A, B, C) and female (D, E, F) rats. Panel G: Representative RNAscope images for Vglut2+ (red) and PV+ (green) Arc expression (white) in the PFC. Data are expressed as mean ± SEM of 6 animals per group (20–30 cells per group). **$p < 0.01$; ***$p < 0.001$. Panels B and E: $^{\&}$PNS x AS interaction ($p < 0.001$ CT + AS vs. CT; $p < 0.05$ CT + AS vs. PNS + AS); Panels C and F: $^{\&}$PNS x AS interaction ($p < 0.01$ PNS + AS vs. PNS; $p < 0.05$ PNS + AS vs. CT + AS). Two-way ANOVA with Tukey's post hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

post-hoc analysis showed that Vglut2+/PV+ activation ratio is increased in PNS animals exposed to AS ($p = 0.004$), as compared to their sham counterpart, while CT rats exposed to AS do not show such alteration.

Regarding females, *Arc* expression in Vglut2+ cells was increased in animals exposed to AS [$F(1,87) = 18.46$, $p < 0.001$; Fig. 5D], regardless of PNS exposure. On the other hand, a significant PNS X AS interaction effect was identified when investigating *Arc* expression in PV+ cells [$F(1,87) = 9.794$, $p = 0.002$; Fig. 5E]. *Post-hoc* analysis revealed that CT rats exposed to AS had increased activation of PV+ cells ($p < 0.001$), an effect that was not found in PNS animals. Last, when examining the Vglut2+/PV+ activation ratio, we observed a significant PNS x AS interaction [$F(1,87) = 11.35$, $p = 0.001$; Fig. 5F]. Similar to the changes observed in male rats, *post-hoc* analysis showed that AS increased the Vglut2/PV activation ratio in PNS animals ($p = 0.014$), an effect that was not found in CT rats.

3.5. Analysis of glucocorticoid-related markers of animals exposed to prenatal stress and modulation by the acute challenge in adolescence

Considering the close relationship between stress exposure and the glucocorticoid system, we investigated possible changes in glucocorticoid signaling within the prefrontal cortex of adolescent rats previously exposed to PNS and challenged with an acute stress. To this purpose, we analyzed the mRNA levels of the Glucocorticoid receptor (*Nr3c1),* its cochaperone FKBP prolyl isomerase 5 *(Fkbp5)*, as well as two genes that are strongly regulated following activation of glucocorticoid receptors, namely Serum/Glucocorticoid Regulated Kinase 1 (*Sgk1*), and Forkhead box O1 (*Foxo1*).

With respect to the analysis of male rats, we observed a significant PNS effect for *Nr3c1* [$F(1,21) = 4.868$, $p = 0.038$; Fig. 6A] and *Fkbp5* [$F(1,21) = 14.91$, $p < 0.001$; Fig. 6B], with an increased expression in the rats exposed to gestational stress independently of the acute challenge. Regarding *Sgk1* mRNA levels, we found a significant AS effect [$F(1,21) = 58.01$, $p < 0.001$; Fig. 6C], with an up-regulation following the acute stress regardless of PNS exposure. Last, a significant PNS effect was observed for *Foxo1* [$F(1,21) = 8.497$, $p = 0.008$; Fig. 6D], whose expression was significantly reduced independently of the acute challenge.

When considering the females, we found a significant PNS x AS interaction for *Nr3c1* mRNA levels [$F(1,21) = 7.366$, $p = 0.013$; Fig. 6E]. *Post-hoc* analysis showed a reduction of its expression in CT animals exposed to AS ($p = 0.003$), as compared to their sham counterpart, an effect that was not observed in PNS animals. We also observed a significant AS effect for *Sgk1* mRNA levels [$F(1,22) = 17.53$, $p < 0.001$; Fig. 6G], since the animals exposed to the acute challenge showed increased gene expression regardless of PNS exposure. Finally, no significant changes were observed for *Fkbp5* (Fig. 6F) and *Foxo1* (Fig. 6H).

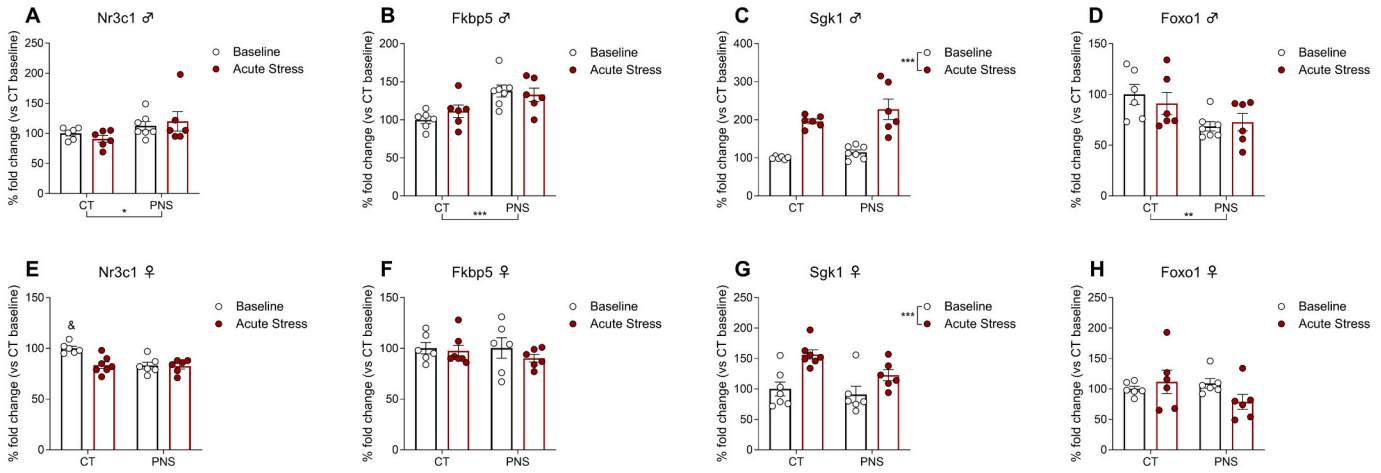

Fig. 6. Analysis of glucocorticoid-related markers in the prefrontal cortex of animals exposed to prenatal stress: modulation by an acute challenge in adolescence. The mRNA levels for Nr3c1, Fkbp5, Sgk1, and Foxo1 were measured in control (CT) and prenatally stressed (PNS) animals that were exposed or not to the acute challenge. The analyses were performed in male (A, B, C, D) and female (E, F, G, H) rats. Data are expressed as mean ± SEM of 6–8 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001. Panel E: &PNS x AS interaction (p < 0.01 CT vs. CT + AS). Two-way ANOVA with Tukey's post hoc test.

3.6. Analysis of antioxidant capacity of animals exposed to prenatal stress and modulation by the acute challenge in adolescence

It is known that redox mechanisms play a significant role in stress-related disorders, and that an antioxidant imbalance may facilitate the negative effects induced under challenging conditions. On these bases, we have decided to investigate the expression of Nuclear factor erythroid 2-related factor 2 (*Nrf2)*, a key player in the antioxidant defense, and two of its downstream genes, named Glutamate–cysteine ligase catalytic subunit 1 (*Gclc1)*, and Kelch-like ECH-associated protein 1 (*Keap1)* in the PFC of animals exposed to prenatal stress, under resting conditions or in response to the acute challenge.

Regarding the analysis of male animals, we observed a significant AS effect for *Nrf2* [F (1,21) = 5.484, $p = 0.029$; Fig. 7A], with increased mRNA levels after the acute challenge, regardless of PNS exposure. Conversely, no significant changes of *Gclc1* (Fig. 7B) and *Keap1* (Fig. 7C) gene expression were found either as a consequence of PNS or AS exposure.

When investigating the females, we found a significant PNS x AS interaction for *Nrf2* [F (1,22) = 6.087, $p = 0.021$; Fig. 7D] as well as for *Gclc1* gene expression [F (1,22) = 5.350, $p = 0.030$; Fig. 7E]. *Post-hoc* analysis revealed a significant reduction of *Nrf2* and *Gclc1* mRNA levels in PNS animals exposed to the acute challenge, as compared to the baseline PNS group ($p = 0.002$ and $p = 0.020$, respectively), and such effects were not observed in CT rats exposed to AS. Last, we observed a significant effect of AS for *Keap1* gene expression [F (1,22) = 13.89, $p = 0.001$; Fig. 7F], with a significantly decreased expression following the acute challenge, regardless of PNS exposure.

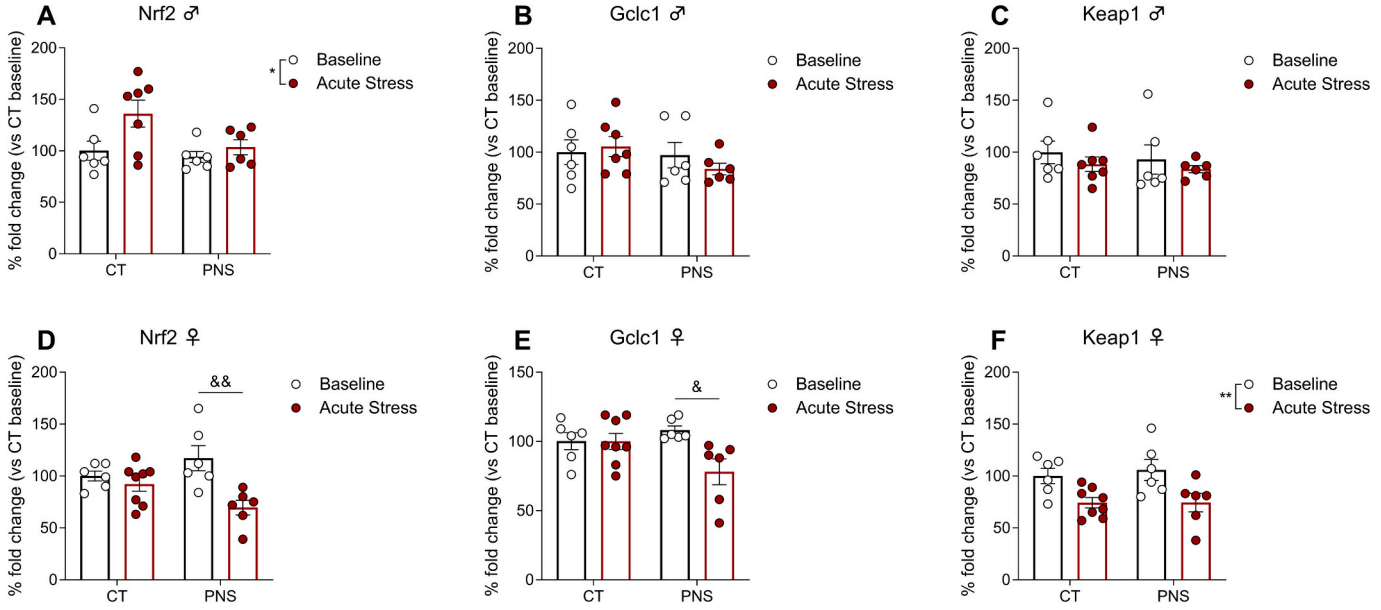

Fig. 7. Analysis of markers for the antioxidant capacity in the prefrontal cortex of animals exposed to prenatal stress: modulation by an acute challenge in adolescence.
The mRNA levels for Nrf2, Gclc1, and Keap1 were measured in control (CT) and prenatally stressed (PNS) animals that were exposed or not to the acute challenge. The analyses were performed in male (A, B, C) and female (D, E, F). Data are expressed as mean ± SEM of 6–8 animals per group. *p < 0.05; **p < 0.01; Panel D: &PNS x AS interaction (p < 0.01 PNS + AS vs. PNS); Panel E: &PNS x AS interaction effect (p < 0.05 PNS + AS vs. PNS) Two-way ANOVA with Tukey's post hoc test.

4. Discussion

In the present study, we demonstrate that the emotional dysregulation of adolescent animals previously exposed to prenatal stress is associated with an altered ability to cope under challenging conditions. Indeed, while adolescent rats exhibit a widespread activation of different brain regions in response to an acute swim stress, some differences between CT and PNS rats can be observed primarily in the modulation of the PFC. We found a larger z-activation score in the PFC of PNS males following exposure to the acute challenge, an effect mainly due to increased *Arc* mRNA levels in those animals. Subsequent analysis of *Arc* expression within glutamatergic and GABAergic cells in the PFC revealed a significant imbalance of E/I activation following AS exposure in males and females previously exposed to PNS. Additionally, alterations in stress-related and antioxidant markers were identified in the PFC of both sexes following exposure to PNS and AS. These findings suggest that PNS exposure may prime the brain for an altered responsiveness to a secondary challenge, with major effects in the modulation of the PFC.

At the behavioral level, we confirmed that only a subset of PNS animals show impaired sociability and anhedonic-like behavior. This set of data goes in line with previous studies that investigated the effects of PNS on mood-related behaviors (Weinstock, 2017). Indeed, while using a different statistical approach in examining the behavioral outcomes of PNS, a recent study conducted in our laboratory with a different rat strain (Wistar), showed that only a subset of PNS animals show impaired sociability and increased anxiety-like behavior during adolescence (Creutzberg et al., 2023a). This highlights that the neurobiological and behavioral consequences of PNS are heterogenous, and not all subjects will exhibit uniform responses to gestational stressors.

The primary objective of this study was to investigate the responsiveness of PNS animals to an acute challenge, namely a forced swim stress, during adolescence that represents a highly relevant time frame for the onset of mental disorders. Even though PNS animals per se did not show significant changes in corticosterone levels under resting conditions, as expected, exposure to AS resulted in increased corticosterone levels for both males and females. While these data may suggest that PNS exposure did not alter HPA axis responsiveness, our assessment was limited to a single time point at sacrifice, which may not fully capture possible HPA-related changes, as previously demonstrated under stress conditions (Creutzberg et al., 2021).

At the molecular level, all the brain regions investigated show a significant activation following the acute challenge, both in males and females. While previous research has linked these regions to various structural and functional alterations induced by PNS exposure (Nolvi et al., 2023; Humphreys et al., 2020; Lemaire et al., 2000), our findings indicate that exposure to gestational stress did not produce major differences in the ability of dHIPP, vHIPP, and AMY in responding to the AS. Additionally, although there were variations in the expression of IEGs within those regions, it was consistently observed that *cFos* expression was upregulated in response to AS. This aligns with its well-stablished role as a highly responsive activity-dependent gene, frequently utilized as a marker of cellular activity following a wide range of stimuli (Yap and Greenberg, 2018).

When focusing on the PFC, we observed that PNS exposure primed this brain region of males for an altered activation following AS during adolescence. Given the well-stablished role of the PFC in exerting top-down control, alterations in its activity could potentially influence the functionality of several brain regions (Malik et al., 2022; Paneri and Gregoriou, 2017). Interestingly, activation of the PFC in females exposed to AS is impaired in animals that experienced PNS, compared to those that were not exposed to stress during pregnancy. Such difference is primarily driven by an inability to up-regulate *Npas4* and *cFos* mRNA levels in females, which highlights significant sex-differences in brain responsiveness following PNS exposure (Luoni et al., 2016; Marchisella et al., 2021). These findings are consistent with previous studies suggesting that females exhibit heightened susceptibility to stress early in development, which may increase their risk for developing mood disorders later in life (Goodwill et al., 2019; Mengelkoch and Slavich, 2024). The blunted response to the acute challenge could be indicative of disrupted brain function. A summary of all the sex-specific differences in the PFC identified in our study can be found in the supplementary table 3.

The functionality of the PFC is significantly shaped by the interplay between glutamatergic and GABAergic neurons. Thus, maintaining a balance between E/I neurotransmission is necessary for proper functioning and responsiveness of this brain region. Accordingly, while the response to environmental challenges is dependent on an integrated regulation of E/I systems, an imbalance has been associated with stress-induced disorders (Marchisella et al., 2021; Sohal and Rubenstein, 2019). With that in mind, we sought to explore whether the alterations in PFC activity were linked to specific neuronal populations, namely glutamatergic and GABAergic cells. Recognizing that *Arc* is a reliable marker of brain activity, which plays a key role in regulating the expression of over a thousand genes involved in neuronal plasticity and intrinsic excitability processes, we have selected this gene for the cell-type specific transcriptional analysis (Leung et al., 2022). Our findings revealed that PNS increased *Arc* mRNA levels in Vglut2+ neurons in males, while no changes were observed in PV+ cells. Interestingly, upon exposure to the acute challenge a similar up-regulation of *Arc* mRNA levels in Vglut2+ neurons was observed in both males and females, whereas an increase of *Arc* expression in PV+ cells was only found in CT animals exposed to AS. When evaluating the Vglut2+/PV+ activation ratio, a significant E/I imbalance in PNS males and females exposed to AS was evident. In accordance with our data, an E/I imbalance within the PFC is known to be a consequence of stress exposure and may represent a trait marker of several mental disorders (Sohal and Rubenstein, 2019; Liu et al., 2021; Lee et al., 2017; Bittar and Labonté, 2021). Nevertheless, it is important to acknowledge that alterations on E/I balance may vary based on phenotypic traits, which could be induced by distinct stressors, as well as sex and species (Liu et al., 2021).

The ability to respond under challenging conditions depends on the integration of multiple signals involving different systems (von Ziegler et al., 2022). Hence, to corroborate our finding, we also investigated potential differences in the modulation of glucocorticoid-related and antioxidant markers. While male animals show some changes in glucocorticoid-dependent genes due to the prenatal condition, the expression of *Sgk1* that is highly sensitive to glucocorticoid receptor activation was up-regulated following AS independently from PNS exposure, suggesting that the adverse experience did not produce overt changes in the ability to activate glucocorticoid signaling. Regarding *Foxo1* gene expression, we found a downregulation in males exposed to PNS, an effect that has been previously shown in the PFC of animals exposed to chronic unpredictable stress (Liu et al., 2020).

Interestingly, the analysis of the antioxidant response in the PFC highlighted significant sex-dependent changes due to the prenatal condition. Indeed, PNS females exposed to AS showed reduced expression of *Nrf2* and *Gclc1,* indicating an impaired antioxidant capacity following the acute challenge, an effect that was not observed in CT animals. This could suggest a heightened susceptibility to oxidative stress, a factor that may contribute to brain dysfunction under stressful conditions (Sandberg et al., 2014; Baxter and Hardingham, 2016).

There are limitations that must be considered when interpreting our data. Firstly, we investigated the domains of negative valence and social processes, but our analysis did not address the full spectrum of behavioral impairments that could originate from PNS exposure. Secondly, considering that our goal was to measure a rapid brain responsiveness following AS exposure, we decided to focus on mRNA analysis, since transcriptional mechanisms represent a point of convergence of different pathways that may orchestrate a response to the acute challenge (von Ziegler et al., 2022). However, translational mechanisms as well as post-translational modifications (such as protein phosphorylation) can also

be relevant in stress response. Lastly, due to the limitation in the use of animals, we had to restrict our analyses to one post-stress time point that may not be ideal for all the systems under investigation.

In conclusion, our study strengthens the existing evidence that PNS induces mood-related impairments in male and female animals at adolescence. Furthermore, gestational stress alters the brain responsiveness to a secondary stressor. This suggests that the consequences of the adverse experience during gestation are not limited to behavioral disruptions but could lead to a deficit in coping strategies during critical time windows, which may be highly relevant for the susceptibility to develop mental disorders.

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Ethical statement

All procedures included in this study comply with the ARRIVE guidelines and were approved by the Italian Health Ministry under protocol n. 752/2020, in accordance with the Italian legislation on animal experimentation (Decreto Legislativo 116/92) in conformity with the rules and principles of the EU Directive 2010/63/EU.

CRediT authorship contribution statement

Rodrigo Orso: Writing – original draft, Investigation, Formal analysis. **Kerstin Camile Creutzberg:** Writing – review & editing, Investigation, Formal analysis. **Veronica Begni:** Writing – review & editing, Investigation. **Giulia Petrillo:** Investigation. **Annamaria Cattaneo:** Writing – review & editing. **Marco Andrea Riva:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

M.A.R. has received compensation as speaker/consultant from Angelini, Lundbeck, Otzuka, Sumitomo Pharma, and Sunovion, and he has received research grants from Sumitomo Pharma. A.C. has received compensation as a speaker from Sumitomo Pharma. All the other authors declare no financial interests or potential conflicts of interest.

Data availability

Data will be made available upon request to the corresponding author M.A.R (m.riva@unimi.it).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2024.111162.

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