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# Inflammatory status along the brain-liver axis in animals vulnerable to prenatal stress: sex-related implications for stress-induced comorbidities

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The prevalence of mood disorders is constantly increasing, with exposure to stress early in life (ELS) as one of the major risk factors. Recent studies reported that ELS can increase the risk for mental disorders, but also for several cardiometabolic conditions, often in comorbidity. However, biological processes underlying these negative outcomes with a sex dependent effect are still poorly understood. Here, we used the preclinical model of prenatal stress (PNS) mimicking early in life adversities to investigate the presence of an abnormal inflammatory response as a possible mechanism leading to the onset of a vulnerable phenotype for mental and metabolic disorders in the offspring. We showed that adolescent male rats, classified as vulnerable to PNS by a two-step cluster analysis, based on three different behavioral tests, have brain microglia hyperactivation in the dorsal hippocampus. We then focused on liver, as a key organ involved in the development of several metabolic disorders and strictly communicating with the brain via immune-inflammatory pathways. We found that rats showing a vulnerable behavioral phenotype also showed abnormal inflammatory response in the liver. Moreover, liver inflammation is correlated with an increased expression of leptin receptor, an important adipokine involved in several metabolic processes. Overall, this study suggests that male but not female rats exposed to PNS and showing a vulnerable phenotype are characterized by brain and liver pro-inflammatory status, pointing out the need to target the inflammatory system via pharmacological or non-pharmacological strategies to reduce the risk for both mental and physical disorders in individuals exposed to ELS.

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## INTRODUCTION

The prevalence of mood disorders is worldwide increasing [1], particularly during adolescence, which is a critical vulnerable time window for the onset of several psychiatric disorders [2]. Although different treatment options are available, about 30–50% of patients do not respond [3], and some of them develop treatment resistant depression, overall indicating the urgent need to identify novel targets for interventions.

It is well known that exposure to stress early in life (ELS) is one of the major risk factors for the development of negative outcomes later in life [4, 5] such as depression [6], psychosis [7], and anxiety disorders [8]. Moreover, ELS may also increase the risk to develop several cardiometabolic conditions [9], such as insulin resistance [10], obesity [11] and type 2 diabetes [12]. However, it is well known that not all the individuals who have been exposed to ELS develop negative outcomes and this could be due to an interaction of several factors, such as the genetic background, the environment, and the biological sex [13]. Therefore, while some individuals exposed to adverse experiences become more vulnerable and develop pathological conditions, others develop coping strategies becoming more resilient to the consequences to stress [14].

Among the biological mechanisms involved in the effect of ELS on mood and metabolism, inflammation has been recognized as a key biological system. Indeed, several preclinical and clinical studies showed that ELS experiences are associated with the presence of high levels of inflammatory markers [15–18] and that patients with mental disorders, such as depression [19, 20], generalized anxiety disorder [21], post-traumatic stress disorder [22] or metabolism dysfunction [23–25] are characterized by altered immune system functioning and by the presence of higher levels of several inflammatory markers.

Within the context of stress exposure, inflammation, mental and metabolic disorders comorbidities, the brain-liver axis has been identified as a dynamic and crucial interplay between the brain and liver which is involved both in the physiological and pathological processes modulated by ELS [26].

To support the existence of the brain-liver axis communication and its impact also on mood, several evidences demonstrated that hepatic diseases, such as nonalcoholic fatty liver disease (NAFLD) and hepatic encephalopathy, are characterized by the presence of a pro-inflammatory status in the liver and that those inflammatory mediators released by the liver can enter into the brain and trigger

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brain inflammatory responses [27, 28], increasing therefore the well known associated risk for mental disorders [28–30]. The increased inflammatory response due to ELS exposure alters also important processes in the liver, including glucose and lipid metabolism [30, 31], increasing the risk for several metabolic disorders.

Among several signaling molecules involved in the brain–liver interplay, leptin has emerged as an important player. Leptin is an adipokine which was initially investigated for its central role in regulating food intake and body weight [32], although more recent evidence has also demonstrated that leptin upregulates several inflammatory molecules such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6), which are associated with the development of insulin resistance and type 2 diabetes [33].

Nevertheless, the specific causal mechanisms leading to mental and metabolic comorbidity and to the development of a vulnerable or resilient phenotype in association with ELS, are still not well understood, limiting the development of preventive and therapeutic strategies. Moreover, previous studies suggest that male rodents may exhibit heightened sensitivity to stress due to differences in HPA axis regulation and neuroimmune interactions [34], thus potentially amplifying the link between stress exposure and inflammatory responses. Consequently, the association between stress, brain and hepatic inflammation, in the context of behavioral and metabolic outcomes still need more investigation.

Therefore, in this study we aimed to evaluate how a vulnerable behavioral phenotype emerging as consequences to exposure to ELS could be characterized by the presence of a pro-inflammatory status both in the brain and in the liver, contributing to explain the role of ELS in predisposing the development of mental and metabolic disorders and of their comorbidity.

## MATERIALS AND METHODS

### Experimental design and animal housing

Adult nulliparous male and female Wistar rats were purchased from the Center for Experimental Biological Models (CeMBE) at the Pontifical Catholic University of Rio Grande do Sul, Brazil, and were left undisturbed in the animal facility for 10 days before the beginning of the experiment. Animals were kept in an environment with controlled temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity ( $55 \pm 5\%$ ) under a 12 h/12 h light/dark cycle (lights on at 6 am) with food and water *ad libitum* during the whole experiment. After acclimatization, animals were mated (1 male and 2 females) for 48 h. At gestational day (GD) 14 dams were single-housed and randomly allocated to the control or to the prenatal stress (PNS) group. Dams from the PNS group were exposed to a restraint stress protocol during the last week of pregnancy (GD14 to delivery) as previously published [35], while control dams were left undisturbed. PNS dams were placed into transparent Plexiglas cylinders (20 cm length  $\times$  9 cm diameter  $\times$  9 cm height) 3 times a day for 45 min (starting at 9 am, 12 pm, and 5 pm  $\pm$  2 h) under bright light (1500 lux). At postnatal day (PND) 0, the day of birth, litters were culled to 8 pups (4 males and 4 females). Pups were then left undisturbed to prevent unnecessary manipulations until PND21 when they were weaned and housed in groups of 2/3 per cage. All procedures included in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institute of Health (NIH) and were approved by the Ethics Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul, under the Ethical Approval Code #8922.

### Behavioral assessment

Behavioral assessment, which included social interaction (SI), sucrose preference (SP) and novelty suppressed feeding (NSF) tests, was performed on adolescent offspring (PND35–39) during the day light phase as described in detail in our recent paper (see Creutzberg and Begni et al. [36]). All the tests were video-recorded, and each video was then analyzed by two independent researchers that were blind to the animal's condition. The final behavioral score was calculated as the mean of the score calculated by the two evaluators. See details on behavioral tests in the Supplementary Material.

### RNA isolation and gene expression analyses

All animals were sacrificed by decapitation at PND42. The brain and the liver were quickly collected. The brain was free-hand-dissected to obtain ventral (VH) and dorsal (DH) hippocampus. Liver was dissected too. Tissues were snap-frozen in dry ice and stored at  $-80^\circ\text{C}$  until molecular analyses. Total RNA was extracted from VH, DH and liver from all the animals using the RNeasy Mini Kit (Qiagen, Hilden, Germany) (VH and DH) or AllPrep DNA/RNA/miRNA universal kit (Qiagen) (liver) according to the manufacturer's protocol. RNA concentration was measured at the NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) or Nanodrop Technologies, Wilmington, DE, USA) and further diluted for quantitative real-time polymerase chain reaction (qRT-PCR) on the CFX384 Real-Time system (Bio-Rad Laboratories, Hercules, CA, USA). All the samples were run in triplicates and  $\beta$ -Actin and GAPDH were used as housekeeping genes. Primers and probes were purchased from Thermo Fisher Scientific or Eurofins Genomics (Luxembourg City, Luxembourg), and their ID's or sequences are shown in Table S1. For gene expression data, Pfaffl method was used to determine the relative expression ratio of each gene of interest in PNS animals as compared to controls [37].

### Measurement of inflammatory markers in liver by Luminex assay

Liver tissues were homogenized by using a lysis buffer containing 100 mmol/L Tris, 1% Triton X-100, 150 mmol/L NaCl, 35 mg/mL PMSF, 10 mmol/L  $\text{Na}_2\text{VO}_4$ , 10 mmol/L  $\text{Na}_4\text{P}_2\text{O}_7$  and 4 mmol/L EDTA (Tissue Extraction Reagent I, ThermoFisher) to extract total proteins. The ProcartaPlex Rat Th Complete Panel, 14plex (Invitrogen, Carlsbad, CA, USA, EPX140-30120-901) was used to detect and quantify 14 different analytes (cytokines, chemokines and growth factors) in the liver using the Bio-Plex Multiplex Immunoassay System (Bio-Rad). The panel included pro-inflammatory cytokines (interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-5, IL-6, IL-12P70, IL-13, IL-17 $\alpha$ , interferon  $\gamma$  [IFN- $\gamma$ ], TNF- $\alpha$ , granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte colony-stimulating factor [G-CSF]) and anti-inflammatory cytokines (IL-4 and IL-10). The ProcartaPlex 96-well plate and the liver samples were prepared according to the manufacturer's instructions. When the signal was below the limit of detection and displayed as "OOR <=" (out of range below) it was considered non-available (N/A) and excluded from the dataset.

### Behavioral cluster analyses

To identify animals vulnerable and resilient to stress based on behavioral assessment, a two-step cluster analysis was performed. It considered the latency in eating food in the NSF, the social preference in the SI, and the sucrose preference in the SP test. The cluster analysis was performed as described by Creutzberg and Begni et al. [36] using the Schwarz information criterion (BIC) and the log-likelihood method as a distance measure [38] without predefining the number of clusters. A detailed description of behavioral outcomes is provided in our recently published paper [36].

### Statistical analysis

Data from qRT-PCR and Luminex assay were analyzed using IBM SPSS Statistics v.27 and GraphPad Prism 9. Student's t-test was used to analyze the differences between the control and PNS groups with stress as a variable. To examine possible differences between vulnerable, resilient and control animals, one-way ANOVA was performed with stress as a factor applying Bonferroni correction. Data are presented as group mean  $\pm$  standard error of the mean (SEM). The graphs represent individuals as dots, and  $p$ -value  $< 0.05$  was considered statistically significant. A z-score was calculated considering gene expression of pro-inflammatory and anti-inflammatory cytokines to provide an integrated inflammatory status. The individual z-score was determined applying the following formula:  $z = (x - \mu) / \sigma$ , where  $x$  is the relative expression ratio of each gene,  $\mu$  is the mean of control group, and  $\sigma$  is the population standard deviation of the control group. The z-scores were then determined by averaging the individual z-scores of all genes of interest and we considered the relative expression ratio for pro-inflammatory genes (IL-1 $\beta$ , MIF, CD68 in liver and C3, C4b, CX3CR1, iNOS, CD68 in brain) and the reciprocal for anti-inflammatory genes (IL-4 and IL-10 in liver). The z-score was only calculated if the qRT-PCR results of at least three genes were available for the same animal. Correlation analyses between hepatic inflammation and leptin receptor expression were calculated by using Pearson or Spearman correlation analyses.

## RESULTS

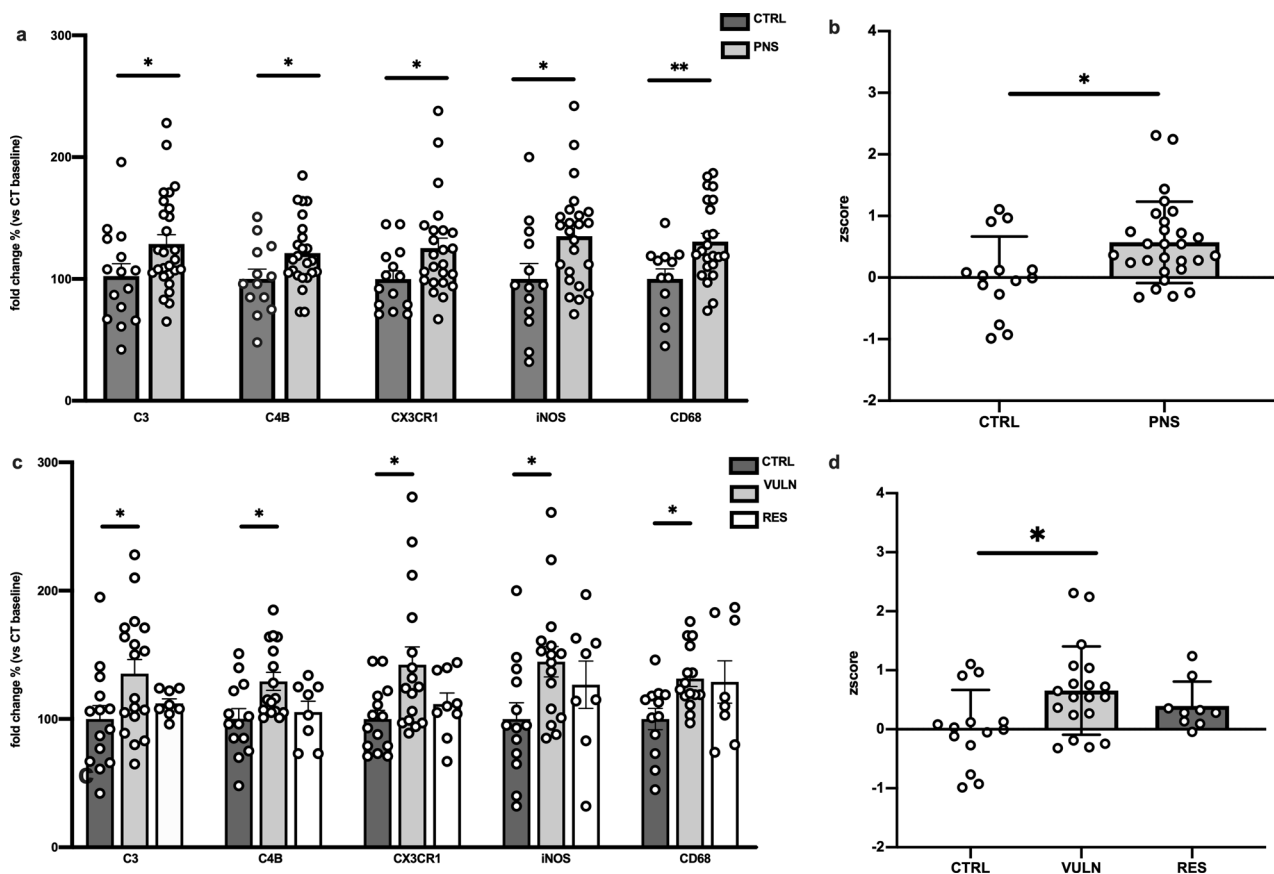
### Behavioral clustering identifies resilient and vulnerable rats exposed to PNS

As reported in our recent paper [36], in the comparison between control and PNS-exposed animals, we observed that stress exposure induced impairments in sociability, a reduction in sucrose preference, and increased latency to eat food in NSF both in male and female rats. In the same paper, a two-step cluster analysis identified two primary clusters (CL1 and CL2) with a strong separation between them (silhouette measure of cohesion and separation  $> 0,5$ ). Within the PNS group, 30% of animals were classified as belonging to CL1, while the remaining 70% were assigned to CL2. In the CTRL group, the majority of animals fell into CL1 (72%), while the remaining 28% were categorized as part of CL2. The highest predictor importance was attributed to the latency to eat in the NSF test, followed by the preference for social interaction measured in the social interaction test. Sucrose preference had minimal predictive relevance for cluster separation. Animals assigned to CL1 exhibited lower anxiety-like behaviors in the NSF test and a higher social interaction ratio compared to those in CL2 and were classified as resilient to PNS exposure, while those in CL2 were categorized as vulnerable. Indeed, a subsequent comparison between controls, vulnerable and resilient rats confirmed that only vulnerable rats displayed significant dysfunctions in anxiety-related and social behaviors. All these data were clearly described and reported in a work by Creutzberg and Begni et al. [36]. This division was used to perform the following molecular analyses.

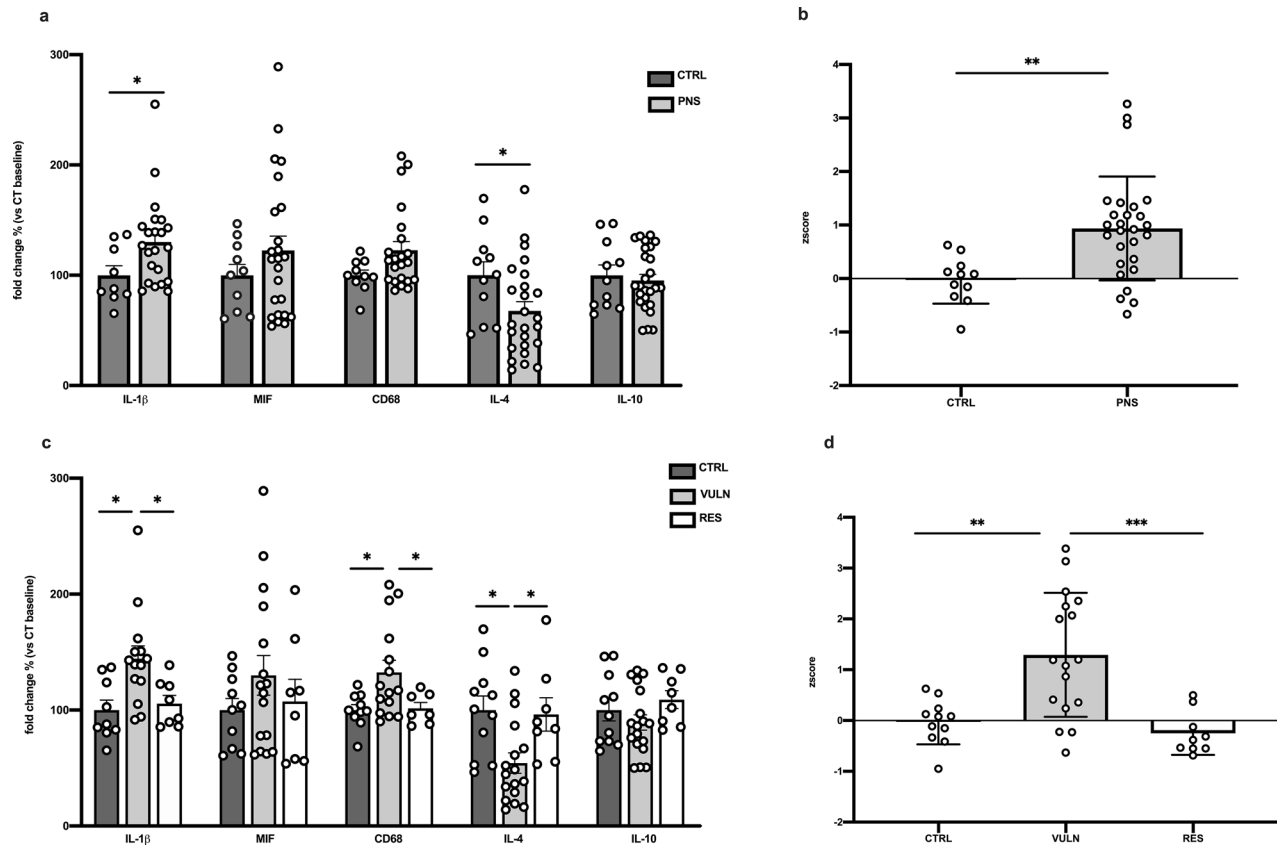
### PNS exposure leads to an activation of microglia cells in the dorsal hippocampus of adolescent male animals with a 'vulnerable' phenotype

Our first aim was to evaluate whether PNS exposure could lead to microglia activation towards a pro-inflammatory phenotype. Therefore, we evaluated the expression levels of a panel of genes associated with microglia activation, namely Cluster of Differentiation 68 (CD68), the CX3C motif chemokine receptor 1 (CX3CR1), the Complement component 4b (C4b), the Complement component 3 (C3) and the inducible nitric oxide synthase (iNOS), both in the dorsal and in the ventral hippocampus of adolescent male rats exposed or not to PNS. In the dorsal hippocampus, we found a significant upregulation of C3, C4b, CX3CR1, iNOS and CD68 in PNS animals as compared to controls (C3: +28,3%,  $p = 0,0398$ ; C4b: +21,3%,  $p = 0,0398$ ; CX3CR1: +25,4%,  $p = 0,0377$ ; iNOS: +35,2%  $p = 0,0227$ ; CD68: +30,7%,  $p = 0,0095$ ) (Fig. 1a), indicating a stress-induced activation of microglia cells in this brain region, which was confirmed also by calculating the microglia activation composite z-score ( $p = 0,0137$ ) (Fig. 1b).

Since exposure to PNS produces a behavioral phenotype only in a percentage of the exposed animals (Creutzberg et al., 2024), we next assessed if the changes in microglial function were associated with such vulnerability. Interestingly we found that C3, C4b, CX3CR1, iNOS and CD68 were significantly upregulated in vulnerable and not resilient PNS animals, with PNS resilient animals showing indeed a pattern of modulation similar to controls (C3: +35,4%,  $p = 0,0489$ ; C4b: +29,3%,  $p = 0,0290$ ; CX3CR1: +42,2%,  $p = 0,0416$ ; iNOS: +44,5%,  $p = 0,0453$ ; CD68:



**Fig. 1** Gene expression analysis of microglia activation related markers in the dorsal hippocampus of male animals exposed to prenatal stress. The mRNA levels for C3, C4b, CX3CR1, iNOS and CD68 were measured in control (CTRL) and prenatally stressed group (PNS) (a) or after the separation in vulnerable (VULN) and resilient (RES) (c). The z-score was calculated based on the mRNA levels of C3, C4b, CX3CR1, iNOS and CD68 genes considering whole PNS group (b) or after the separation in vulnerable (VULN) and resilient (RES) (d). Statistical analysis for panels a, b: t-test, \* $p < 0,05$  and \*\* $p < 0,01$  ( $n = 14$  to  $30$  per group). Statistical analysis for panels c, d: one-way ANOVA, Tukey's post hoc, \* $p < 0,05$  ( $n = 9$  to  $19$  per group). Data are expressed as mean  $\pm$  SEM and individuals are represented as dots.



**Fig. 2** Gene expression analysis of inflammatory markers in the liver of male animals exposed to prenatal stress. The mRNA levels for IL-1 $\beta$ , MIF, CD68, IL-4 and IL-10 were measured in control (CTRL) and prenatally stressed group (PNS) (a) or after the separation in vulnerable (VULN) and resilient (RES) (c). The z-score was calculated based on the mRNA levels of IL-1 $\beta$ , MIF, CD68, IL-4 and IL-10 genes considering whole PNS group (b) or after the separation in vulnerable (VULN) and resilient (RES) (d). Statistical analysis for panels a, b: t-test, \* $p < 0,05$  and \*\* $p < 0,01$  ( $n = 14$  to 30 per group). Statistical analysis for panels c, d: one-way ANOVA, Tukey's post hoc, \* $p < 0,05$ , \*\* $p < 0,01$  and \*\*\* $p < 0,001$  ( $n = 9$  to 19 per group). Data are expressed as mean  $\pm$  SEM and individuals are represented as dots.

+31,5%,  $p = 0,0454$  in vulnerable animals compared to controls and C3: +12,1%,  $p = 0,4936$ ; C4b: +5,3%,  $p = 0,9999$ ; CX3CR1: +11,7%,  $p = 0,9230$ ; iNOS: +26,8%,  $p = 0,4380$ ; CD68: +28,9%,  $p = 0,1075$  in resilient animals compared to controls) (Fig. 1c). This effect was confirmed when we calculated the microglia activation composite z-score (Fig. 1d) whose value was significantly higher only in vulnerable animals compared to controls ( $p = 0,0237$ ). Similar analyses were performed in the ventral hippocampus, although no effect of PNS was observed, also when considering PNS resilient and vulnerable animals (see details in the Supplementary Material and shown in figure S1).

#### PNS exposure does not activate microglia cells in the hippocampus of female animals

To evaluate possible sex differences in microglia activation, we have investigated the expression of the same genes analyzed in males, also in female animals. As shown in supplementary figure S2a, we did not find significant changes in the expression of microglia activation related genes after PNS exposure in dorsal hippocampus, although a trend was observed (all  $p > 0,05$ ) when considering the whole PNS-exposed group as well as the subdivision into vulnerable or resilient animals, no significant changes were reported in vulnerable animals compared to controls (all  $p > 0,05$ ) and in resilient animals compared to controls (all  $p > 0,05$ ) (Figures S2c). Similar analyses were performed also in the ventral hippocampus, and similarly, no effect of PNS was observed, also when considering animals divided into resilient and vulnerable (see details in the Supplementary Material and in figure S3).

#### Liver inflammatory status is altered in adolescent male animals vulnerable to PNS exposure

To investigate the effect of PNS exposure on liver inflammation we evaluated the expression levels of different inflammatory mediators, including the interleukin-1 beta (IL-1 $\beta$ ), the macrophage inhibitory factor (MIF), the interleukin-4 (IL-4), the interleukin-10 (IL-10) and CD68. We found a significant upregulation of IL-1 $\beta$  in the liver of PNS male animals as compared to controls, and a significant downregulation of IL-4, but no significant changes in MIF, CD68 and IL-10 expression (IL-1 $\beta$ : +30,4%,  $p = 0,0452$ ; IL-4: -32,3%,  $p = 0,0415$ ; MIF: +17,3%,  $p = 0,5146$ ; CD68: +22,7%,  $p = 0,1634$ ; IL-10: -9,0%,  $p = 0,6547$ ) (Fig. 2a). The composite inflammatory z-score, that was calculated with aforementioned genes, showed an increased pro-inflammatory status in the liver of animals exposed to PNS, as compared to controls ( $p = 0,0034$ ) (Fig. 2b). Interestingly, we found that the pro-inflammatory status of the liver was also related to the vulnerability to PNS exposure.

Indeed, we found a significant upregulation of IL-1 $\beta$  and CD68 in the liver of vulnerable animals, when compared to controls (IL-1 $\beta$ : +44,3%,  $p = 0,0125$ ; CD68: +32,5%,  $p = 0,0343$ ) and to resilient animals (IL-1 $\beta$ : +38,7%,  $p = 0,0273$ ; CD68: +30,9%,  $p = 0,0343$ ) (Fig. 2c). We also found a significant downregulation of IL-4 only in the liver of vulnerable animals as compared to controls (IL-4: -45,7%,  $p = 0,0112$ ) and resilient animals (IL-4: -41,9%,  $p = 0,0403$ ) (Fig. 2c). Composite inflammatory z-score confirmed an increased pro-inflammatory status in PNS vulnerable animals compared to controls ( $p = 0,0011$ ) and resilient animals ( $p = 0,0006$ ) (Fig. 2d).

Interestingly, we found a positive although not significant association between the composite inflammatory z-score in the



liver and inflammatory z-score in the dorsal hippocampus of male animals exposed to PNS ( $r = 0,37$ ;  $p = 0,078$ ) (data not shown).

To evaluate possible sex differences, the same panel of genes analyzed in males was measured in female animals. However, similarly to the hippocampus, no significant changes were found in inflammatory markers expression as a consequence of PNS exposure (all  $p > 0,05$ ) (Fig. S4a). Moreover, no significant changes were found when dividing PNS exposed animals into vulnerable or resilient ones (See details in the Supplementary Material and in figure S4b).

Considering that no effect was observed in the liver of female animals, we decided to perform subsequent protein analyses only in males and not in females.

In particular, protein levels of 14 inflammatory mediators (cytokines and chemokines) were measured in liver samples of male rats by using Luminex assay. We observed a significant upregulation of TNF- $\alpha$ , IL-17 $\alpha$ , GM-CSF, IL-1 $\alpha$ , IL-5, IL-13, IFN- $\gamma$ , and IL-2 in PNS male animals as compared to controls (TNF- $\alpha$ :  $p = 0,0001$ ; IL-17 $\alpha$ :  $p = 0,0011$ ; GM-CSF:  $p = 0,0050$ ; IL-1 $\alpha$ :  $p = 0,0214$ ; IL-5:  $p = 0,0010$ ; IL-13:  $p = 0,0128$ ; IFN- $\gamma$ :  $p = 0,0002$ ; IL-2:  $p = 0,0017$ ) (Fig. 3a–c). No significant alterations in IL-4 and IL-10 levels in PNS animals as compared to controls were observed (IL-4:  $p = 0,3765$ ; IL-10:  $p = 0,1017$ ) (Fig. 3a, b). See details in Supplementary Materials in table S2.

When animals were divided according to the behavioral phenotype, we found that IL-5, IL-17 $\alpha$ , IL-2, IL-1 $\alpha$  were significantly upregulated only in vulnerable and not resilient PNS animals as compared to controls (IL-5:  $p = 0,0014$ ; IL-17:  $p = 0,0014$ ; IL-2:  $p = 0,0042$ ; IL-1 $\alpha$ :  $p = 0,0147$ ) (Fig. 4a–c). We also found an upregulation in the levels of TNF- $\alpha$ , GM-CSF and IFN- $\gamma$  both in vulnerable (TNF- $\alpha$ :  $p = 0,0002$ ; GM-CSF:  $p = 0,0086$ ; IFN- $\gamma$ :  $p = 0,0045$ ) and resilient animals (TNF $\alpha$ :  $p = 0,0163$ ; GM-CSF:  $p = 0,9393$ ; IFN- $\gamma$ :  $p = 0,0101$ ) (Fig. 4a, b), whereas IL-4 and IL-10 were not significantly modulated among the three groups. See details in Supplementary Materials in table S3.

#### **PNS vulnerable animals show alterations in the liver leptin receptor that are associated with the inflammatory status**

As we found a consistent pro-inflammatory status in the liver from animals vulnerable to PNS and considering the key role of leptin in inflammatory responses [39, 40] and metabolic features, we explored possible alterations in leptin signaling in association with the pro-inflammatory status in PNS-exposed animals.

Interestingly, we found that stress exposure induced a significant upregulation in leptin receptor (LepR) in liver samples from PNS males, as compared to controls (LepR: +230,3%;  $p = 0,0001$ ) (Fig. 5a). Conversely, liver samples from female PNS animals showed no significant changes (LepR: +62,2%;  $p = 0,0816$ ) (Fig. 5a), in line with the absence of a pro-inflammatory status.

Moreover, when we divided animals into vulnerable or resilient, we observed a significant upregulation of LepR only in vulnerable males as compared to both resilient (LepR: +136,0%;  $p = 0,0136$ ) and control animals (LepR: +270,0%;  $p = 0,0001$ ) (Fig. 5b). Conversely, no significant differences in the expression of LepR were found in female rats when we divided them according to the phenotype (Fig. 5b).

Considering that leptin signaling cascade is involved in the production of several inflammatory molecules, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 [33], we tested possible associations between the expression of liver leptin receptor and the levels of the measured inflammatory molecules. Interestingly, as shown in Fig. 5, we found a positive and significant association between LepR expression and the composite inflammatory z-score in the liver and in the dorsal hippocampus of male animals exposed to PNS ( $r = 0,4995$ ,  $p = 0,0211$  and  $r = 0,3615$ ,  $p = 0,0019$ ) (Fig. 5c, d), suggesting a relationship between this adipokine and the pro-inflammatory status in the liver and in the dorsal hippocampus. Interestingly, this correlation is more pronounced in the group of

vulnerable male animals (Fig. 6a) compared to resilient ones (Fig. 6b), as a more significant positive association was observed between LEPTR and composite inflammatory z-score in vulnerable males ( $r = 0,633$ ,  $p = 0,0011$ ).

#### **DISCUSSION**

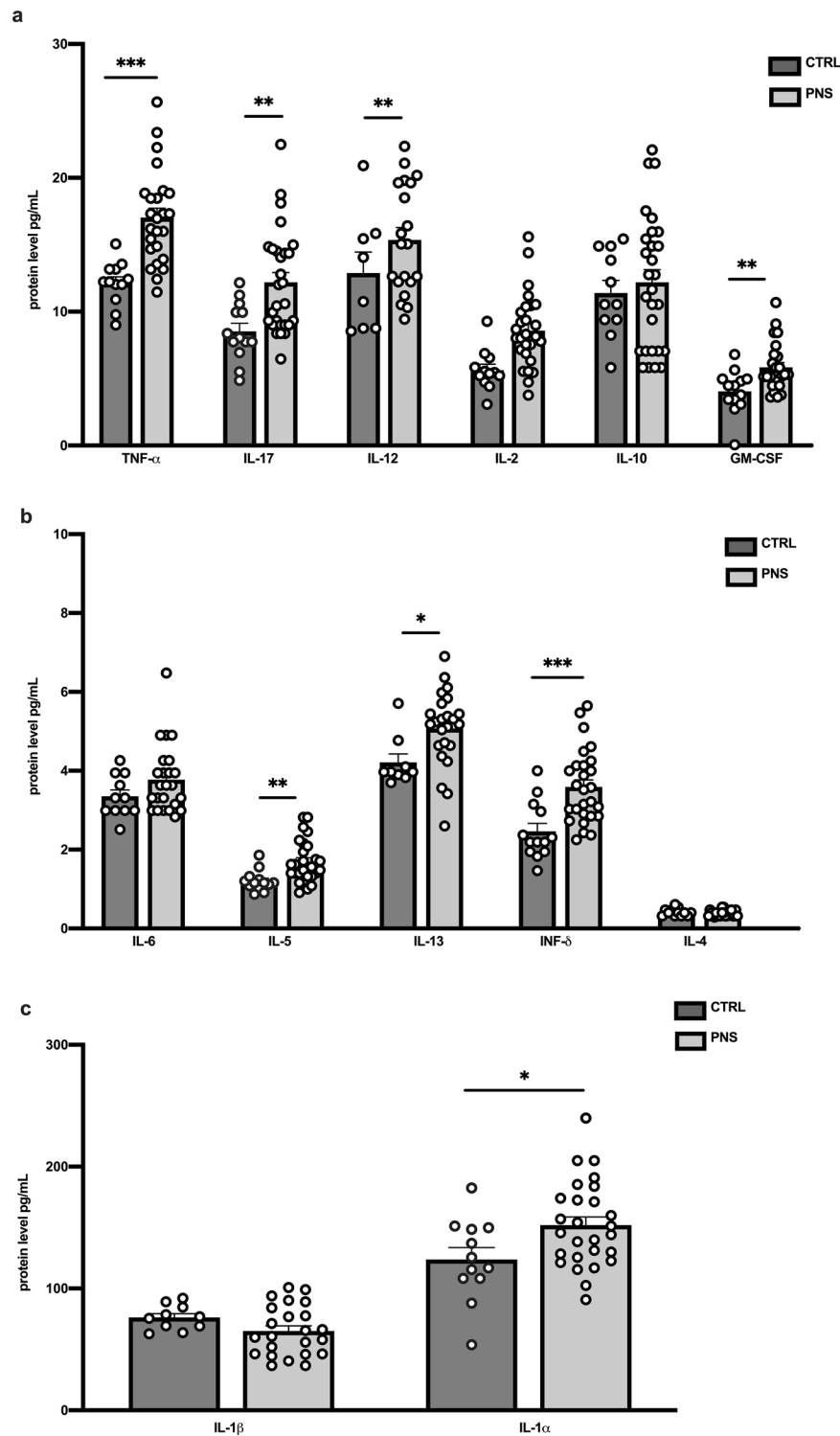
In this study, we delved into the impact of prenatal stress on the communication between the liver and brain, potentially influencing the susceptibility to mental and metabolic disorders due to early-life adversities. In particular, by using an animal model of PNS which mimics exposures to adversities during the first period of life, we showed that adolescent animals that develop emotional dysregulation as consequence of PNS are characterized by the presence of a proinflammatory status both in the hippocampus and also in the liver, pointing to the inflammatory system as a key biological process contributing to an enhanced risk to develop altered behaviors and metabolic disorders.

We found that exposure to PNS produced a significant increase of microglia markers expression in the dorsal hippocampus of adolescent male rats, with a prominent effect in the expression levels of genes encoding for C3 and C4 proteins. Importantly, such effect was observed only in PNS-exposed animals that developed a vulnerable phenotype, and not in resilient animals suggesting a relationship between such changes and the psychopathologic consequences of the adverse experience. C3 and C4 belong to the complement cascade, a set of proteins able to support a strong inflammatory response in association with the immune system. Complement proteins participate also in synaptic pruning, a process carried out by activated microglia and deregulated in several psychiatric conditions such as schizophrenia and depression [41], underscoring their significance in regulating the physiological development and functioning of the central nervous system [42]. Interestingly, the activation of C3 and C4 is corroborated by the presence of an upregulation of CD68, CX3CR1 and iNOS, indicating an overall activation of microglial cells. CD68 is a lysosomal marker, strictly involved in phagocytosis processes [43], while CX3CR1 is necessary to interact with neurons [44] and iNOS is upregulated to produce oxygen reactive species at the infection site. The presence of a microglia hyperactivation status may alter synaptic pruning [45] thus interfering with normal brain development [46].

To investigate the effect of PNS on metabolic function we focused our attention to the liver, which represents the organ most involved in the metabolic processes. Given the influence of glucocorticoids on immune cell functionality, stress can potentially impact the inflammatory response within the liver [47]. Moreover, during the adaptive response to stress, glucocorticoids regulate multiple aspects of energy metabolism including gluconeogenesis and glycogen storage in the liver [47].

We found a significant increase both in the gene expression and protein levels of pro-inflammatory markers in the liver of male vulnerable rats. Interestingly, we also found that leptin receptor expression was increased in the liver of animals exposed to PNS. Leptin is an adipokine, which was initially investigated for its central role in regulating food intake and body weight [32], and leptin also upregulates inflammatory molecules such as tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6).

The intricate connection between liver and brain goes beyond their individual functions and involves a complex interaction where cytokines may play a key role [30, 48]. As mentioned, cytokines produced in the liver are not only involved in promoting peripheral inflammation, and therefore in enhancing the risk for several metabolic disorders such as insulin resistance and diabetes type 2 [33], but also in contributing to brain inflammation with a potential relevance for the development of mental disorders [27, 28]. Numerous studies have highlighted that individuals with chronic liver diseases often experience altered behaviors,

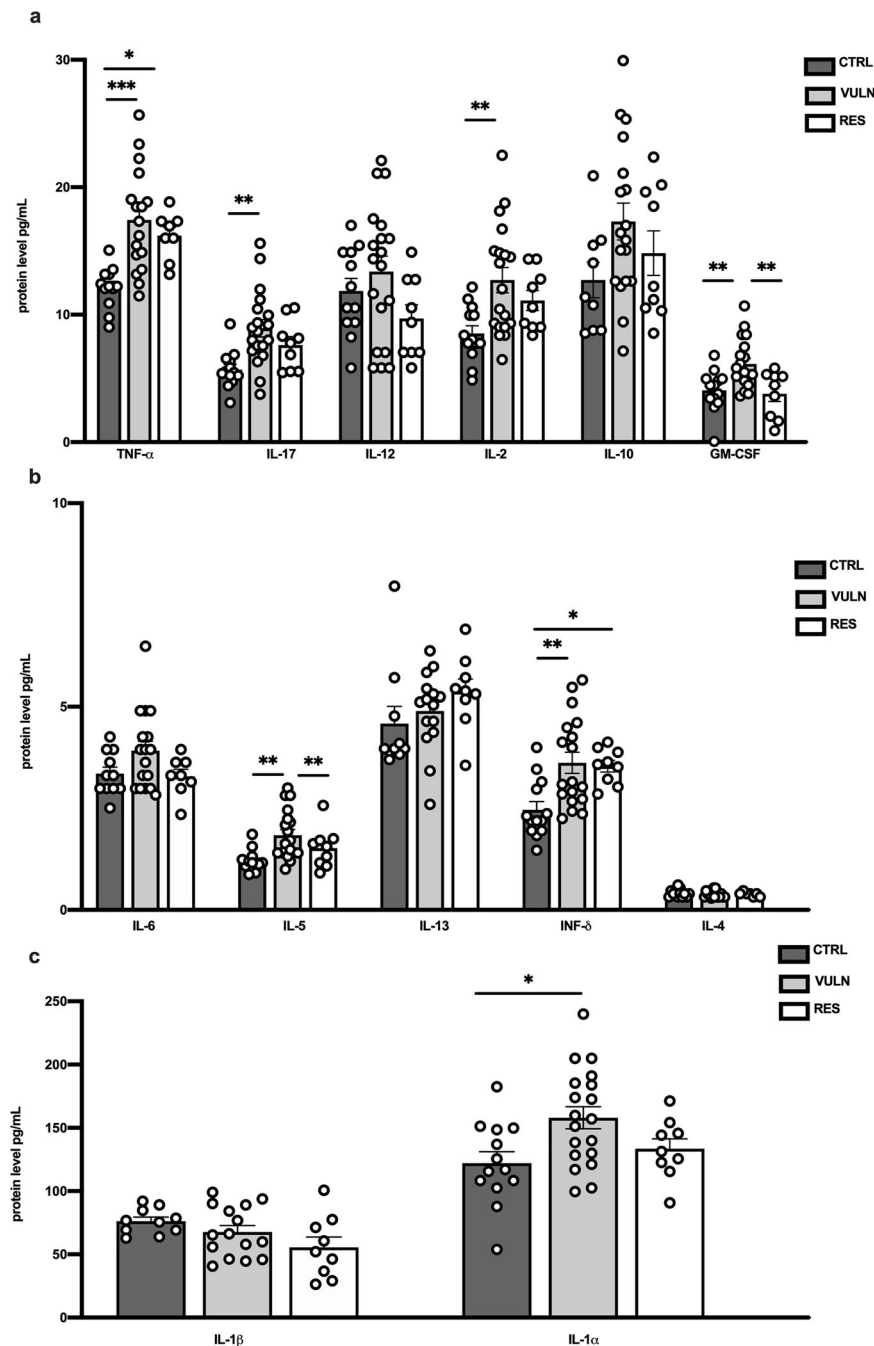


**Fig. 3 Protein expression analysis of inflammatory markers in the liver of male animals exposed to prenatal stress.** The protein levels for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17 $\alpha$ , GM-CSF, IL-1 $\alpha$ , IL-5, IL-12P70, IL-13, INF- $\gamma$ , IL-2, IL-4, IL-10 were measured in control (CTRL) and prenatally stressed group (PNS) (a–c). Statistical analysis for panels a, b, c: t-test, \* $p < 0,05$ , \*\* $p < 0,01$  and \*\*\* $p < 0,001$  ( $n = 13$  to 28 per group). Data are expressed as mean  $\pm$  SEM and individuals are represented as dots.

including depressive symptoms and other psychiatric manifestations, significantly impacting their overall Quality of Life (QoL) [49, 50].

Several signaling pathways have been described as potential link between systemic inflammation and brain function [51, 52]. Among these mechanisms, a neuronal pathway, involving afferent

fibers of the vagus nerve, that innervates the liver has been described. Peripheral immune responses could activate vagal nerve via pro-inflammatory cytokines, through receptors expressed on vagal nerve endings. After activation, vagal afferents carry stimuli to the brain leading to changes in brain functions and behavior [52]. It has been also suggested that, when systemic

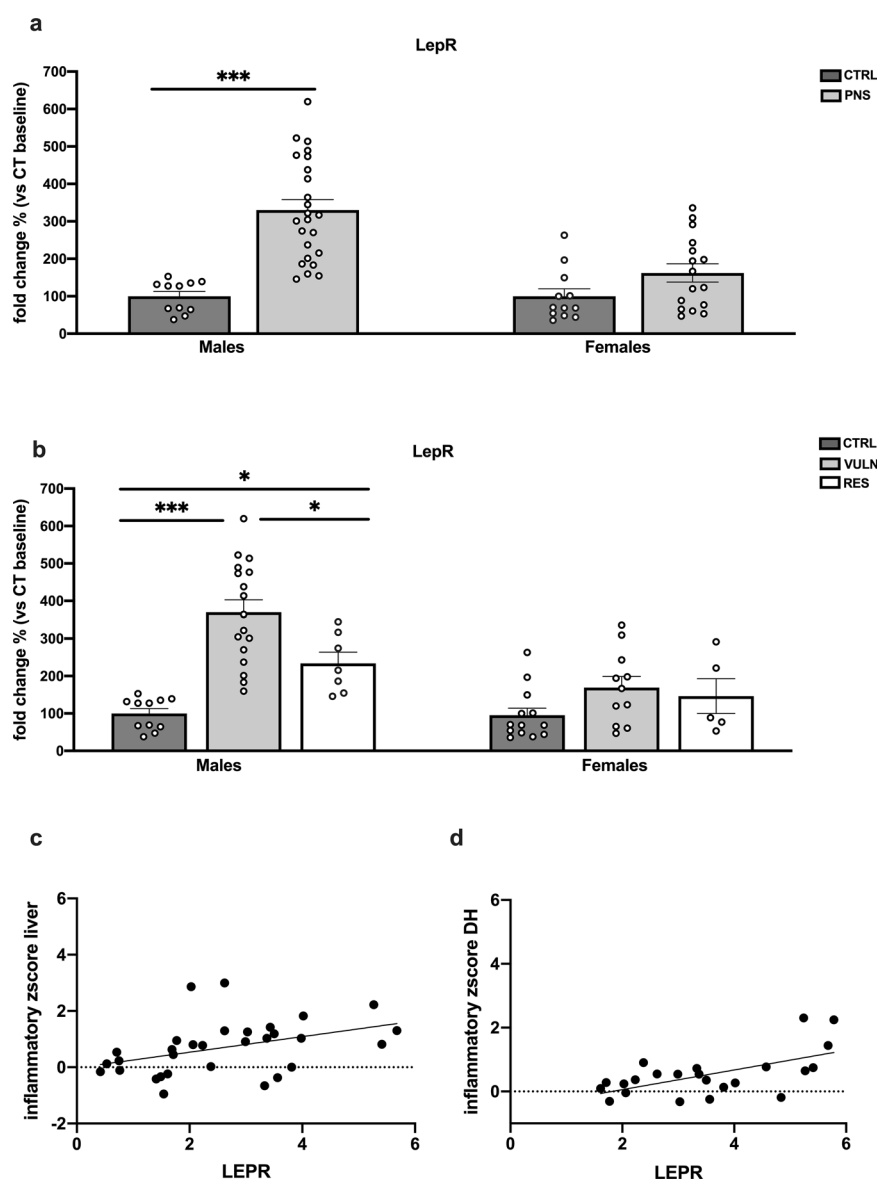


**Fig. 4 Protein expression analysis of inflammatory markers in the liver of vulnerable and resilient male animals.** The protein levels for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17 $\alpha$ , GM-CSF, IL-1 $\alpha$ , IL-5, IL-12P70, IL-13, INF- $\gamma$ , IL-2, IL-4, IL-10 were measured after the separation in vulnerable (VULN) and resilient (RES) (a–c). Statistical analysis for panels a, b, c: one-way ANOVA, Tukey's post hoc, \* $p < 0,05$ , \*\* $p < 0,01$  and \*\*\* $p < 0,001$  ( $n = 9$  to 19 per group). Data are expressed as mean  $\pm$  SEM and individuals are represented as dots.

inflammation is present, immune cells reach the brain and activate the Cerebral Endothelial Cells (CECs) [49], leading to stimulation of secondary messenger production, which are released within the brain parenchyma and activate resident immune cells in the brain (e.g., astrocytes and microglia) [49, 51]. Resident brain immune cells activated in this way, can themselves release cytokines that alter neurotransmission and behavior [53].

As mentioned, neuroinflammation could be strictly linked with peripheral inflammatory processes coming from the liver as consequence of stress exposure, however the link between stress exposures, hepatic inflammation and behavioral alterations is still poorly investigated. However, it is known that glucocorticoids,

released after stress exposure, regulate multiple aspects of energy metabolism in the liver [47] and cytokines produced in the brain can have an impact on some liver's metabolic activities such as glucose regulation, lipid metabolism, and detoxification [54]. Among several possible mediators of the hepatic inflammation found in PNS animals, we have focused our attention on leptin, an adipokine with roles in food intake and energy metabolism, which participates also in inflammatory responses [33]. The activity of leptin and its close link with several pro-inflammatory mediators underlie the ability of this molecule to promote and sustain low-grade inflammation, as consequence of stress exposure, which could ultimately favor the development of both metabolic and



**Fig. 5** Gene expression analysis of Leptin receptor in the liver of male animals exposed to prenatal stress. The mRNA levels for LepR were measured in control (CTRL) and prenatally stressed group (PNS) (a) in males and females or after the separation in vulnerable (VULN) and resilient (RES) (b) in males or females. Pearson's correlation analysis was performed to correlate expression of Leptin receptor and composite inflammatory z-score in liver (c) and in the dorsal hippocampus (d). Statistical analysis for panel a: t-test, \*\*\* $p < 0.001$  ( $n = 14$  to 30 per group). Statistical analysis for panel b: one-way ANOVA, Tukey's post hoc, \* $p < 0.05$  and \*\*\* $p < 0.001$  ( $n = 14$  to 30 per group). Data are expressed as mean  $\pm$  SEM and individuals are represented as dots. Statistical analysis for panel c: Pearson's correlation.

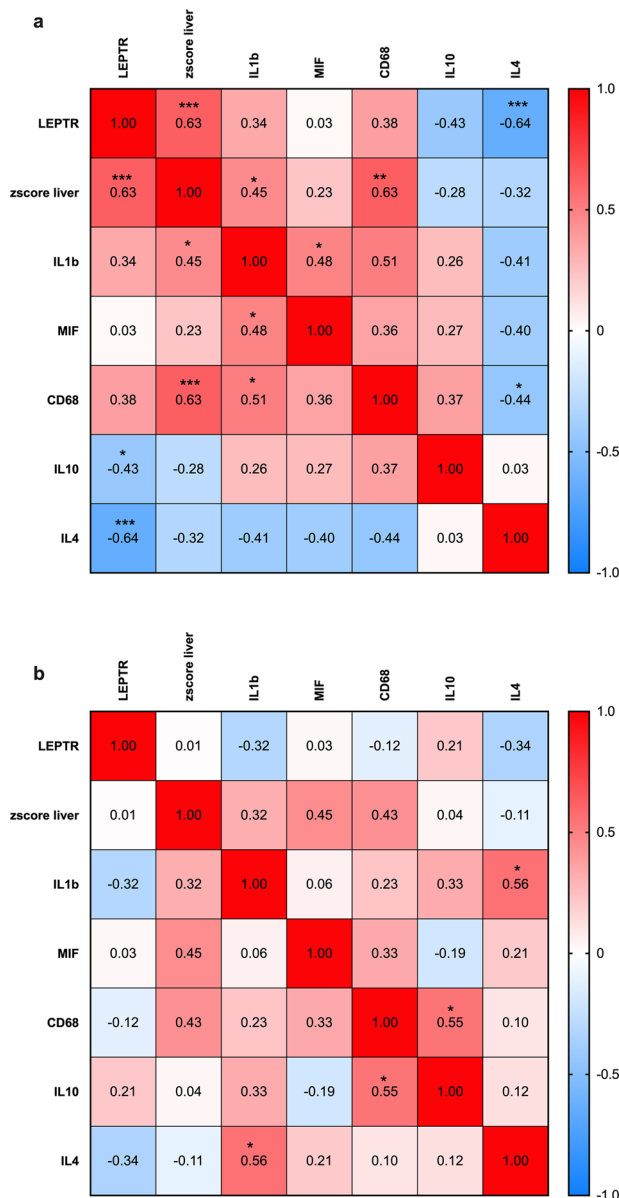
psychiatric disorders [40]. Our findings support the notion that levels of LepR exhibit a positive correlation with hepatic composite inflammatory z-score and inflammatory z-score in the dorsal hippocampus in male rats subjected to PNS, suggesting the potential involvement of leptin signaling in this inflammatory cascade. Moreover, pro-inflammatory mediators such as TNF- $\alpha$  and IL-1, which upregulate leptin expression, contribute to the generation of a loop of acute phase reactants that influence each other in promoting the development of chronic peripheral inflammation [40]. Interestingly, a dysregulation of leptin signaling was found both in patients diagnosed with mood disorders [55, 56], even in comorbidity with metabolic disorders [57, 58], and it has been suggested as a possible predictive marker for metabolic syndrome [59].

One important aspect of our study was the sex specificity of the inflammatory changes as a consequence of PNS exposure, both in the brain and in the liver, since only male animals vulnerable to

the adverse experience show such alterations, although both sexes show significant behavioral alterations [36]. This suggests that behavioral alterations in females may not be directly driven by inflammatory changes alone. While inflammation is a contributing factor in males, behavioral outcomes following PNS in both sexes are the result of complex, multifactorial mechanisms, where above to immune responses, also hormonal influences, and neurodevelopmental processes that interact in a sex-dependent manner could be involved.

Sex dimorphisms in the regulation of inflammatory responses exist and need to be clarified also in the context of our results. Indeed, microglia cells from males or females behave very differently. In males, microglia cells are always in a ready to intervene against any possible harmful stimulus for the brain, conversely, female microglia, less extended and slightly slower, can play a more protective role against possible damage to brain cells. To support this, a series of experiments on mice, conducted





**Fig. 6** Heat map of correlation analysis of leptin receptor and inflammatory markers in the liver of vulnerable and resilient male animals. Heat map of correlations between leptin receptor, inflammatory markers, and composite inflammatory z-score after the separation in vulnerable (VULN) (a) and resilient (RES) (b) males in the liver. As shown on the right, red indicates the strongest positive correlations, while blue indicates the strongest negative correlations. Statistical analysis for panels a and b: Spearman's correlation.

by Susanne A. Wolf and colleagues, have first observed that in the brain of male animals, microglia cells are more abundant and have a larger cell body [60], likely to indicate their reactivity status.

While our study offers valuable insights, it is essential to acknowledge certain limitations that may impact the interpretation of our results. First, we did not check the estrous cycle of female animals that could affect molecular analyses. Moreover, although we have measured expression level of leptin receptor to investigate the alterations in the related signaling, we do not have data on leptin in the blood, limiting the discussion on potential implications of leptin as circulating markers associated with metabolic alterations in these animals.

In conclusion, our findings indicate that the onset of a vulnerable phenotype as consequence to exposure to PNS is associated with the presence of enhanced inflammatory status in the hippocampus and in the liver, where a pivotal role in causing such inflammatory status is played by leptin signaling. Overall, we sustain an implication of a low-grade multiorgan inflammation in the onset and development of mood and metabolic disorders and/or their comorbidity.

## DATA AVAILABILITY

The data that support the findings of this study are available upon reasonable request.

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## AUTHOR CONTRIBUTIONS

ID: investigation, formal analysis, writing – original draft. GP: investigation, formal analysis, writing – original draft. VB: investigation. KCC: investigation. RO: investigation. RGO: methodology, supervision. MAR: supervision, review & editing. AC: conceptualization, supervision, writing, review & editing.

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## COMPETING INTERESTS

MAR has received compensation as speaker/consultant from Angelini, Lundbeck, Otsuka, and Sumitomo Pharma, and he has received research grants from Sumitomo Pharma. All the other authors declare no financial interests or potential conflicts of interest.

## ETHICS

All animal procedures were conducted in accordance with the institutional guidelines for the care and use of animals. This study did not involve human participants or human tissue samples.

## ADDITIONAL INFORMATION

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