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High-fat diet at adulthood interacts with prenatal stress, affecting both brain inflammatory and neuroendocrine markers in male rats

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Response to Editor

1)Author contributions need to be explicitly included in a statement in the manuscript itself

Done

2) Provide an abbreviations list

Done

3) Provide a clear data sharing statement, and we strongly encourage that you place your data on a public access repository (e.g. Figshare).

Done

4) When revising the manuscript, please **embolden** or underline major changes to the text so they are easily identifiable and DO NOT leave 'track change' formatting marks in your paper.

Done

5) Please ensure that you provide a text and a figure file for the **Graphical Abstract** (as detailed in the instructions below).

Done

6) When carrying out your revisions please refer to the **checklist below**.

Done

Response to reviewers

Reviewer #1

In this paper, Berry et al examine the effects of prenatal stress and the effects of a second-adult challenge (HFD) on the inflammatory and neural plasticity phenotype of adult offspring using gene expression profiles. They conclude that overall PNS decreases the pro-inflammatory phenotype and likewise decreases neuronal plasticity. By contrast, a HFD increases the vulnerability of developing a pro-inflammatory phenotype. In general, I believe that the methods were performed properly and that there was good discussion explaining the possible implications of their findings. However, there were many issues with the paper in its current form, with my biggest hesitation around over-interpretation of the results:

Major concerns:

1) I don't believe many of their conclusions are warranted based on the data they present. For example, they conclude that PNS reduces brain plasticity setting the stage for increased vulnerability for further insults, with a HFD promoting a pro-inflammatory phenotype. However, the data doesn't show this. In the PNS-HFD group, the proinflammatory markers are never significantly different from the Control-CD group. If anything, the HFD doesn't increase the pro-inflammatory phenotype it rather resets it to control levels. I think these conclusions should be toned down to reflect the data more accurately. Also, the title should be changed to reflect this.

*We thank the reviewer for pointing out this issue. The title has been changed as follows: "**Prenatal stress interacts with an adult metabolic challenge affecting brain inflammatory and neuroendocrine markers in male rats**".*

*This sentence in the abstract "HFD unveiled a pro-inflammatory phenotype by inducing a specific increase in the above-mentioned cytokines, suggesting that PNS might impinge upon the same mechanisms underlying vulnerability to metabolic challenges at adulthood" has been changed as follows: **"Overall, PNS reduced the expression of Bdnf and Tnf-α while HFD administered at adulthood counteracted this effect suggesting that PNS impinges upon the same pathways regulating responses to a metabolic challenge at adulthood".***

The Discussion and the Concluding remarks have been amended by toning down the effect of HFD on the pro-inflammatory phenotype of the PNS rats. See below.

*"…HFD feeding, experienced at adulthood, was able to unmask a pro-inflammatory phenotype, inducing an increase in many pro-inflammatory cytokines in those animals that had experienced PNS." This sentence has been changed as follows: "…**moreover, HFD feeding, experienced at adulthood, induced an increase in pro-inflammatory cytokines in those animals that had experienced PNS**."*

"Therefore, we can hypothesise that the increase in the pro-inflammatory cytokines observed as assessed in the prefrontal cortex (Il-1b and Tnf-a)…".

*This sentence has been changed as follows: "**Here we observed a similar blunted activation upon PNS with HFD triggering a response only in PNS subjects (increased Il-1β and Tnf-α in the prefrontal cortex and Tnf-α in the dorsal and ventral hippocampus).**"*

"We have here provided evidence for PNS to reduce brain plasticity (first hit) setting the stage for increased vulnerability to further insults during life (HFD, second hit) promoting a pro-inflammatory phenotype".

*This sentence has been changed as follows: "**We have here provided evidence for PNS to reduce brain plasticity (first hit) setting the stage for increased vulnerability to further insults during life (HFD, second hit).**"*

2) The data relating to ghrelin signaling seems out of place. There is no mention of ghrelin signaling or its significance in the introduction. I think there should be more context/rationale mentioned as to why the authors chose to specifically examine ghrelin in the introduction. Ghrelin is not a unique link connecting mood disorders (HPA axis function) and metabolic disorders. In fact, there are a plethora of neuroendocrine signals connecting the two pathophysiologies, including leptin, FGF21, endocannabinoids, etc. It seems out of context to focus in on ghrelin without providing further context.

We thank the reviewer for the thorough suggestion, we have added the following at the end of the Introduction paragraph

"We specifically assessed in these brain regions levels of Bdnf, Tnf-α, Il-6 and Il-β.

Stress powerfully affects both mood and energy homeostasis. Such effects are achieved not only through the interaction between GC hormones and their receptors (that regulate HPA axis function in addition to glucose metabolism) but also by triggering a multitude of signalling cascades reciprocally modulating one another in a regional-, temporal, and functional-dependent manner (Balsevich et al., 2019). Among these, ghrelin is a peptide hormone produced in the stomach and involved in the signalling of meal initiation. Its action is mediated through the growth hormone secretagogue receptor (Ghs-R) that is found to be highly expressed in several brain regions including the hypothalamus, pituitary gland and hippocampus (see (Zarouna, 2015) and references therein). Both Ghrelin and Ghs-R are modulated by stress (Patterson et al., 2010); there is evidence that ghrelin may decrease anxiety-like and depressive-like behaviours in mice (Lutter et al., 2008) moreover, it appears to be involved in in food anticipatory activity and consumption (Blum et al., 2009; Verhagen et al., 2011) as well as in mood disorders (Zarouna, 2015). Interestingly, both genetic variants within ghrelin and GC signalling pathways have been associated with obesity, stress-related mental disorders, or both (see (Balsevich et al., 2019) and references therein). To this regard, we also investigated changes in the expression levels of Ghs-R as well as of Nr3c1 (encoding for the glucocorticoid receptors - GR) and the GC co-chaperon Fkbp5 (encoding for the FK506 binding protein 51) in all the above-mentioned brain regions."

*Moreover we have added in the concluding remarks the following sentence "**Further investigation are thus warranted to assess more in detail the role played by these molecules in setting the stage for co-morbidity between metabolic and psychiatric disorders. In addition, the role of additional co-regulators, such as endocannabinoids should be tested (Balsevich, 2019).***

3) Although the authors provide sufficient explanation as to why PNS decreases a pro-inflammatory phenotype in the discussion, I still believe that this largely disagrees with previously published data (typically PNS and ELS increase pro-inflammatory phenotypes and risk for mood disorders). I think these conflicting results should be discussed more.

*We thank the reviewer for the suggestion and the following has been added to the Discussion paragraph: "**Worth to notice, we have previously shown that upon PNS, male rats were characterized by increased corticosterone levels under basal conditions, this effect was associated with a decrease in reactive oxygen species (ROS) as well as a with decreased NF-kB signalling in the hippocampus suggesting a lower set-point under basal conditions in PNS male rats (Anacker et al., 2013). Here we observed a similar blunted activation upon PNS with HFD triggering a response only in PNS subjects (increased Il-1β and Tnf-α in the prefrontal cortex and Tnf-α in the dorsal and ventral hippocampus). To our knowledge this is one of the first times that such a reduction in inflammatory mediators is described a result of PNS. Because cytokines have been shown to modulate hippocampal development and plasticity (Bourgognon and Cavanagh, 2020; Goshen et al., 2007) a decreased inflammatory profile as a result of a "first hit" (PNS) might indeed set the stage for an increased response to a "second hit" (metabolic challenge)."***

4) The study used a candidate approach for both brain regions examined as well as genes examined. Both seemed somewhat arbitrary and not inclusive. For example, why not assess the amygdala, which plays a critical role in the stress response, emotionality, and mood disorders?

We thank the reviewer for the thorough question. Indeed, the aim of this study was to investigate the contribution of prenatal stress to increase the vulnerability to later life metabolic and mood disorders. To this regard, we focused on specific brain regions such as the prefrontal cortex, hippocampus and hypothalamus that are well-known to play a role in mood disorders and that also integrate mood and metabolic signals (hippocampus and hypothalamus). We agree with this reviewer that the amygdala is a very interesting region to study and future research will also try to focus on this area. As far as gene expression is concerned also in this case, we focused on those signalling cascades known to be deeply involved in the modulation of mood and metabolism.

Further, the discussion does mention why GR and Fkbp5 were selected for examination. However, there are major gaps in interpreting these findings. How do changes in either GR or Fkbp5 affect GR sensitivity? The authors should examine circulating CORT levels under baseline or after a challenge or perform a DEX/CRH test to assess whether GR sensitivity is altered. I understand that this may not be possible at this point, however given the direction of changes across different brain regions, it is difficult to determine the significance. With that said, the authors do discuss the possible implications in the discussion. But they don't adequately discuss how FKBP5/GR signaling relates to metabolic vulnerability. think the rationale for selecting these candidates should again be stated in the introduction.

*As suggested by this reviewer, we have introduced the rationale for studying FKBP5/GR signalling in the Introduction paragraph (see above answer to Q2). With regard to HPA axis reactivity in relation to changes in expression levels of Fkbp5 and GR sensitivity, the Discussion paragraph already reported that we have previously shown that PNS male rats were characterised by elevated GC levels when compared to controls (Anacker et al., 2013); this issue has been further strengthened (see also answer to Q3). Moreover, we have now provided evidence that levels of Fkbp5 are positively associated to the time spent in the open arms of the EPM (see Figure 6). "**Interestingly, we also showed that levels of Fkbp5 in the dorsal hippocampus were positively related to the time spent in the open arm of the EPM and that this positive correlation is lost as a result both of PNS as well as of HFD. This result might suggest that under physiological conditions Fkbp5 is required to engage animals in the proper exploration of novel environments through a fine modulation of the GR receptors and that this balance can be greatly affected by both early life stressors as well as by metabolic challenges."** This sentence has been added to the Discussion paragraph.*

We also added further consideration with regard to this issue in the Concluding remarks (see below)

*"**Thirdly, genomic and epigenomic regulation of glucocorticoids in the brain may affect mood and metabolism but is based upon the coordinated activity of many chaperone proteins, in addition to proper circadian and ultradian fluctuation of hormone release (Gray et al., 2017)."**.*

5) The correlation graphs between immobility in the EPM and gene expression data should not be included in this study. It is not possible to effectively evaluate the methodology here when the EPM was not included in this study but in a previously published study. Moreover, why did they just correlate to immobility in the EPM? In general time spent in the open and closed arms are the major readouts. Is this the only parameter that significantly correlated? I think this data should be removed.

We thank the reviewer for pointing out this issue. Following this suggestion, and suggestions from reviewer 2, we have briefly added the EPM methodology to the text and carried out a regression analysis between Fkbp5 levels in the dorsal hippocampus and time spent in the open arm of the EPM. We believe that the regression analyses reported in this paper are very important to strengthen data deriving from the analysis of gene expression in the brain area investigated and to characterise the effects of changes in the selected genes. Furthermore, we would like to stress that this paper and Panetta et al. 2017 should be considered as closely related publications since they refer to the same experimental subjects and they complement one-another.

6) The GR and FKBP5 gene expression data should be represented in the form of a graph (not table) like all other data. It is much easier to visualize/interpret graphs than figures

Done

7) The hypothalamus gene expression data should also be included in the graphs, despite many regions showing no change in gene expression from PNS or HFD in the hypothalamus, there were a few cases where there was a change in the hypothalamus (i.e. Fkbp5). Regardless, I think the data should be graphically represented.

Done

8) In the methods, they mention the groups including both males and females. In this study and with the specific research questions, only males are relevant. I think it is confusing to mention females. It only raises questions. I would omit methods related to females. Also, in lines 131-135, they mention methods for a previous paper. This should not be included here. It can be discussed in the discussion in terms of relevance to the current study.

We thank this reviewer for the suggestion; the method section has been modified by removing details about female rats as well as those concerning gene expression levels previously assessed; see below:

"By this time, n=20 CTRL and n=21 PNS males and n=20 CTRL and n=19 PNS females were assigned either to high-fat diet (HFD) or control diet (CD) regimen for 8 weeks. The final number of animals per group was: n=10 CTRL -CD, n=10 CTRL -HFD, n=10 PNS-CD and n=11 PNS-HFD for the male offspring; n=10 CTRL -CD, n=10 CTRL -HFD, n=10 PNS-HFD and n=9 PNS-CD for the female offspring. After 4 weeks on the respective diets, all animals underwent a number of metabolic and behavioural assessments; they were successively sacrificed, brain were dissected out and the ventral hippocampus used to assess Bdnf long 3'UTR, hypothalamus used to assess levels of, LepR, Adipo-R1 and Adipo-R2; total Bdnf expression was assessed in both areas. Results from the above-mentioned studies have been recently published in Panetta et al. 2017. Here we investigated selectively in male animals' brains the expression levels of Il-6, Il-1β, Tnf-α, Nr3c1, Fkbp5, Ghs-R, in the dorsal hippocampus, ventral hippocampus, hypothalamus and prefrontal cortex; total Bdnf was investigated only in the dorsal hippocampus and prefrontal cortex (since it was already investigated in the other two areas, (Panetta et al., 2017)."

This paragraph has been modified as follows: ***"By this time, n=20 CTRL and n=21 PNS males were assigned either to high-fat diet (HFD) or control diet (CD) regimen for 8 weeks. The final number of animals per group was: n=10 CTRL -CD, n=10 CTRL -HFD, n=10 PNS-CD and n=11 PNS-HFD. After 4 weeks on the respective diets, all animals underwent a number of metabolic and behavioural assessments; they were successively sacrificed and brains dissected out to investigate the expression levels of Il-6, Il-1β, Tnf-α, Nr3c1, Fkbp5, Ghs-R, in the dorsal hippocampus, ventral hippocampus, hypothalamus and prefrontal cortex; total Bdnf was investigated only in the dorsal hippocampus and prefrontal cortex (since it was already investigated in the other two areas, (Panetta et al., 2017)."***

9) Lines 397-398: Chronic CORT or DEX decreases Fkbp5 methylation and increases FKBP5 expression in mouse models of both HFD and stress (Scharf et al. 2011). Scharf et al. 2011 does NOT show this. They show FKBP5 expression in brain regions after 24h food deprivation or 1x dexamethasone treatment. Correct references should be provided or this statement should be corrected.

We thank this reviewer for pointing out this issue. The sentence, and the associated reference, have been removed from the text.

10) Lines 407-410: "the hippocampal increase in Fkbp5 as a result of HFD might suggest that this metabolic challenge (second hit) might impact cognitive functions in our animal model. This piece of data is further strengthened by a similar increase in the levels of Nr3c1 in the ventral hippocampus of all HFD rats." Perhaps it is my interpretation, but does this make sense? If you have increase Fkbp5, you in theory decrease GR sensitivity. Therefore, wouldn't the increase in GR offset the increase in FKBP5?

We thank the reviewer for the thorough remarks. Regulation of glucocorticoids is a process relying on the intricate coordination of many chaperone proteins in addition to proper circadian and ultradian fluctuation of hormone release. We have changed the sentence to more clearly indicate that a cross-regulation between a metabolic challenge and Nr3c1/Fkbp5 appears from these data, strengthening the notion that indeed these mediators are involved in "sensing" environmental challenges, be these of psychological nature or metabolic.

"Changes in Nr3c1 and Fkbp5 in the hippocampus a result of HFD indicate a cross-regulation between a metabolic challenge and Nr3c1/Fkbp5 balance, strengthening the notion that indeed these molecules are involved in "sensing" environmental challenges, be these of psychological or metabolic nature".

We have also added the following sentence to the concluding remarks (see also above Q4). ***"Thirdly, genomic and epigenomic regulation of glucocorticoids in the brain may affect mood and metabolism but is based upon the coordinated activity of many chaperone proteins, in addition to proper circadian and ultradian fluctuation of hormone release (Gray et al. 2017). Thus, additional analyses should be performed in future studies to assess the interrelationship between psychological and metabolic stressors. In addition, given the observed changes of Ghs-R and Nr3c1 in the dorsal hippocampus following a HFD, future studies should be devoted to better explore the effects of HFD and of PNS, and their interaction, on brain plasticity".***

Minor concerns:

1) For the assignment of pups to CD/HFD and control/PNS groups, how many pups from each litter were represented in each group? This information should be provided given it is best practice to have only 1-2 pups from each litter in each group.

*"**Groups were composed by 1 pup/litter (CTRL-CD; CTRL-HFD; PNS-CD; PNS-HFD)**".*

This sentence has been added to the methods section.

2) Typo, line 108: Thus, the aim of this study…….

Done

3) Typo, line 158: …offspring were weighed……and brain were removed……

Done

4) Line 196: post hoc test just missed statistical significance: ADD P-value

*The sentence has been rephrased to make it clearer "**A strong PNS x HFD interaction was found (F(1,34)=4.655; p=0.0381) showing that PNS exposed animals were characterised by decreased levels of this cytokine, while the administration of postnatal HFD counteracted the effects of the PNS (post-hoc comparisons just missed statistical significance)."***

5) Lines 199-202: A significant PNSxHFD interaction was founds. But this wasn't the case (p = 0.0580). Maybe change statistical analysis so that statistical significance is set at 0.05; statistical tendency is set at p < 0.1. For interactions at p<0.1, we also examine lower order main effects.

*We thank the reviewer for the suggestion; the following sentence has been added to the Statistic paragraph "**Statistical tendency was set at p < 0.1. For interactions at p<0.1, we also examined lower order effects**". Moreover, we have changed the word "significant" into "tendency" or "nearly significant" throughout the text, when appropriate, according with the above mentioned specification in the Statistic paragraph.*

6) Line 272: p = 0.0582 was described as significant

See above

7) In Figure 3 (for Bdnf), should there not be 2 separate asterisks? One for PNS vs Control, and One for PNS vs PNS-HFD?

Done

8) It is not explained what Nr3c1 or Fkbp5 are/encode in the intro, methods, or results. Then in line 276, they mention GR mRNA. This should be clarified.

Done

9) Typo, line 349:……level of the IL-1B in the DORSAL hippocampus

Done

10) A lot of the referenced articles in the introduction disagree with the current findings. For example:

a. Bdnf is negatively regulated by pro-inflammatory cytokines. Yet in general there was a decrease of both Bdnf and pro-inflammatory cytokines by PNS, suggesting this is not responsible for the regulation of Bdnf in their study.

We agree with this thorough observation. As suggested by these reviewers, we have toned-down the emphasis on the inflammatory effects observed in order to avoid over-interpretation. Thus, our data rather than pointing directly to changes in the inflammatory phenotype overall suggest a general reduced activation of those pathways possibly involved in brain plasticity. In agreement with this view, we observed a general decrease both in Bdnf and the pro-inflammatory cytokines in PNS male rats.

b. In general they exemplify many studies where PNS leads to a pro-inflammatory phenotype. Are there studies where it reduces the pro-inflammatory phenotype?

See answer to Q3

Yet they did not introduce other key aspects of the paper, such as FKBP5/GR signaling and ghrelin signaling in reference to mood disorders and metabolic regulation. I think the introduction should be re-worked to account for their rationale to look at specific candidates.

See answer to Q2

Reviewer # 2

Comments to the Author

This nice study by Berry et al. describes the central and site-specific effects of prenatal stress (PNS) in combination with a subsequent dietary challenge (HFD) on the mRNA expression level of inflammatory-, stress response-, mood- and metabolic marker genes. Level of mRNA expression were assessed via qPCR in punches of brain areas known to play key roles in neuro psychopathology and homeostasis. An Elevated Plus Maze test, conducted in a previous study by Panetta et al., set base for the correlation analyses of neuroplasticity marker Bdnf and pro-inflammatory marker Il1-β, both of which were inversely correlated with the duration of immobility in the maze. Further, Berry et al. could clearly show interaction effects between the PNS and subsequent dietary challenge on the expression level of nearly all examined marker genes inducing a pro-inflammatory phenotype. This study further helps to disentangle the complex interplay of stress and metabolic control highlighting the key role of central inflammatory processes in this context. However, I do have a number of minor concerns that should be addressed before this manuscript is suitable for publication.

Comments:

1) In the abstract (lines 38 – 40) and in the beginning of the discussion (327 – 330) the authors state that the changes observed in the expression level of Fkbp5 and Nr3c1 underlie the metabolic vulnerability previously observed in Panetta et al. However, the discussion (lines 379 – 422) focuses on the role of Fkbp5 and Nr3c1 in HPA-axis regulation and states that changes of these two marker genes rather reflect a higher stress vulnerability (lines 401 – 402) and diminished cognitive abilities (lines 408 – 409) without discussing how they induce the observed metabolic vulnerability. Therefore, it is very surprising that there is no linear regression analysis of immobility time in the EPM and Fkbp5/Nr3c1 expression level shown. Please, include these and properly discuss all results, including the role of Fkbp5 and Nr3c1 in metabolic control.

*We thank the reviewer for the thorough comment. We have now provided evidence that levels of Fkbp5 are positively associated to the time spent in the open arms of the EPM (see Figure 6). "**Interestingly, we also showed that levels of Fkbp5 in the dorsal hippocampus were positively related to the time spent in the open arm of the EPM and that this positive correlation is lost as a result both of PNS as well as of HFD. This result might suggest that under physiological conditions Fkbp5 is required to engage animals in the proper exploration of novel environments through a fine modulation of the GR receptors and that this balance can be greatly affected by both early life stressors as well as by metabolic challenges."** This sentence has been added to the discussion paragraph. Moreover, we have added the following sentence to the Discussion paragraph to stress the involvement of Fkbp5 and of Nr3c1 in metabolic regulations: "**Glucocorticoids have also an important function in metabolic regulations as they mobilize glucose to fuel the energy demands of the stress response and furthermore promote energy storage, feeding, and weight gain. Thus, it is possible to hypothesize that both psychological as well as metabolic stress might be able to affect HPA axis functionality (Balsevich et al., 2019)".***

2) The last sentence of the abstract in line 42 - 43: "[…] leading to a greater vulnerability to psychopathology." is very unexpected as it is not stated in the abstract that the behavioral phenotype was assessed and correlated to mRNA expression level. Further, it is too stringent as the results of this study as well as Panetta et al. show that there are also metabolic complications arising from both PNS and HFD. Please rephrase the last sentence and mention the behavioral assessment already in the abstract.

*We thank the reviewer for pointing out this issue. The abstract has been modified as follows: **"Furthermore, HFD and PNS affected the expression of both Nr3c1 and Fkbp5, two neuroendocrine mediators involved in the response to stress, metabolic challenges and in the modulation of behavioural plasticity (as shown by***

the correlation between Fkbp5 and the time spent in the open arms of the elevated plus-maze).”. *Results have been reported and discussed. (see methods and Discussion paragraphs).*

3) As “first hit” and “second hit” are mentioned a couple of times in terms of prenatal and subsequent adult stressors, please elaborate on the two-hit model including references in the introduction and try to imply it also in the discussion.

We thank the reviewer for the comment. We have added the following sentences to the introduction: ***“In fact, according to a “two-hit model” of vulnerability to diseases, stress experienced during early developmental phases, might affect brain development leading to a reduced ability to cope with further stressors during life (Daskalakis et al. 2013)”; “Based on the “two hit model” of diseases, we hypothesized that PNS….”***

See also graphical abstract and answer to Q3, Reviewer#1

4) Please mention the sort of PNS that was applied already in line 100 of the introduction.

*The term “PNS” has been changed with “****prenatal restraint stress”***

5) Further indicate the motivation/reason for the choice of the specific prenatal stress paradigm used (in the context of this study) in the methods section.

*This specification has been added to the methods section “****This stress has been selected since it is one of the most well characterised prenatal stressors with well-known effects on developmental trajectories of the offspring”.***

6) Reference Maccari et al. 1995 which is mentioned in line 147 - 148 is missing in the reference section.

Done

7) In line 135 of the methods section it is stated that experiments were selectively performed in males but it is not explained why. Given the fact that females were used in this cohort (see Panetta et al.) it is even more surprising that they were not included in this data set. It should be clearly explained why females were excluded in this study and stated as a major limitation of this work.

*We thank the reviewer for pointing out this issue. Although, this was specified in the introduction, (“In a previous study we have shown that male rats who underwent PNS were more vulnerable than females to the effects of a metabolic challenge experienced at adulthood (high-fat diet feeding - HFD), while females showed an overall greater plasticity, possibly mediated by increased total Bdnf mRNA expression levels both in the hippocampus and in the hypothalamus”) we have also added the following sentence for the sake of clarity “****A growing body of evidence suggests that the effects of PNS are sex-specific and that males’ foetal brain might be more vulnerable to changes in the inflammatory mediators overall showing learning deficits and decreased brain plasticity particularly with regard to the hippocampus prefrontal cortex (McCarthy, 2019; Weinstock, 2007)”.***

8) Please describe the Elevated Plus Maze (EPM) test briefly in the methods section as this is an important readout of this study and explain why exactly immobility time in the maze was chosen as a "a parameter indicative of increased emotionality" (line 180 – 181) and why this readout "can be related with reduced behavioral plasticity" (line 244). It is important for the overall message of this study to explain why this specific readout of the EPM indicates emotionality and plasticity and to reference this properly.

*We thank this reviewer for the suggestion. We have briefly added the EPM methodology to the methods section as follows: "**Briefly, the apparatus was made of Plexiglas and consisted of two opposite open arms and two arms closed by transparent walls (50×10×40 cm). Each rat was placed in the central area of the maze and video-recorded for 5 minutes under dim light conditions. Each session was recorded and behavioral analysis was carried out using a commercial software (''The Observer 3.0'', Noldus, The Netherlands) (see (Panetta et al., 2017) for further details")**.*

*In the absence of a significant effect of percent time in open arms (Panetta et al. 2017) we have focuses on "immobility time" as a proxy for emotionality and we have correlated this behavioural outcome with Il-1beta and BDNF. We mentioned behavioral plasticity since we know that both BDNF and IL-1beta are specifically involved in behavioral plasticity especially in the hippocampus. However, following suggestion from reviewer #1 (see Q4) we have also added regression plot of Fkbp5 and time spent in the open arm. The following has been added to the text at the end of the "Animal and experimental design" paragraph: "**To better characterise the effects of possible changes in gene expression levels, regression analyses were carried out by taking into account specific outcomes deriving from the Elevated Plus-Maze (EPM), as previously performed in (Panetta et al., 2017). We focused on immobility time since this is a well-known proxy for emotionality and reactivity to novel environment (Fernandes and File, 1996) and correlated it with Bdnf and Il-1β since both these mediators are involved in behavioural plasticity (Bourgognon and Cavanagh, 2020; Cirulli and Alleva, 2009; Goshen et al., 2007). Moreover, we also assessed the ability of Fkbp5 to affect emotionality in the EPM by correlating expression levels of this gene in the dorsal hippocampus to the time spent in the open arms of the maze."***

9) In figures 1,3 and 5 the significant interaction effects of PNS and HFD are neither visually indicated nor are they described in the figure legends. However, the two-way ANOVA is initially the most important statistical analysis of the data sets to show combined effects of both stressors.

We thank the reviewer for pointing out this issue. We have now specified the significant post-hoc comparisons within the PNS X HFD interaction in each figure legends to increase their readout.

10) In figure 1, there is a main effect of the HFD in dorsal hippocampus indicated ($$), however in the figure legend the description of this statistical effect is completely missing. Please indicate this effect in figure legend

Done

Further, adjust graph colour for Il-6.

Done

11) The indicated significance in figure 3 (dorsal hippocampus) is not comprehensible as to my understanding there is a post-hoc effect shown here between CTRL and PNS-HFD. However, in the manuscript lines 255 –

256, it is stated that there's significant effects in PNS vs. CTRL and PNS vs. PNS-HFD, which are not indicated in the figure. Please, clarify this.

We thank this reviewer for pointing out this issue. The figure has been changed also in agreement with Reviewer #1

12) Concerning tables 1 and 2, it is not obvious why the authors would choose tables over graphs here, given the fact that collection and analysis of this data set was the same as for the other markers. Preferably, graphs should be chosen to visualize results for Fkbp5 and Nr3c1. Tables could be included for all shown data sets in supplementary material, including missing results for hypothalamus of figures 1, 3 and 5.

Graphs have been now provided and Tables deleted

13) In lines 322 – 323 it is stated that: "PNS, per se, affected brain plasticity by decreasing the expression levels of the neurotrophin Bdnf and pro-inflammatory cytokines…". However, in lines 252 – 253 the authors state the exact opposite of this: "[…] did not affect levels of Bdnf per se […]." Please be cautious with such phrasings and rewrite either of these two sentences.

We agree with this reviewer and the two sentences have been rephrased to make clear that, in the PNS x HFD interaction, the decrease in Bdnf refers to the comparison PNS-CD vs. CTRL-CD, see below:

*"**overall PNS and postnatal HFD did not affect levels of Bdnf** (F(1, 36)= 3.678; 1.964;p= 0.0631; 0.1696), main effect of PNS and HFD respectively)"*

"PNS-CD rats were characterised by reduced brain plasticity showing decreased expression levels of the neurotrophin Bdnf and of pro-inflammatory cytokines in a number of brain regions"

14) In figure 5, the word "decreased" is missing in the figure legend.

Done

High-fat diet at adulthood interacts with prenatal stress, affecting both brain inflammatory and neuroendocrine markers in male rats

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Figures: *7*

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Abstract

Prenatal stress (PNS) affects foetal programming and, through an interaction with subsequent challenges, can increase vulnerability to mood and metabolic disorders. We have previously shown that, following PNS, adult male rats are characterised by increased vulnerability to a metabolic stressor experienced at adulthood (8-week-high-fat diet - HFD). In this study, we specifically assessed whether PNS might interact with an adult metabolic challenge to induce an inflammatory phenotype. Changes in the expression levels of inflammatory (*Il-1β, Tnf-α*, *Il-6*) and of stress response mediators (*Nr3c1*, *Fkbp5*) as well as of mood and metabolic regulators (*Bdnf, Ghs-R*) were investigated in the hippocampus, prefrontal cortex and hypothalamus, brain regions involved in the pathogenesis of depression and prone to inflammation in response to stress. **Overall, PNS reduced the expression of *Bdnf* and *Tnf-α,* while HFD administered at adulthood counteracted this effect suggesting that PNS impinges upon the same pathways regulating responses to a metabolic challenge at adulthood. Furthermore, HFD and PNS affected the expression of both *Nr3c1* and *Fkbp5*, two neuroendocrine mediators involved in the response to stress to metabolic challenges and in the modulation of the emotional profile (as shown by the correlation between *Fkbp5* and the time spent in the open arms of the elevated plus-maze).** Overall, these results indicate that the same metabolic and neuroendocrine effectors engaged by PNS are affected by metabolic challenges at adulthood, **providing some mechanistic insight into the well-known comorbidity between mood and metabolic disorders**.

***Keywords*:** Animal model, Prenatal stress, High-fat diet, Neuroinflammation, Mood disorders, Co-morbidity.

45 **LIST OF ABBREVIATIONS**

46 BDNF= Brain-Derived Neurotrophic Factor

47 CD= Control Diet

48 CRP= C-Reactive Protein

49 CTRL= control

50 EPM= Elevated Plus-Maze

51 EU= European

52 GC= Glucocorticoids

53 GD= Gestational Day

54 GHS-R= Ghrelin Receptor

55 GR= Glucocorticoid Receptor

56 HFD= High-Fat Diet

57 HPA= Hypothalamic–Pituitary–Adrenal

58 IL= interleukin

59 LTP= Long-Term Potentiation

60 NF-kB= Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

61 PND= Post-Natal Day

62 PNS= Prenatal Stress

63 ROS= Reactive Oxygen Species

64 SEM= Standard Error Mean

65

1. INTRODUCTION

Mood disorders are common conditions with major public health implications. It is estimated that each year around 40% of the EU population suffers from a mental disorder. Adjusted for age and comorbidity, this corresponds to 164.8 million people affected (Health Organization Regional Office for Europe, 2015). Among the risk factors that might trigger the onset of psychiatric conditions, or an exacerbation of symptoms, stressful life events play a pivotal role. **In fact, according to a "two-hit model" of vulnerability to diseases, stress experienced during early life phases, might affect brain development leading to a reduced ability to cope with further stressors during life (Daskalakis et al., 2013).** To this regard, a suboptimal intrauterine environment, prompted by maternal stress (ranging from poor socio-economic status and maternal obesity to depression or maltreatments) may predispose the offspring to lifelong negative health outcomes, including neuropsychiatric disorders as well as metabolic and immune dysregulations (Barker, 1995; Berry et al., 2015; Boersma et al., 2013; Cattane et al., 2020; Cirulli et al., 2020; Krontira et al., 2020).

Consistent evidence suggests that depressed patients are characterised by alterations in the functional activity of the immune system, affecting both peripheral and central tissues and that early life stress represents an important factor influencing the inflammatory status (Cattaneo et al., 2015; Dantzer et al., 2008; Dowlati et al., 2010; Liu et al., 2012; Valkanova et al., 2013). As an example, Danese and colleagues reported increased blood C-reactive protein (CRP) levels in maltreated children, an effect that was larger in those subjects that also developed depression later in life (Danese et al., 2009, 2008). Likewise, Slopen and co-workers reported increased CRP and interleukin-6 (IL-6) in adolescents who experienced early life adversities (Slopen et al., 2014). Offspring of women who experienced stressful life events during pregnancy show higher levels of IL-1β, IL-4, IL-5, IL-6, and IL-8 in umbilical cord blood at delivery (Andersson et al., 2016). Interestingly, it has been recently proposed that inflammation may act as a key mediator linking exposure to prenatal stress (PNS) and enhanced vulnerability to psychopathology in the offspring (Hantsoo et al., 2019).

Preclinical studies in rodent models provide strong support to the above mentioned clinical findings, showing for example that PNS exposure causes extended inflammation in the foetal brain (Ślusarczyk et al., 2015). To this regard, Gur and colleagues found increased levels of IL-1β in the placenta of mouse dams undergoing stress during pregnancy and in the foetal brain of their offspring; this was also associated with decreased levels of the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) specifically in the amygdala (Chen et al., 2020; Gur et al., 2017) suggesting that increased expression of placental immune responsive genes upon maternal stress might be considered as a potential mechanism (Bronson and Bale, 2014; Mueller and Bale, 2008).

Despite the large body of clinical and preclinical evidence showing associations among PNS, the hyperactivation of the immune system and an enhanced vulnerability to mood disorders, cause-effect mechanisms are still largely unknown, and the prenatal environment may not act alone to set the stage for long-term onset of mood disorders. Interestingly, psychiatric conditions and metabolic pathologies are often found

to co-occur within the same individual showing a feed-forward pattern overall suggesting the activation of shared mechanisms/pathways (Cattane et al., 2020; Cirulli et al., 2020; Milaneschi et al., 2019). Among these, BDNF and glucocorticoids (GC) hormones have been identified as main effectors of brain plasticity and metabolic function in response to stressful events. Thus, it is possible that these actors may represent both effectors as well as targets of stress - during sensitive developmental periods - leading, in the developing organism, to a remodelling of the mechanisms associated with stress responsiveness (Cattaneo et al., 2015; Cirulli and Alleva, 2009; McEwen, 2000). The neurotrophin BDNF plays a pivotal role in brain and behavioural plasticity as well as in the control of energy homeostasis (Cirulli and Alleva, 2009; Marosi and Mattson, 2014); its expression is finely tuned by GC in response to acute or chronic stressful stimuli (Cirulli, 2017;Cirulli & Alleva, 2009) and it has been also suggested to be negatively regulated by pro-inflammatory cytokines (Barrientos et al., 2004; Bilbo et al., 2008). Thus, BDNF appears as a sensitive target as well as a biomarker of stress response as it could actively contribute to maintain brain health/homeostasis in different challenging conditions. To this regard, the interplay between the activation of stress-related pathways by adverse experiences during development and adult metabolic challenges might be crucial in shaping individual vulnerability to stress (Nederhof and Schmidt, 2012).

A growing body of evidence suggests that the effects of PNS are sex-specific and that males' foetal brain might be more vulnerable to changes in the inflammatory mediators overall showing learning deficits and decreased brain plasticity particularly with regard to the hippocampus prefrontal cortex (McCarthy, 2019; Weinstock, 2007). Indeed, in a previous study we have shown that male rats who underwent PNS were more vulnerable than females to the effects of a metabolic challenge experienced at adulthood (high-fat diet feeding - HFD), while females showed an overall greater plasticity, possibly mediated by increased total *Bdnf* mRNA expression levels both in the hippocampus and in the hypothalamus. **Based on the "two hit model" of diseases,** we hypothesized that PNS might disrupt the intrauterine environment affecting brain developmental trajectories leading, in turn, to increased brain inflammation and reduced plasticity in the offspring (first hit). We also hypothesized that a metabolic challenge such as a HFD, experienced at adulthood, might interact with inflammatory pathways to exacerbate the PNS-induced pro-inflammatory phenotype. Thus, **the** aim of this study was to investigate the effect of a second hit - represented by an HFD - specifically in male rats. We focused on selected brain regions - characterised by high degree of sexual dimorphism (Handa et al., 1994) - such as the dorsal and the ventral hippocampus, the prefrontal cortex and the hypothalamus known to be involved in the pathogenesis of depression and to be prone to inflammatory status in response to external challenges. **We specifically assessed in these brain regions levels of *Bdnf*, *Tnf-α*, Il-6 and *Il-β*.**

Stress powerfully affects both mood and energy homeostasis. Such effects are achieved not only through the interaction between GC hormones and their receptors (that regulate HPA axis function in addition to glucose metabolism) but also by triggering a multitude of signalling cascades reciprocally modulating one another in a regional-, temporal, and functional-dependent manner (Balsevich et al., 2019). Among these, ghrelin is a peptide hormone produced in the stomach and involved in the signalling of meal

initiation. Its action is mediated through the growth hormone secretagogue receptor (Ghs-R) that is found to be highly expressed in several brain regions including the hypothalamus, pituitary gland and hippocampus (see Zarouna, 2015 and references therein). Both Ghrelin and Ghs-R are modulated by stress (Patterson et al., 2010); there is evidence that ghrelin may decrease anxiety-like and depressive-like behaviours in mice (Lutter et al., 2008) moreover, it appears to be involved in in food anticipatory activity and consumption (Blum et al., 2009; Verhagen et al., 2011) as well as in mood disorders (Zarouna, 2015). Interestingly, both genetic variants within ghrelin and GC signalling pathways have been associated with obesity, stress-related mental disorders, or both (see Balsevich et al., 2019 and references therein). To this regard, we also investigated changes in the expression levels of *Ghs-R* as well as of *Nr3c1* (encoding for the glucocorticoid receptors - GR) and the GC co-chaperon *Fkbp5* (encoding for the FK506 binding protein 51) in all the above-mentioned brain regions.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

Twelve adult nulliparous female (230–260 g) and 6 male Sprague-Dawley rats (400 g) were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, animals were pair-housed with same sex conspecifics under standard laboratory conditions (see Panetta et al. 2015 for further details). Pellet food (Altromin-R, Rieper, Italy) and tap water were continuously available. Following one week of adaptation, two females and one male were mated for 24 hours. To assess pregnancy, changes in body weight were monitored. Pregnant females were randomly assigned to either the control (CTRL) or prenatal stress (PNS) groups. CTRL rats were left undisturbed throughout gestation, while PNS females underwent a repeated restraint stress procedure during the last third of gestation until the day delivery day (postnatal day 0 - PND-0). On PND-1 all pups were weighted and litters culled to an average of 5 male and 5 female pups. On PND-21 pups were weaned and housed in groups of 2 or 3 same-sex littermates until 2 months of age. **By this time, n=20 CTRL and n=21 PNS males were assigned either to high-fat diet (HFD) or control diet (CD) regimen for 8 weeks. The final number of animals per group was: n=10 CTRL-CD, n=10 CTRL -HFD, n=10 PNS-CD and n=11 PNS-HFD. Groups were composed by 1 pup/litter (CTRL-CD; CTRL-HFD; PNS-CD; PNS-HFD. After 4 weeks on the respective diets, all animals underwent a number of metabolic and behavioural assessments; they were successively sacrificed and brains dissected out to investigate the expression levels of *Il-6*, *Il-1β*, *Tnf-α*, *Nr3c1*, *Fkbp5*, *Ghs-R*, in the dorsal hippocampus, ventral hippocampus, hypothalamus and prefrontal cortex; total *Bdnf* was investigated only in the dorsal hippocampus and prefrontal cortex (since it was already assessed in the other two areas, (Panetta et al., 2017).**

To better characterise the effects of possible changes in gene expression levels, regression analyses were carried out by taking into account specific outcomes deriving from the Elevated Plus-Maze (EPM), as

previously performed in (Panetta et al., 2017). We focused on immobility time since this is a well-known proxy for emotionality and reactivity to novel environment (Fernandes and File, 1996) and correlated it with *Bdnf* and *Il-1β* since both these mediators are involved in behavioural plasticity (Bourgognon and Cavanagh, 2020; Cirulli and Alleva, 2009; Goshen et al., 2007). Moreover, we also assessed the ability of *Fkbp5* to affect emotionality in the EPM by correlating expression levels of this gene in the dorsal hippocampus to the time spent in the open arms of the maze. As for the EPM protocol used, briefly, the apparatus was made of Plexiglas and consisted of two opposite open arms and two arms closed by transparent walls (50×10×40 cm). Each rat was placed in the center of the maze and video-recorded for 5 minutes under dim light conditions. Each session was recorded and behavioral analysis was carried out using a commercial software (''The Observer 3.0'', Noldus, The Netherlands) (see (Panetta et al., 2017) for further details).

All experimental procedures were reviewed by the ethical body of the Istituto Superiore di Sanità for animal welfare and conducted in conformity with the European Directive 2010/63/EU and the Italian legislation on animal experimentation, D.Lgs. 26/2014. They were authorized by the Italian Ministry of Health.

2.2 Dams' stress procedure

Pregnant females (at gestational day - GD - 14) were restrained in a transparent Plexiglas cylinder (7.5×19 cm) under a bright light (6.500 lux) for 45 minutes three times daily at random times (between 9:00 a.m.-5:00 p.m.) during the dark phase until the expected delivery day (GD-21, **see Maccari et al., 1995** for further details). **This stress has been selected since it is one of the most well characterised prenatal stressors with well-known effects on developmental trajectories of the offspring.**

2.3 High-fat diet administration

CTRL and PNS offspring were fed *ad libitum* either with HFD (energy: 5.24 kcal/g; composition: fat 60%, carbohydrate 20% and protein 20%) or CD (energy: 3.3 kcal/g; composition: fat 17%, carbohydrate 60% and protein 23%) starting from two months of age. The HFD diet (D12492) was purchased from Research Diets, Inc., New Brunswick, NJ, USA; the CD was purchased from Altromin-R, Rieper, Italy.

2.4 Tissue collection

At 4 months of age offspring **was** weighted and sacrificed. **Brains were** removed and the dorsal and ventral hippocampus, hypothalamus and prefrontal cortex were dissected out and immediately frozen at -80°C (Panetta et al., 2017).

2.7 Molecular analysis

2.7.1 RNA isolation and Real Time PCR Analyses

Total RNA was isolated using PureZol RNA isolation reagent (Bio–Rad Laboratories, Italy), treated with DNase to avoid DNA contamination and quantified by spectrophotometric analysis. The quantified RNA was analyzed by TaqMan q-RT PCR Instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories) and Applied BioSystem Assays (Gene Expression Assays: *Il6, Il-β, Tnf-α, Bdnf, Nr3c1, Fkbp5,* and *Ghs-R*). Samples were run in triplicate and each target gene analyzed has been normalized to the expression of the housekeeping (HK) gene β-actin (ActB). The expression of target genes was calculated by using to the Ct method (-ΔΔCt method) (Schmittgen and Livak, 2008), where CTRL-CD rats have been used as a reference group.

2.8 Statistical analysis

Data were evaluated by a two-way ANOVA with Diet (HFD vs CD) and Prenatal condition (PNS vs CTRL) as between-subject factors. *Post-hoc* comparisons between groups were performed using the Tukey's test. A linear regression model was used to assess the ability of *Bdnf* levels and of *Il-1β* in the dorsal hippocampus to affect immobility duration (a parameter indicative of increased emotionality) in the EPM test. A level of probability set at $p<0.05$ was used as statistically significant. **Statistical tendency was set at p<0.1. For interactions at p<0.1, we also examined lower order effects.** Data are presented graphically as means ± SEM box plot (observations outside the ranges are represented with dots outside the boxes). **The raw data supporting the conclusions of this article will be made available by the authors upon request without undue reservation.**

3. RESULTS

3.1 Inflammatory mediators *Il-1β*, *Tnf-α* and *Il-6* mRNA expression

Il-1β - We first analyzed the expression of *Il-1β*, a prototype pro-inflammatory cytokine. We observed that PNS resulted in a nearly significant decrease in the dorsal hippocampus while neither a main effect of HFD nor a PNS x HFD interaction effect were found ($F(1,35)=3.818, 0.004, 2.184$; $p= 0.0587, 0.9518, 0.1484$). No main effects of PNS nor of HFD were observed in the ventral hippocampus ($F(1,34)=0.152, 0.335$; $p=0.6995, 0.5665$). **A strong PNS x HFD interaction was found ($F(1,34)=4.655$; $p=0.0381$) PNS exposed animals being characterised by decreased levels of this cytokine, while the administration of postnatal HFD counteracted the effects of the PNS (post-hoc comparisons just missed statistical significance).** In the prefrontal cortex while no main effects of PNS was observed ($F(1,33)=0.969$; $p=0.3321$) postnatal HFD overall increased levels of *Il-1β* (main effect of HFD: $F(1,33)=5.112$, $p=0.0305$). Similarly to what was observed in the ventral hippocampus, a **nearly significant** PNS x HFD interaction was found with decreased

levels of *Il-1β* in PNS animals, this effect being reverted by postnatal administration of HFD (F(1,33)=3.858; p=0.0580, post hoc comparisons: p<0.01, PNS vs. PNS-HFD); (see Figure 1A).

In the hypothalamus, levels of *Il-1β* were not affected by PNS, HFD nor an interaction was found (F(1,36)=0.042, 0.0001633, 0.070; p=0.8386, 0.9899, 0.7934 respectively for PNS, HFD and PNS x HFD, see Figure 1A).

Tnf-α - In the dorsal hippocampus no main effects of PNS nor of HFD were observed (F(1,37)=1.288, 0.209; p=0.2638, 0.6506). By contrast, a significant interaction was found between PNS and HFD (F(1,37)=5.772; p=0.0214). In particular, PNS animals were characterised by decreased levels of *Tnf-α,* although this effect was no longer observed in PNS-HFD animals that showed levels of this cytokine comparable to those observed in CTRL animals (post hoc comparisons: p<0.05, PNS vs. CTRL).

No main effects of PNS nor of HFD were found in the ventral hippocampus (F(1,34)=0.033, 2.941; p=0.8573, 0.0955, respectively for PNS and HFD), while a PNS x HFD interaction was found showing that administration of postnatal HFD in the PNS group selectively increased levels of this cytokine ((F(1,34)=6.747; p=0.0138); post hoc comparisons: PNS vs. HFD-PNS, p<0.01).

No main effects of PNS nor of HFD were observed in the prefrontal cortex (F(1,33)= 0.079, 1.648; p=0.7809, 0.2082 respectively for PNS and HFD) however, as also observed for the ventral hippocampus a PNS x HFD interaction effect was found showing that the administration of postnatal HFD in the PNS group selectively increased levels of this cytokine (F(1,33)=7.946; p=0.0081); post hoc comparisons: PNS vs. HFD-PNS, p<0.01); (see Figure 1B).

Levels of *Tnf-α* in the hypothalamus (see Figure 1B) were not affected neither by PNS nor by postnatal HFD (F(1, 35)=0.286, 0.702, 2.111; p= 0.5964; 0.4079; 0.1551 respectively for PNS, HFD and PNS x HFD).

Il-6 - Postnatal HFD decreased levels of *Il-6* in the dorsal hippocampus (main effect of HFD: F(1,37=0.814; p=0.0066), whereas no effect of the PNS nor of the PNS x HFD interaction effect were found (PNS main effect: F(1,37)=1.387; p=0.2465; PNS x HFD interaction: F(1,37)=0.164; p=0.6881).

Levels of *Il-6* did not change as a result of PNS, HFD or their interaction neither in the ventral hippocampus (F(1,36)=0.759, 2.631, 0.029; p=0.3893, 0.1135, 0.8647 respectively for PNS, HFD and PNS x HFD) nor in the prefrontal cortex (F(1,35)=0.144, 0.053, 0.202 respectively for PNS, HFD and PNS x HFD); (see Figure 1C). PNS decreased nearly significantly the levels of *Il-6* also in the hypothalamus (F(1,36)=3.889; p=0.0563), but no effect of HFD nor an interaction PNS x HFD were found to be significant (F(1,36)=0.903, 0.757; p=0.3482, 0.3899, respectively for HFD and PNS x HFD).

----------FIGURE 1 about here----------

Interestingly, when expression levels of *Il-1β* in the dorsal hippocampus were related to immobility duration in the EPM (a test run in a companion paper on the same experimental animals, see (Panetta et al., 2017)), we

observed a significant strong negative association linking these two parameters only in the PNS-CD animals, overall suggesting that reduced levels of this cytokine can be related with reduced behavioral plasticity (see discussion; CTRL-CD: $F(1,8)=0.007$; $p=0.9367$; $R^2=0.01$; CTRL-HFD: $F(1,8)=3.461$; $p=0.1051$; $R^2=33.1$; PNS-CD: $F81,8)= 7.538$; $p=0.0287$; $R^2=51.8$; PNS-HFD: $F(1,9)= 1.739$; $p=0.2237$, $R^2=17.9$; see Figure 2).

----------FIGURE 2 about here----------

3.2 Neuronal plasticity marker - *Bdnf* mRNA expression

We first investigated the expression of *Bdnf*, a neurotrophin that is considered a prototype marker of plasticity. Within the dorsal hippocampus, **overall PNS and postnatal HFD did not affect levels of *Bdnf*** ($F(1, 36)= 3.678$; 1.964;$p= 0.0631$; 0.1696), main effect of PNS and HFD respectively), although we observed a significant interaction between PNS and postnatal HFD ($F(1, 36)=5.606$; $p=0.0234$). More in detail, PNS decreased *Bdnf* expression, while the postnatal administration of HFD was able to counteract this effect (PNS vs. CTRL, $p<0.05$; PNS vs. PNS-HFD, $p<0.05$). In the prefrontal cortex no effect of PNS, HFD or their interaction was observed ($F(1, 36)=0.200$, 1.377, 0.174; $p=0.6572$; 0.2482; 0.6794, respectively for PNS, HFD and PNS x HFD); (see Figure 3).

----------FIGURE 3 about here----------

Interestingly, levels of *Bdnf* in the dorsal hippocampus were negatively associated with the time spent immobile in the EPM (a test run in a companion paper on same experimental animals, see (Panetta et al., 2017)) only in the PNS-CD group (CTRL-CD: $F(1,9)=0.730$; $p=0.4261$; $R^2= 0.081$; CTRL-HFD: $F(1,9)= 0.090$; $p=0.7713$; $R^2=0.011$; PNS-CD: $F(1,8)=11.084$; $p=0.0127$; $R^2=0.612$; PNS-HFD: $F(1,9)=0.716$; $p=0.4221$; $R^2= 0.082$), supporting a role of this neurotrophin in the emotional phenotype (see Figure 4).

----------FIGURE 4 about here----------

3.3 Stress response mediators - *Nr3c1*, *Fkbp5* mRNA expression

Nr3c1 - In the dorsal hippocampus the postnatal HFD decreased the levels of *Nr3c1* when compared to controls and a similar **nearly significant** effect was observed for PNS (main effect: $F(1,36)=3.827$, 7.063, $p=0.0582$; 0.0117 respectively for PNS, HFD). Moreover, a significant PNS x HFD interaction was found showing that both PNS-CD and CTRL-HFD as well as the combination PNS-HFD were characterised by a decrease of GR mRNA levels ($F(1,36)=6.228$; $p=0.0173$ and PNS x HFD; post hoc comparisons $p<0.05$).

In the ventral hippocampus levels of *Nr3c1* were increased a result of postnatal HFD (main effect: $F(1,36)=10.405$; $p=0.0027$), while no effects of PNS nor of the PNS x HFD interaction were found ($F(1, 36)=0.011$, 0.258; $p= 0.9167$; 6146). In the prefrontal cortex the postnatal HFD overall increased the levels of *Nr3c1* (main effect of HFD: $F(1, 37)=12.453$; $p=0.0011$); no effects of PNS nor of the PNS x HFD interaction

were observed ((F(1, 37)=1.001, 0.702; p=0.3236; 0.4076). No effects of PNS, HFD nor of their interaction were found in the hypothalamus (F(1, 37)=0.157, 0.009, 1.067; p= 0.6943; 0.9257; 0.3084); (**see Figure 5**).

Fkbp5 - In the dorsal hippocampus the postnatal HFD increased the levels of *Fkbp5* (F(1,35)=10.331;p=0.0028), while no effect of PNS nor of the PNS x HFD interaction was observed (F(1,35)=1.502, 0.958; p= 0.2285; 0.3343). No effect of PNS, HFD or their interaction was found in the ventral hippocampus (F(1,36)=0.781, 3.222, 0.007; p=0.3828; 0.0811; 0.9359). In the prefrontal cortex, PNS increased the levels of *Fkbp5* (F(1,37)=5.244; p=0.0278), while no effect of HFD nor of the PNS x HFD interaction was observed (F(1,37)=1.219, 2.019; p=0.2767; 0.1637). A main effect of the postnatal HFD was also observed In the hypothalamus, the Postnatal HFD decreased levels of *Fkbp5* (F(1,37)=9.156; p=0.0045); no effect of PNS nor of the PNS x HFD interaction was observed (F(1,37)=0.094, 3.353; p= 0.7608; 0.0751); (see **Figure 5**).

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Interestingly, levels of *Fkbp5* in the dorsal hippocampus were positively related with increased time spent in the open arm of the EPM (a test run in a companion paper on same experimental animals, see (Panetta et al., 2017)) only in the CTRL-CD group (CTRL-CD: F(1,8)=26.617; p=0.0013; R^2= 0.792; CTRL-HFD: F(1,9)= 0.104; p=0.7713; R^2=0.013; PNS-CD: F(1,8)=0.253; p=0.6307; R^2=0.035; PNS-HFD: F(1,9)=0.503; p=0.6307; R^2= 0.4982). This result might suggest that *Fkbp5* is physiologically required to engage animals in the proper exploration of novel environments through a fine modulation of GR receptors (see Figure 6).

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3.4 Metabolic/mood regulator marker - *Ghs-R* mRNA expression

Ghs-R - In the dorsal hippocampus the postnatal HFD overall decreased levels of *Ghs-R* (F(1,33)=1,948; p=0.0007). By contrast, PNS increased *Ghs-R* levels (F(1,33)=5.192; p=0.0293), but no interaction between PNS x HFD was observed (F(1,33)=0.290; p= 0.5941). In the ventral hippocampus HFD overall increased the levels of *Ghs-R* (F(1,32)=6.278; p=0.0175), while no main effect of PNS was observed (F(1,32)=0.593; p=0.4468). However, *Ghs-R* was increased in the CTRL-HFD and in the PNS-CD groups (interaction between PNS and HFD: F(1,32)=14.778; p=0.0005, post hoc: CTRL vs. CTRL-HFD, p<0.01; CTRL vs. PNS, p<0.01). In the prefrontal cortex, PNS increased the levels of *Ghs-R* (F(1,29)=5.865; p=0.0219) and no effect of HFD was observed (F(1,29)=2.191; p=9.1496). However, in this brain area, *Ghs-R* was increased in the CTRL-HFD and in the PNS-CD groups (interaction between PNS and HFD: F(1,29)=6.870; p= 0.0138, post hoc: CTRL vs. CTRL-HFD, p<0.05; CTRL vs. PNS, p<0.01); (**see Figure 7**). As for the hypothalamus, neither PNS nor HFD affected levels of *Ghs-R* (F(1,34)=3.155, 3.033, 0.907; p=0.0846; 0.0906; 0.3477).

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4. DISCUSSION

In the present study we assessed the long-term effects of exposure to PNS (a first hit) on foetal brain programming with regard to neuronal plasticity and brain inflammation. Moreover, we used a metabolic challenge (HFD) at adulthood (second hit) to assess potential interaction effects with the PNS-induced phenotype. We have previously shown (Panetta et al., 2017) that although PNS resulted in a strong metabolic liability in male rats fed with HFD at adulthood it also dampened the negative effects driven by such a metabolic challenge on the emotional phenotype as assessed in the EPM. Here we extended such body of evidence by providing information on the possible mechanisms underlying this interaction. **PNS-CD rats were characterised by reduced brain plasticity showing decreased expression levels of the neurotrophin *Bdnf* and of pro-inflammatory cytokines in a number of brain regions; moreover, HFD feeding, experienced at adulthood, induced an increase in pro-inflammatory cytokines in those animals that had experienced PNS**. This suggests that PNS might impinge upon the same mechanisms underlying vulnerability to metabolic challenges at adulthood. Furthermore, HFD greatly affected the expression levels of the main effectors of HPA axis function (the glucocorticoid receptor - GR - gene *Nr3c1* and of the GR's co-chaperone *Fkbp5*) an effect possibly underlying the metabolic vulnerability previously observed in male rats.

In our study, we found that PNS animals were characterised by differential expression of mRNA levels of *Il-1β* and *Tnf-α* in almost all the brain regions investigated except for the hypothalamus, an area that, in our experimental condition, appeared to be resilient to inflammatory changes driven by both pre- and postnatal stressors. While *Tnf-α* was tightly modulated upon pre- and postnatal stressors, changes in *Il-1β* were observed only when considering the interaction with the second hit (HFD), suggesting that the former cytokine (*Tnf-α*) might be considered as a reliable marker of stress adaptation in response to a prenatal challenge. As for *Il-6*, no effect was observed upon PNS in neither of the brain areas considered, though HFD overall decreased its expression levels in the dorsal hippocampus. TNF-α, IL-1β and IL-6 are the main activators of the HPA and are in turn modulated (inhibited) by GC hormones. As described by O'Connors and colleagues these cytokines hold differential sensitivity for adrenal steroids with TNF-α being the most sensitive to such inhibition in the range of physiological levels, IL-1β being second and IL-6 being the most resistant (O'Connor et al., 2000).

There is general consensus that elevated inflammation can exacerbate or even give rise to depressive symptoms or may be associated to other psychiatric conditions (Dantzer et al., 2018, 2008). However, cytokines are also constitutively released in the healthy brain by resident myeloid cells to keep proper synaptic plasticity. As an example, the modulation of both IL-1β and TNF-α plays an important role in the processes of LTP and synaptic scaling (a form of homeostatic plasticity) (Rizzo et al., 2018; Salim et al., 2012). Here we found that lower mRNA levels of *Il-1β* in the **dorsal** hippocampus of PNS rats were associated to increased time spent immobile in the EPM (as previously assessed in (Panetta et al., 2017)). Moreover, and in line with our previous work (Panetta et al., 2017), PNS rats were characterised by decreased levels of the neurotrophin *Bdnf* (in the dorsal

hippocampus); this decrease was also associated to an increase in the time spent immobile in the EPM, suggesting that PNS might affect foetal brain programming by reducing neuronal plasticity. Interestingly, the interaction between PNS and adult HFD (PNS-HFD group) resulted in increased levels of *Il-1β* and *Tnf-α* mRNA. Alboni and colleagues have recently provided evidence for increased rather than decreased levels of pro-inflammatory cytokines in the brain of stressed mice treated with the antidepressant fluoxetine. Such an effect was associated to increased BDNF levels and stronger LTP in the hippocampus (Alboni et al., 2017, 2016), suggesting that brain plasticity may also be related to the activation of basal metabolism that in turn is positively associated to the ability to properly mount and control inflammatory responses. To this regard, we cannot exclude that prolonged exposure to the HFD or to a stronger metabolic insult, such as a western pattern diet (rich in fats and sugar), should lead to an overall excessive brain inflammation (not observed in this study) with main consequences on emotional/cognitive behaviour. **Worth to notice, we have previously shown that upon PNS, male rats were characterized by increased corticosterone levels under basal conditions, this effect was associated with a decrease in reactive oxygen species (ROS) as well as a with decreased NF-kB signalling in the hippocampus suggesting a lower set-point under basal conditions in PNS male rats (Anacker et al., 2013). Here we observed a similar blunted activation upon PNS with HFD triggering a response only in PNS subjects (increased Il-1β and Tnf-α in the prefrontal cortex and Tnf-α in the dorsal and ventral hippocampus). To our knowledge this is one of the few instances in which a reduction in inflammatory mediators is described a result of PNS. Because cytokines have been shown to modulate hippocampal development and plasticity (Bourgognon and Cavanagh, 2020; Goshen et al., 2007) a decreased expression profile as a result of a "first hit" (PNS) might set the stage for an increased response to a "second hit" (metabolic challenge).** Obesity is characterised by low-grade systemic and central inflammation and TNF-α and IL-1β appear to be key players in this condition and in the associated pathologies (Mighiu et al., 2012). Although the hypothalamus is a well-known area playing a main role in the crosstalk between brain and periphery in metabolic pathologies associated to obesity, other brain regions have been shown to be affected by high-fat diet- or obesity-induced inflammation such as the hippocampus, cortex, brainstem, or amygdala (Guillemot-Legris and Muccioli, 2017). There are data to suggest that the effects of a metabolic challenge may be different depending on the region of interest dealing with brain plasticity (hippocampus) or metabolic regulation and homeostasis (hypothalamus) (Rasgon and McEwen, 2016). Thus, our data also indicate that PNS might set the stage for a differential regulation of central inflammatory mediators in specific brain regions, an effect that can be unmasked only upon the occurrence of a second hit, such as the HFD at adult age.

Hyperactivity of the HPA axis and increased circulating GC have been implicated in the pathogenesis of mood and anxiety disorders suggesting an impairment in the ability of the HPA axis to self-regulate its function by shutting down the system (Binder, 2009; Holsboer, 2000; Pariante and Miller, 2001). **Glucocorticoids have also an important function in metabolic regulations as they mobilize glucose to fuel the energy demands of the stress response and furthermore promote energy storage, feeding, and weight gain. Thus, it is possible to hypothesize that both psychological as well as metabolic stress might be able to affect HPA**

axis functionality (Balsevich et al., 2019). A growing body of evidence suggests that levels of *Fkbp5* mRNA GR co-chaperone have been associated with higher levels of circulating cortisol and reduced negative feedback inhibition of the stress response associated with a depressive phenotype (Binder, 2009). We have previously shown that, following PNS, male rats were characterised by elevated GC levels when compared to controls (Anacker et al., 2013); **in this study we show elevated levels of *Fkbp5* upon PNS in the prefrontal cortex and upon HFD administration in the dorsal hippocampus, suggesting that both psychological and metabolic stressors affect this mediator. When we looked at the hypothalamus, *Fkbp5* expression levels were reduced in the HFD group, possibly accounting for a compensatory mechanism. Interestingly, we also showed that levels of *Fkbp5* in the dorsal hippocampus were positively related to the time spent in the open arm of the EPM and that this positive correlation is lost as a result both of PNS as well as of HFD. This result might suggest that under physiological conditions *Fkbp5* is required to engage animals in the proper exploration of novel environments through a fine modulation of the GR receptors and that this balance can be greatly affected by both early life stressors as well as by metabolic challenges.**

The prefrontal cortex is one of the most important cortical area among the network of regions being involved in the pathogenesis of depression that plays a main role in the ability to process positive and negative emotions (Harms et al., 2017; Kaya and McCabe, 2019). Thus, the observed increase in levels of *Fkbp5* in this brain area should not be surprising and may underlie a condition of potential increased vulnerability to stress.

Changes in *Nr3c1* and *Fkbp5* in the hippocampus, a result of HFD, indicate a cross-regulation between a metabolic challenge and *Nr3c1/Fkbp5* balance, strengthening the notion that indeed these molecules are involved in "sensing" environmental challenges, be these of psychological or metabolic nature. We also found that HFD increased the levels of *Nr3c1* in the prefrontal cortex; by contrast, changes in *Fkbp5* in this brain area were observed only as a result of PNS. These results possibly suggest a fine and complex multiple level regulation of the GR-negative feedback in this specific brain region highlighting the role of this brain area in mood disorders such as depression. Transcriptional control of GR relies in part upon the DNA methylation status at multiple alternative initiation sites that are tissue specific in fact, *Nr3c1* gene is characterised by an unusually complex promoter structure (Turner et al., 2010). This might possibly explain the unexpected decrease in the dorsal hippocampus observed as a result of HFD or the lack of effects observed in the hypothalamus (see also concluding remarks).

When we investigated levels of *Ghs-R* we found a specific decrease upon HFD in the dorsal hippocampus while an increase was observed in both the ventral hippocampus and the prefrontal cortex in CTRL-HFD and PNS-CD animals. It has become increasingly apparent that the dorsal and the ventral portions of the hippocampus are preferentially involved in different physiological functions the first playing a role in the regulation of cognitive processes (spatial memory) the second in the modulation of emotions - such that they have been defined the cold and the hot hippocampus respectively - (Fanselow and Dong, 2010). Chen and co-workers provided evidence that infusion of ghrelin in the dorsal hippocampus enhanced synaptic plasticity and spatial memory (Chen et al., 2020). By contrast, Kanoski and colleagues showed that ghrelin delivery to the ventral but not the dorsal hippocampus increased the ability of environmental food-related cues to stimulate

meal initiation and to enhance motivation to obtain it by increasing food intake frequency (Kanoski et al., 2013) confirming that this hippocampal sub-region might be of importance in feeding/appetitive rewarding behaviours. To this regard it is interesting to note that we have previously found an increase in caloric intake upon HFD (Panetta et al., 2017). Recently Guo and colleagues have shown that GHS-R knock-out mice were characterised by improved abilities to cope to social stressors, decreased emotionality and depressive-like behaviours (Guo et al., 2019). Thus, we can hypothesize that both the pre-natal and the postnatal stressors might act by increasing the ghrelin signal in two brain regions of main importance for the ability to process emotions (the ventral hippocampus and the prefrontal cortex) setting the stage for increased vulnerability to mood disorders.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We have here provided evidence that PNS can affect brain development (first hit) setting the stage for increased vulnerability to further insults during life (HFD, second hit). In particular, we observed important changes in the expression levels of GR, their chaperons and *Ghs-R* in response to both PNS and HFD, confirming that the there is an important overlap between pathways and effectors involved in the regulation of emotions and in food intake and metabolic balance. **Further investigations are warranted to assess more in detail the role played by these molecules in setting the stage for co-morbidity between metabolic and psychiatric disorders. In addition, the role of additional co-regulators, such as endocannabinoids should be tested (Balsevich et al., 2019).**

There are some limitations of the current study that should be mentioned. First, we examined here the expression level of selected genes of interest but did not complement this with the levels of the corresponding proteins, the functional end products of the genes. Secondly, we have used real-time PCR in combination with biopsy punching as a quantitative approach instead of in situ hybridization. Due to this approach we had to make a selection of brain areas and lose anatomical resolution. **Thirdly, genomic and epigenomic regulation of glucocorticoids in the brain may affect mood and metabolism but is based upon the coordinated activity of many chaperone proteins, in addition to proper circadian and ultradian fluctuation of hormone release (Gray et al., 2017). Future studies should assess the interrelationship between psychological and metabolic stressors. In addition, given the observed changes of *Ghs-R* and *Nr3c1* in the dorsal hippocampus following a HFD, further research should be devoted to explore more in depth the effects of HFD and of PNS, and their interaction, on brain plasticity.**

493 **Conflict of Interest Statement**

494 The authors have nothing to disclose.

495

496 **Author's contribution**

497 **AB and CM analysed, interpreted data and wrote the manuscript; MM and AC collected all gene**
498 **expression data; FC and MR designed the experiment and provided data interpretation.**

499

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REFERENCES

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N., Wolfer, D.P., Limatola, C., Tremblay, M.È., Maggi, L., Branchi, I., 2016. Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain. Behav. Immun. 58, 261–271. https://doi.org/10.1016/j.bbi.2016.07.155

Alboni, S., Van DIjk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Mol. Psychiatry 22, 552–561. https://doi.org/10.1038/mp.2015.142

Anacker, C., Cattaneo, A., Luoni, A., Musaelyan, K., Zunszain, P.A., Milanesi, E., Rybka, J., Berry, A., Cirulli, F., Thuret, S., Price, J., Riva, M.A., Gennarelli, M., Pariante, C.M., 2013. Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. Neuropsychopharmacology 38, 872–883. https://doi.org/10.1038/npp.2012.253

Andersson, N.W., Li, Q., Mills, C.W., Ly, J., Nomura, Y., Chen, J., 2016. Influence of prenatal maternal stress on umbilical cord blood cytokine levels. Arch. Womens. Ment. Health 19, 761–767. https://doi.org/10.1007/s00737-016-0607-7

Balsevich, G., Abizaid, A., Chen, A., Karatsoreos, I.N., Schmidt, M. V., 2019. Stress and glucocorticoid modulation of feeding and metabolism. Neurobiol. Stress. https://doi.org/10.1016/j.ynstr.2019.100171

Barker, D.J.P., 1995. Intrauterine programming of adult disease. Mol. Med. Today. https://doi.org/10.1016/S1357-4310(95)90793-9

Barrientos, R.M., Sprunger, D.B., Campeau, S., Watkins, L.R., Rudy, J.W., Maier, S.F., 2004. BDNF mRNA expression in rat hippocampus following contextual learning is blocked by intrahippocampal IL-1β administration. J. Neuroimmunol. 155, 119–126. https://doi.org/10.1016/j.jneuroim.2004.06.009

Berry, A., Panetta, P., Luoni, A., Bellisario, V., Capoccia, S., Andrea Riva, M., Cirulli, F., 2015. Decreased Bdnf expression and reduced social behavior in periadolescent rats following prenatal stress. Dev. Psychobiol. 57, 365–373. https://doi.org/10.1002/dev.21297

Bilbo, S.D., Barrientos, R.M., Eads, A.S., Northcutt, A., Watkins, L.R., Rudy, J.W., Maier, S.F., 2008. Early-life infection leads to altered BDNF and IL-1β mRNA expression in rat hippocampus following learning in adulthood. Brain. Behav. Immun. 22, 451–455. https://doi.org/10.1016/j.bbi.2007.10.003

Binder, E.B., 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology. https://doi.org/10.1016/j.psyneuen.2009.05.021

Blum, I.D., Patterson, Z., Khazall, R., Lamont, E.W., Sleeman, M.W., Horvath, T.L., Abizaid, A., 2009. Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. Neuroscience 164, 351–359. https://doi.org/10.1016/j.neuroscience.2009.08.009

Boersma, G.J., Lee, R.S., Cordner, Z.A., Ewald, E.R., Purcell, R.H., Moghadam, A.A., Tamashiro, K.L., 2013. Prenatal stress decreases Bdnf expression and increases methylation of Bdnf exon IV in rats. Epigenetics 9, 437–447. https://doi.org/10.4161/epi.27558

Bourgognon, J.-M., Cavanagh, J., 2020. The role of cytokines in modulating learning and memory and brain plasticity. Brain Neurosci. Adv. 4, 239821282097980. https://doi.org/10.1177/2398212820979802

Bronson, S.L., Bale, T.L., 2014. Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment. Endocrinology 155, 2635–2646. https://doi.org/10.1210/en.2014-1040

Cattane, N., Räikkönen, K., Anniverno, R., Mencacci, C., Riva, M.A., Pariante, C.M., Cattaneo, A., 2020. Depression, obesity and their comorbidity during pregnancy: effects on the offspring's mental and physical health. Mol. Psychiatry. https://doi.org/10.1038/s41380-020-0813-6

Cattaneo, A., Macchi, F., Plazzotta, G., Veronica, B., Bocchio-Chiavetto, L., Riva, M.A., Pariante, C.M., 2015. Inflammation and neuronal plasticity: A link between childhood trauma and depression pathogenesis. Front. Cell. Neurosci. https://doi.org/10.3389/fncel.2015.00040

Chen, H.J., Antonson, A.M., Rajasekera, T.A., Patterson, J.M., Bailey, M.T., Gur, T.L., 2020. Prenatal stress causes intrauterine inflammation and serotonergic dysfunction, and long-term behavioral deficits through microbe- and CCL2-dependent mechanisms. Transl. Psychiatry 10, 1–12. https://doi.org/10.1038/s41398-020-00876-5

Cirulli, F., 2017. Interactions between early life stress and metabolic stress in programming of mental and metabolic health. Curr. Opin. Behav. Sci. https://doi.org/10.1016/j.cobeha.2016.12.009

Cirulli, F., Alleva, E., 2009. The NGF saga: From animal models of psychosocial stress to stress-related psychopathology. Front. Neuroendocrinol. https://doi.org/10.1016/j.yfrne.2009.05.002

Cirulli, F., Musillo, C., Berry, A., 2020. Maternal Obesity as a Risk Factor for Brain Development and Mental Health in the Offspring. Neuroscience. https://doi.org/10.1016/j.neuroscience.2020.01.023

Danese, A., Moffitt, T.E., Harrington, H.L., Milne, B.J., Polanczyk, G., Pariante, C.M., Poulton, R., Caspi, A., 2009. Adverse childhood experiences and adult risk factors for age-related disease: Depression, inflammation, and clustering of metabolic risk markers. Arch. Pediatr. Adolesc. Med. 163, 1135–1143. https://doi.org/10.1001/archpediatrics.2009.214

Danese, A., Moffitt, T.E., Pariante, C.M., Ambler, A., Poulton, R., Caspi, A., 2008. Elevated inflammation levels in depressed adults with a history of childhood maltreatment. Arch. Gen. Psychiatry 65, 409–416. https://doi.org/10.1001/archpsyc.65.4.409

Dantzer, R., Cohen, S., Russo, S.J., Dinan, T.G., 2018. Resilience and immunity. Brain. Behav. Immun. 74, 28–42. https://doi.org/10.1016/j.bbi.2018.08.010

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: When the immune system subjugates the brain. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn2297

Daskalakis, N.P., Bagot, R.C., Parker, K.J., Vinkers, C.H., de Kloet, E.R., 2013. The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. Psychoneuroendocrinology 38, 1858–1873. https://doi.org/10.1016/j.psyneuen.2013.06.008

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., Lanctôt, K.L., 2010. A Meta-Analysis of Cytokines in Major Depression. Biol. Psychiatry 67, 446–457. https://doi.org/10.1016/j.biopsych.2009.09.033

Fanselow, M.S., Dong, H.W., 2010. Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? Neuron. https://doi.org/10.1016/j.neuron.2009.11.031

Fernandes, C., File, S.E., 1996. The influence of open arm ledges and maze experience in the elevated plus-maze, in: Pharmacology Biochemistry and Behavior. Elsevier Inc., pp. 31–40. https://doi.org/10.1016/0091-3057(95)02171-X

Goshen, I., Kreisel, T., Ounallah-Saad, H., Renbaum, P., Zalzstein, Y., Ben-Hur, T., Levy-Lahad, E., Yirmiya, R., 2007. A dual role for interleukin-1 in hippocampal-dependent memory processes. Psychoneuroendocrinology 32, 1106–1115. https://doi.org/10.1016/j.psyneuen.2007.09.004

Gray, J.D., Kogan, J.F., Marrocco, J., McEwen, B.S., 2017. Genomic and epigenomic mechanisms of glucocorticoids in the brain. Nat. Rev. Endocrinol. https://doi.org/10.1038/nrendo.2017.97

600 Guillemot-Legris, O., Muccioli, G.G., 2017. Obesity-Induced Neuroinflammation: Beyond the
601 Hypothalamus. Trends Neurosci. https://doi.org/10.1016/j.tins.2017.02.005

602 Guo, L., Niu, M., Yang, J., Li, L., Liu, S., Sun, Y., Zhou, Z., Zhou, Y., 2019. GHS-R1a Deficiency
603 Alleviates Depression-Related Behaviors After Chronic Social Defeat Stress. Front. Neurosci. 13.
604 https://doi.org/10.3389/fnins.2019.00364

605 Gur, T.L., Shay, L., Palkar, A.V., Fisher, S., Varaljay, V.A., Dowd, S., Bailey, M.T., 2017. Prenatal stress
606 affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult
607 female offspring. Brain. Behav. Immun. 64, 50–58. https://doi.org/10.1016/j.bbi.2016.12.021

608 Handa, R.J., Burgess, L.H., Kerr, J.E., O'keefe, J.A., 1994. Gonadal steroid hormone receptors and sex
609 differences in the hypothalamo-pituitary-adrenal axis. Horm. Behav. 28, 464–476.
610 https://doi.org/10.1006/hbeh.1994.1044

611 Hantsoo, L., Kornfield, S., Anguera, M.C., Epperson, C.N., 2019. Inflammation: A Proposed Intermediary
612 Between Maternal Stress and Offspring Neuropsychiatric Risk. Biol. Psychiatry.
613 https://doi.org/10.1016/j.biopsych.2018.08.018

614 Harms, M.B., Birn, R., Provencal, N., Wiechmann, T., Binder, E.B., Giakas, S.W., Roeber, B.J., Pollak,
615 S.D., 2017. Early life stress, FK506 binding protein 5 gene (FKBP5) methylation, and inhibition-
616 related prefrontal function: A prospective longitudinal study. Dev. Psychopathol. 29, 1895–1903.
617 https://doi.org/10.1017/S095457941700147X

618 Health Organization Regional Office for Europe, W., 2015. The European Mental Health Action Plan 2013-
619 2020.

620 Holsboer, F., 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23,
621 477–501. https://doi.org/10.1016/S0893-133X(00)00159-7

622 Kanoski, S.E., Fortin, S.M., Ricks, K.M., Grill, H.J., 2013. Ghrelin signaling in the ventral hippocampus
623 stimulates learned and motivational aspects of feeding via PI3K-Akt signaling. Biol. Psychiatry 73,
624 915–923. https://doi.org/10.1016/j.biopsych.2012.07.002

625 Kaya, S., McCabe, C., 2019. What role does the prefrontal cortex play in the processing of negative and
626 positive stimuli in adolescent depression? Brain Sci. 9. https://doi.org/10.3390/brainsci9050104

627 Krontira, A.C., Cruceanu, C., Binder, E.B., 2020. Glucocorticoids as Mediators of Adverse Outcomes of
628 Prenatal Stress. Trends Neurosci. https://doi.org/10.1016/j.tins.2020.03.008

629 Liu, Y., Ho, R.C.M., Mak, A., 2012. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble
630 interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: A meta-
631 analysis and meta-regression. J. Affect. Disord. https://doi.org/10.1016/j.jad.2011.08.003

632 Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S.A., Anderson, J.G., Jung, S., Birnbaum, S.,
633 Yanagisawa, M., Elmquist, J.K., Nestler, E.J., Zigman, J.M., 2008. The orexigenic hormone ghrelin
634 defends against depressive symptoms of chronic stress. Nat. Neurosci. 11, 752–753.
635 https://doi.org/10.1038/nn.2139

636 Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses
637 the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. 15, 110–
638 116. https://doi.org/10.1523/jneurosci.15-01-00110.1995

639 Marosi, K., Mattson, M.P., 2014. BDNF mediates adaptive brain and body responses to energetic challenges.
640 Trends Endocrinol. Metab. https://doi.org/10.1016/j.tem.2013.10.006

641 McCarthy, M.M., 2019. Stress during pregnancy: Fetal males pay the price. Proc. Natl. Acad. Sci. U. S. A.
642 https://doi.org/10.1073/pnas.1916909116

643 McEwen, B.S., 2000. Effects of adverse experiences for brain structure and function, in: Biological
644 Psychiatry. Elsevier, pp. 721–731. https://doi.org/10.1016/S0006-3223(00)00964-1

645 Mighiu, P.I., Filippi, B.M., Lam, T.K.T., 2012. Linking inflammation to the brain-liver axis. Diabetes.
646 https://doi.org/10.2337/db12-0330

647 Milaneschi, Y., Simmons, W.K., van Rossum, E.F.C., Penninx, B.W., 2019. Depression and obesity:
648 evidence of shared biological mechanisms. Mol. Psychiatry. https://doi.org/10.1038/s41380-018-0017-
649 5

650 Mueller, B.R., Bale, T.L., 2008. Sex-specific programming of offspring emotionality after stress early in
651 pregnancy. J. Neurosci. 28, 9055–9065. https://doi.org/10.1523/JNEUROSCI.1424-08.2008

652 Nederhof, E., Schmidt, M. V., 2012. Mismatch or cumulative stress: Toward an integrated hypothesis of
653 programming effects. Physiol. Behav. https://doi.org/10.1016/j.physbeh.2011.12.008

654 O'Connor, T.M., O'Halloran, D.J., Shanahan, F., 2000. The stress response and the hypothalamic-pituitary-
655 adrenal axis: From molecule to melancholia. QJM - Mon. J. Assoc. Physicians.
656 https://doi.org/10.1093/qjmed/93.6.323

657 Panetta, P., Berry, A., Bellisario, V., Capoccia, S., Raggi, C., Luoni, A., Longo, L., Riva, M.A., Cirulli, F.,
658 2017. Long-term sex-dependent vulnerability to metabolic challenges in prenatally stressed rats. Front.
659 Behav. Neurosci. 11. https://doi.org/10.3389/fnbeh.2017.00113

660 Pariante, C.M., Miller, A.H., 2001. Glucocorticoid receptors in major depression: Relevance to
661 pathophysiology and treatment. Biol. Psychiatry. https://doi.org/10.1016/S0006-3223(00)01088-X

662 Patterson, Z.R., Ducharme, R., Anisman, H., Abizaid, A., 2010. Altered metabolic and neurochemical
663 responses to chronic unpredictable stressors in ghrelin receptor-deficient mice. Eur. J. Neurosci. 32,
664 632–639. https://doi.org/10.1111/j.1460-9568.2010.07310.x

665 Rasgon, N.L., McEwen, B.S., 2016. Insulin resistance - A missing link no more. Mol. Psychiatry.
666 https://doi.org/10.1038/mp.2016.162

667 Rizzo, F.R., Musella, A., De Vito, F., Fresegna, D., Bullitta, S., Vanni, V., Guadalupi, L., Stampanoni Bassi,
668 M., Buttari, F., Mandolesi, G., Centonze, D., Gentile, A., 2018. Tumor Necrosis Factor and Interleukin-
669 1β Modulate Synaptic Plasticity during Neuroinflammation. Neural Plast.
670 https://doi.org/10.1155/2018/8430123

671 Salim, S., Chugh, G., Asghar, M., 2012. Inflammation in anxiety, in: Advances in Protein Chemistry and
672 Structural Biology. Academic Press Inc., pp. 1–25. https://doi.org/10.1016/B978-0-12-398314-
673 5.00001-5

674 Slopen, N., Koenen, K.C., Kubzansky, L.D., 2014. Cumulative adversity in childhood and emergent risk
675 factors for long-term health. J. Pediatr. 164, 631-638.e2. https://doi.org/10.1016/j.jpeds.2013.11.003

676 Ślusarczyk, J., Trojan, E., Głombik, K., Budziszewska, B., Kubera, M., Lasoń, W., Popiołek-Barczyk, K.,
677 Mika, J., Wędzony, K., Basta-Kaim, A., 2015. Prenatal stress is a vulnerability factor for altered
678 morphology and biological activity of microglia cells. Front. Cell. Neurosci. 9.
679 https://doi.org/10.3389/fncel.2015.00082

680 Turner, J.D., Alt, S.R., Cao, L., Vernocchi, S., Trifonova, S., Battello, N., Muller, C.P., 2010. Transcriptional
681 control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem. Pharmacol. 80,
682 1860–8. https://doi.org/10.1016/j.bcp.2010.06.037

683 Valkanova, V., Ebmeier, K.P., Allan, C.L., 2013. CRP, IL-6 and depression: A systematic review and meta-
684 analysis of longitudinal studies. J. Affect. Disord. https://doi.org/10.1016/j.jad.2013.06.004

685 Verhagen, L.A.W., Egecioglu, E., Luijendijk, M.C.M., Hillebrand, J.J.G., Adan, R.A.H., Dickson, S.L.,

2011. Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity. Eur. Neuropsychopharmacol. 21, 384–392. https://doi.org/10.1016/j.euroneuro.2010.06.005

Weinstock, M., 2007. Gender differences in the effects of prenatal stress on brain development and behaviour. Neurochem. Res. https://doi.org/10.1007/s11064-007-9339-4

Zarouna, S., 2015. Mood disorders: A potential link between ghrelin and leptin on human body? World J. Exp. Med. 5, 103. https://doi.org/10.5493/wjem.v5.i2.103

FIGURE LEGENDS

GRAPHICAL ABSTRACT. Based on the "two hit model" of the developmental origin of diseases, PNS (first hit) affects foetal brain developmental trajectories; signalling pathways related to brain plasticity and function (*Bdnf*, *Tnf-α and Il-β*) are down-set potentially providing increased liability to later-life mood and metabolic disorders. A later-life challenge such as HFD feeding (second hit), experienced at adulthood, might impinge upon the same signalling pathways primed by PNS leading to enhanced responses to the metabolic challenge. The hippocampus and prefrontal cortex, being main targets of glucocorticoids, are finely modulated by PNS and readily respond to the HFD metabolic stress.

FIGURE 1. Overall subjects who experienced prenatal stress (PNS-CD) were characterised by decreased m-RNA levels of both *Il-1β* (panel A) and *Tnf-α* (panel B) in almost all the brain regions investigated; *Il-6* showed a decrease in its expression levels upon HFD in the dorsal hippocampus (panel C). *Il-1β* showed statistical significance only in the prefrontal cortex while *Tnf-α* was tightly modulated upon pre- and postnatal stressors. Data are presented graphically as means ± SEM box plot (observations outside the ranges are represented with dots outside the boxes). Post hoc comparisons: **$$p<0.01, main effect of diet (dorsal hippocampus, *Il-6*); post hoc comparisons: *p<0.05, interaction effect (dorsal hippocampus, *Tnf-α*: PNS-CD vs. CTRL-CD); **p<0.01, interaction effect (ventral hippocampus, *Tnf-α*: PNS-CD vs. PNS-HFD;** Prefrontal cortex, ***Tnf-α* and *Il1-β*: PNS-CD vs. PNS-HFD).** Number of subjects:8-10 within each experimental group.

FIGURE 2. Male rats exposed to prenatal stress (PNS) were characterised by an inverse relation between levels of *Il-1β* in the dorsal hippocampus and immobility duration as assessed in the elevated plus maze test (EPM). *p<0.05**.** Number of subjects: 6-10 within each experimental group.

FIGURE 3. Prenatal stress decreased *Bdnf* m-RNA levels specifically in the dorsal hippocampus suggesting a reduced neuronal plasticity. This effect is reversed by HFD administration at adulthood. Data are presented graphically as means ± SEM box plot (observations outside the ranges are represented with dots outside the boxes). Post hoc comparisons *p<0.05, **interaction effect in the dorsal hippocampus, PNS-CD vs. CTR-CD and PNS-HFD**. Number of subjects is between 9-11 within each experimental group.

FIGURE 4. Male rats exposed to prenatal stress (PNS) were characterised by an inverse relation between levels of *Bdnf* in the dorsal hippocampus and immobility duration as assessed in the elevated plus maze test (EPM). Post hoc comparisons *p<0.05. Number of subjects: 9-10 within each experimental group.

FIGURE 5. Independently from the prenatal condition, post-natal HFD administration increases levels of *Nr3c1* in the ventral hippocampus and prefrontal cortex by contrast, in the dorsal hippocampus a significant interaction between PNS and HFD results in decreased levels of this gene both as a result of pre- and post-natal stressors. No change in *Nr3c1* was observed in the hypothalamus as a result of PNS nor of HFD (see panel A). As for *Fkbp5*, HFD independently from the prenatal condition, increased its expression levels in the dorsal hippocampus while a decrease was observed in the hypothalamus; no change was observed in the ventral hippocampus. PNS overall increased levels of Fkbp5 specifically in the prefrontal cortex. $$p<0.01, main effect of diet (*Nr3c1*: ventral hippocampus and prefrontal cortex; Fkbp5: dorsal hippocampus and hypothalamus; *Fkbp5*: dorsal hippocampus; hypothalamus); £p<0.05, main effect of PNS (*Fkbp5*: prefrontal cortex); post hoc comparisons: **p<0.01, interaction effect (*Nr3c1*: CTRL-CD vs. CTRL-HFD, PNS-CD and PNS-HFD); Number of subjects:8-10 within each experimental group.

FIGURE 6. Control subjects are characterized by a direct relationship between expression levels of *Fkbp5* in the dorsal hippocampus and the time spent in the open arms of the Elevated Plus Maze, overall suggesting a permissive role for explorative behavior of this GC-related genes under physiological conditions. Post hoc comparisons **p<0.01. Number of subjects: 6-9 within each experimental group.

FIGURE 7. Levels of *Ghs-R* were found to be specifically **decreased** upon HFD in the dorsal hippocampus while an increase was observed in both the ventral hippocampus and the prefrontal cortex in CTRL-HFD and PNS-CD animals. Main effect of HFD: **main effect of diet**, $$ p<0.01; post hoc comparisons **for the interaction effects**: ***p<0.05: prefrontal cortex, CTRL-CD vs. CTRL-HFD; **p<0.01: ventral hippocampus, CTRL-CD vs. CTRL-HFD and PNS-CD, prefrontal cortex, CTRL-CD vs. PNS-CD**. Number of subjects: 8-10 within each experimental group.

High-fat diet at adulthood interacts with prenatal stress, affecting both brain inflammatory and neuroendocrine markers in male rats

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Abstract

Prenatal stress (PNS) affects foetal programming and, through an interaction with subsequent challenges, can increase vulnerability to mood and metabolic disorders. We have previously shown that, following PNS, adult male rats are characterised by increased vulnerability to a metabolic stressor experienced at adulthood (8-week-high-fat diet - HFD). In this study, we specifically assessed whether PNS might interact with an adult metabolic challenge to induce an inflammatory phenotype. Changes in the expression levels of inflammatory (*Il-1β, Tnf-α*, *Il-6*) and of stress response mediators (*Nr3c1*, *Fkbp5*) as well as of mood and metabolic regulators (*Bdnf, Ghs-R*) were investigated in the hippocampus, prefrontal cortex and hypothalamus, brain regions involved in the pathogenesis of depression and prone to inflammation in response to stress. Overall, PNS reduced the expression of *Bdnf* and *Tnf-α,* while HFD administered at adulthood counteracted this effect suggesting that PNS impinges upon the same pathways regulating responses to a metabolic challenge at adulthood. Furthermore, HFD and PNS affected the expression of both *Nr3c1* and *Fkbp5*, two neuroendocrine mediators involved in the response to stress to metabolic challenges and in the modulation of the emotional profile (as shown by the correlation between *Fkbp5* and the time spent in the open arms of the elevated plus-maze). Overall, these results indicate that the same metabolic and neuroendocrine effectors engaged by PNS are affected by metabolic challenges at adulthood, providing some mechanistic insight into the well-known comorbidity between mood and metabolic disorders.

***Keywords*:** Animal model, Prenatal stress, High-fat diet, Neuroinflammation, Mood disorders, Co-morbidity.

LIST OF ABBREVIATIONS

BDNF= Brain-Derived Neurotrophic Factor

CD= Control Diet

CRP= C-Reactive Protein

CTRL= control

EPM= Elevated Plus-Maze

EU= European

GC= Glucocorticoids

GD= Gestational Day

GHS-R= Ghrelin Receptor

GR= Glucocorticoid Receptor

HFD= High-Fat Diet

HPA= Hypothalamic–Pituitary–Adrenal

IL= interleukin

LTP= Long-Term Potentiation

NF-kB= Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

PND= Post-Natal Day

PNS= Prenatal Stress

ROS= Reactive Oxygen Species

SEM= Standard Error Mean

1. INTRODUCTION

Mood disorders are common conditions with major public health implications. It is estimated that each year around 40% of the EU population suffers from a mental disorder. Adjusted for age and comorbidity, this corresponds to 164.8 million people affected (Health Organization Regional Office for Europe, 2015). Among the risk factors that might trigger the onset of psychiatric conditions, or an exacerbation of symptoms, stressful life events play a pivotal role. In fact, according to a "two-hit model" of vulnerability to diseases, stress experienced during early life phases, might affect brain development leading to a reduced ability to cope with further stressors during life (Daskalakis et al., 2013). To this regard, a suboptimal intrauterine environment, prompted by maternal stress (ranging from poor socio-economic status and maternal obesity to depression or maltreatments) may predispose the offspring to lifelong negative health outcomes, including neuropsychiatric disorders as well as metabolic and immune dysregulations (Barker, 1995; Berry et al., 2015; Boersma et al., 2013; Cattane et al., 2020; Cirulli et al., 2020; Krontira et al., 2020).

Consistent evidence suggests that depressed patients are characterised by alterations in the functional activity of the immune system, affecting both peripheral and central tissues and that early life stress represents an important factor influencing the inflammatory status (Cattaneo et al., 2015; Dantzer et al., 2008; Dowlati et al., 2010; Liu et al., 2012; Valkanova et al., 2013). As an example, Danese and colleagues reported increased blood C-reactive protein (CRP) levels in maltreated children, an effect that was larger in those subjects that also developed depression later in life (Danese et al., 2009, 2008). Likewise, Slopen and co-workers reported increased CRP and interleukin-6 (IL-6) in adolescents who experienced early life adversities (Slopen et al., 2014). Offspring of women who experienced stressful life events during pregnancy show higher levels of IL-1β, IL-4, IL-5, IL-6, and IL-8 in umbilical cord blood at delivery (Andersson et al., 2016). Interestingly, it has been recently proposed that inflammation may act as a key mediator linking exposure to prenatal stress (PNS) and enhanced vulnerability to psychopathology in the offspring (Hantsoo et al., 2019).

Preclinical studies in rodent models provide strong support to the above mentioned clinical findings, showing for example that PNS exposure causes extended inflammation in the foetal brain (Ślusarczyk et al., 2015). To this regard, Gur and colleagues found increased levels of IL-1β in the placenta of mouse dams undergoing stress during pregnancy and in the foetal brain of their offspring; this was also associated with decreased levels of the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) specifically in the amygdala (Chen et al., 2020; Gur et al., 2017) suggesting that increased expression of placental immune responsive genes upon maternal stress might be considered as a potential mechanism (Bronson and Bale, 2014; Mueller and Bale, 2008).

Despite the large body of clinical and preclinical evidence showing associations among PNS, the hyperactivation of the immune system and an enhanced vulnerability to mood disorders, cause-effect mechanisms are still largely unknown, and the prenatal environment may not act alone to set the stage for long-term onset of mood disorders. Interestingly, psychiatric conditions and metabolic pathologies are often found

to co-occur within the same individual showing a feed-forward pattern overall suggesting the activation of shared mechanisms/pathways (Cattane et al., 2020; Cirulli et al., 2020; Milaneschi et al., 2019). Among these, BDNF and glucocorticoids (GC) hormones have been identified as main effectors of brain plasticity and metabolic function in response to stressful events. Thus, it is possible that these actors may represent both effectors as well as targets of stress - during sensitive developmental periods - leading, in the developing organism, to a remodelling of the mechanisms associated with stress responsiveness (Cattaneo et al., 2015; Cirulli and Alleva, 2009; McEwen, 2000). The neurotrophin BDNF plays a pivotal role in brain and behavioural plasticity as well as in the control of energy homeostasis (Cirulli and Alleva, 2009; Marosi and Mattson, 2014); its expression is finely tuned by GC in response to acute or chronic stressful stimuli (Cirulli, 2017;Cirulli & Alleva, 2009) and it has been also suggested to be negatively regulated by pro-inflammatory cytokines (Barrientos et al., 2004; Bilbo et al., 2008). Thus, BDNF appears as a sensitive target as well as a biomarker of stress response as it could actively contribute to maintain brain health/homeostasis in different challenging conditions. To this regard, the interplay between the activation of stress-related pathways by adverse experiences during development and adult metabolic challenges might be crucial in shaping individual vulnerability to stress (Nederhof and Schmidt, 2012).

A growing body of evidence suggests that the effects of PNS are sex-specific and that males' foetal brain might be more vulnerable to changes in the inflammatory mediators overall showing learning deficits and decreased brain plasticity particularly with regard to the hippocampus prefrontal cortex (McCarthy, 2019; Weinstock, 2007). Indeed, in a previous study we have shown that male rats who underwent PNS were more vulnerable than females to the effects of a metabolic challenge experienced at adulthood (high-fat diet feeding - HFD), while females showed an overall greater plasticity, possibly mediated by increased total *Bdnf* mRNA expression levels both in the hippocampus and in the hypothalamus. Based on the "two hit model" of diseases, we hypothesized that PNS might disrupt the intrauterine environment affecting brain developmental trajectories leading, in turn, to increased brain inflammation and reduced plasticity in the offspring (first hit). We also hypothesized that a metabolic challenge such as a HFD, experienced at adulthood, might interact with inflammatory pathways to exacerbate the PNS-induced pro-inflammatory phenotype. Thus, the aim of this study was to investigate the effect of a second hit - represented by an HFD - specifically in male rats. We focused on selected brain regions - characterised by high degree of sexual dimorphism (Handa et al., 1994) - such as the dorsal and the ventral hippocampus, the prefrontal cortex and the hypothalamus known to be involved in the pathogenesis of depression and to be prone to inflammatory status in response to external challenges. We specifically assessed in these brain regions levels of *Bdnf*, *Tnf-α*, Il-6 and *Il-β*.

Stress powerfully affects both mood and energy homeostasis. Such effects are achieved not only through the interaction between GC hormones and their receptors (that regulate HPA axis function in addition to glucose metabolism) but also by triggering a multitude of signalling cascades reciprocally modulating one another in a regional-, temporal, and functional-dependent manner (Balsevich et al., 2019). Among these, ghrelin is a peptide hormone produced in the stomach and involved in the signalling of meal initiation. Its action is mediated through the growth hormone secretagogue receptor (Ghs-R) that is found to be highly expressed in

several brain regions including the hypothalamus, pituitary gland and hippocampus (see Zarouna, 2015 and references therein). Both Ghrelin and Ghs-R are modulated by stress (Patterson et al., 2010); there is evidence that ghrelin may decrease anxiety-like and depressive-like behaviours in mice (Lutter et al., 2008) moreover, it appears to be involved in in food anticipatory activity and consumption (Blum et al., 2009; Verhagen et al., 2011) as well as in mood disorders (Zarouna, 2015). Interestingly, both genetic variants within ghrelin and GC signalling pathways have been associated with obesity, stress-related mental disorders, or both (see Balsevich et al., 2019 and references therein). To this regard, we also investigated changes in the expression levels of *Ghs-R* as well as of *Nr3c1* (encoding for the glucocorticoid receptors - GR) and the GC co-chaperon *Fkbp5* (encoding for the FK506 binding protein 51) in all the above-mentioned brain regions.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

Twelve adult nulliparous female (230–260 g) and 6 male Sprague-Dawley rats (400 g) were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, animals were pair-housed with same sex conspecifics under standard laboratory conditions (see Panetta et al. 2015 for further details). Pellet food (Altromin-R, Rieper, Italy) and tap water were continuously available. Following one week of adaptation, two females and one male were mated for 24 hours. To assess pregnancy, changes in body weight were monitored. Pregnant females were randomly assigned to either the control (CTRL) or prenatal stress (PNS) groups. CTRL rats were left undisturbed throughout gestation, while PNS females underwent a repeated restraint stress procedure during the last third of gestation until the day delivery day (postnatal day 0 - PND-0). On PND-1 all pups were weighted and litters culled to an average of 5 male and 5 female pups. On PND-21 pups were weaned and housed in groups of 2 or 3 same-sex littermates until 2 months of age. By this time, n=20 CTRL and n=21 PNS males were assigned either to high-fat diet (HFD) or control diet (CD) regimen for 8 weeks. The final number of animals per group was: n=10 CTRL-CD, n=10 CTRL -HFD, n=10 PNS-CD and n=11 PNS-HFD. Groups were composed by 1 pup/litter (CTRL-CD; CTRL-HFD; PNS-CD; PNS-HFD. After 4 weeks on the respective diets, all animals underwent a number of metabolic and behavioural assessments; they were successively sacrificed and brains dissected out to investigate the expression levels of *Il-6*, *Il-1β*, *Tnf-α*, *Nr3c1*, *Fkbp5*, *Ghs-R*, in the dorsal hippocampus, ventral hippocampus, hypothalamus and prefrontal cortex; total *Bdnf* was investigated only in the dorsal hippocampus and prefrontal cortex (since it was already assessed in the other two areas, (Panetta et al., 2017).

To better characterise the effects of possible changes in gene expression levels, regression analyses were carried out by taking into account specific outcomes deriving from the Elevated Plus-Maze (EPM), as previously performed in (Panetta et al., 2017). We focused on immobility time since this is a well-known proxy for emotionality and reactivity to novel environment (Fernandes and File, 1996) and correlated it with *Bdnf* and *Il-1β* since both these mediators are involved in behavioural plasticity (Bourgognon and Cavanagh, 2020;

Cirulli and Alleva, 2009; Goshen et al., 2007). Moreover, we also assessed the ability of *Fkbp5* to affect emotionality in the EPM by correlating expression levels of this gene in the dorsal hippocampus to the time spent in the open arms of the maze. As for the EPM protocol used, briefly, the apparatus was made of Plexiglas and consisted of two opposite open arms and two arms closed by transparent walls (50×10×40 cm). Each rat was placed in the center of the maze and video-recorded for 5 minutes under dim light conditions. Each session was recorded and behavioral analysis was carried out using a commercial software (''The Observer 3.0'', Noldus, The Netherlands) (see (Panetta et al., 2017) for further details).

All experimental procedures were reviewed by the ethical body of the Istituto Superiore di Sanità for animal welfare and conducted in conformity with the European Directive 2010/63/EU and the Italian legislation on animal experimentation, D.Lgs. 26/2014. They were authorized by the Italian Ministry of Health.

2.2 Dams' stress procedure

Pregnant females (at gestational day - GD - 14) were restrained in a transparent Plexiglas cylinder (7.5×19 cm) under a bright light (6.500 lux) for 45 minutes three times daily at random times (between 9:00 a.m.-5:00 p.m.) during the dark phase until the expected delivery day (GD-21, see Maccari et al., 1995 for further details). This stress has been selected since it is one of the most well characterised prenatal stressors with well-known effects on developmental trajectories of the offspring.

2.3 High-fat diet administration

CTRL and PNS offspring were fed *ad libitum* either with HFD (energy: 5.24 kcal/g; composition: fat 60%, carbohydrate 20% and protein 20%) or CD (energy: 3.3 kcal/g; composition: fat 17%, carbohydrate 60% and protein 23%) starting from two months of age. The HFD diet (D12492) was purchased from Research Diets, Inc., New Brunswick, NJ, USA; the CD was purchased from Altromin-R, Rieper, Italy.

2.4 Tissue collection

At 4 months of age offspring was weighted and sacrificed. Brains were removed and the dorsal and ventral hippocampus, hypothalamus and prefrontal cortex were dissected out and immediately frozen at -80°C (Panetta et al., 2017).

205 **2.7 Molecular analysis**

206 **2.7.1 RNA isolation and Real Time PCR Analyses**

207 Total RNA was isolated using PureZol RNA isolation reagent (Bio–Rad Laboratories, Italy), treated with
208 DNase to avoid DNA contamination and quantified by spectrophotometric analysis. The quantified RNA was
209 analyzed by TaqMan q-RT PCR Instrument (CFX384 real time system, Bio-Rad Laboratories) using the
210 iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories) and Applied BioSystem Assays (Gene
211 Expression Assays: *Il6, Il-β, Tnf-α, Bdnf, Nr3c1, Fkbp5,* and *Ghs-R*). Samples were run in triplicate and each
212 target gene analyzed has been normalized to the expression of the housekeeping (HK) gene β-actin (ActB).
213 The expression of target genes was calculated by using to the Ct method (-ΔΔCt method) (Schmittgen and
214 Livak, 2008), where CTRL-CD rats have been used as a reference group.

215

216 **2.8 Statistical analysis**

217 Data were evaluated by a two-way ANOVA with Diet (HFD vs CD) and Prenatal condition (PNS vs CTRL)
218 as between-subject factors. *Post-hoc* comparisons between groups were performed using the Tukey's test. A
219 linear regression model was used to assess the ability of *Bdnf* levels and of *Il-1β* in the dorsal hippocampus to
220 affect immobility duration (a parameter indicative of increased emotionality) in the EPM test. A level of
221 probability set at $p<0.05$ was used as statistically significant. Statistical tendency was set at $p<0.1$. For
222 interactions at $p<0.1$, we also examined lower order effects. Data are presented graphically as means ± SEM
223 box plot (observations outside the ranges are represented with dots outside the boxes). The raw data supporting
224 the conclusions of this article will be made available by the authors upon request without undue reservation.
225

226 **3. RESULTS**

227

228 **3.1 Inflammatory mediators *Il-1β*, *Tnf-α* and *Il-6* mRNA expression**

229 *Il-1β* - We first analyzed the expression of *Il-1β*, a prototype pro-inflammatory cytokine. We observed that
230 PNS resulted in a nearly significant decrease in the dorsal hippocampus while neither a main effect of HFD
231 nor a PNS x HFD interaction effect were found (F (1,35)=3.818, 0.004, 2.184; p= 0.0587, 0.9518, 0.1484).
232 No main effects of PNS nor of HFD were observed in the ventral hippocampus (F(1,34)=0.152, 0.335;
233 p=0.6995, 0.5665). A strong PNS x HFD interaction was found (F(1,34)=4.655; p=0.0381) PNS exposed
234 animals being characterised by decreased levels of this cytokine, while the administration of postnatal HFD
235 counteracted the effects of the PNS (post-hoc comparisons just missed statistical significance).
236 In the prefrontal cortex while no main effects of PNS was observed (F(1,33)=0.969; p=0.3321) postnatal HFD
237 overall increased levels of *Il-1β* (main effect of HFD: F(1,33)=5.112, p=0.0305). Similarly to what was
238 observed in the ventral hippocampus, a nearly significant PNS x HFD interaction was found with decreased

levels of *Il-1β* in PNS animals, this effect being reverted by postnatal administration of HFD (F(1,33)=3.858; p=0.0580, post hoc comparisons: p<0.01, PNS vs. PNS-HFD); (see Figure 1A).

In the hypothalamus, levels of *Il-1β* were not affected by PNS, HFD nor an interaction was found (F(1,36)=0.042, 0.0001633, 0.070; p=0.8386, 0.9899, 0.7934 respectively for PNS, HFD and PNS x HFD, see Figure 1A).

Tnf-α - In the dorsal hippocampus no main effects of PNS nor of HFD were observed (F(1,37)=1.288, 0.209; p=0.2638, 0.6506). By contrast, a significant interaction was found between PNS and HFD (F(1,37)=5.772; p=0.0214). In particular, PNS animals were characterised by decreased levels of *Tnf-α,* although this effect was no longer observed in PNS-HFD animals that showed levels of this cytokine comparable to those observed in CTRL animals (post hoc comparisons: p<0.05, PNS vs. CTRL).

No main effects of PNS nor of HFD were found in the ventral hippocampus (F(1,34)=0.033, 2.941; p=0.8573, 0.0955, respectively for PNS and HFD), while a PNS x HFD interaction was found showing that administration of postnatal HFD in the PNS group selectively increased levels of this cytokine ((F(1,34)=6.747; p=0.0138); post hoc comparisons: PNS vs. HFD-PNS, p<0.01).

No main effects of PNS nor of HFD were observed in the prefrontal cortex (F(1,33)= 0.079, 1.648; p=0.7809, 0.2082 respectively for PNS and HFD) however, as also observed for the ventral hippocampus a PNS x HFD interaction effect was found showing that the administration of postnatal HFD in the PNS group selectively increased levels of this cytokine (F(1,33)=7.946; p=0.0081); post hoc comparisons: PNS vs. HFD-PNS, p<0.01); (see Figure 1B).

Levels of *Tnf-α* in the hypothalamus (see Figure 1B) were not affected neither by PNS nor by postnatal HFD (F(1, 35)=0.286, 0.702, 2.111; p= 0.5964; 0.4079; 0.1551 respectively for PNS, HFD and PNS x HFD).

Il-6 - Postnatal HFD decreased levels of *Il-6* in the dorsal hippocampus (main effect of HFD: F(1,37=0.814; p=0.0066), whereas no effect of the PNS nor of the PNS x HFD interaction effect were found (PNS main effect: F(1,37)=1.387; p=0.2465; PNS x HFD interaction: F(1,37)=0.164; p=0.6881).

Levels of *Il-6* did not change as a result of PNS, HFD or their interaction neither in the ventral hippocampus (F(1,36)=0.759, 2.631, 0.029; p=0.3893, 0.1135, 0.8647 respectively for PNS, HFD and PNS x HFD) nor in the prefrontal cortex (F(1,35)=0.144, 0.053, 0.202 respectively for PNS, HFD and PNS x HFD); (see Figure 1C). PNS decreased nearly significantly the levels of *Il-6* also in the hypothalamus (F(1,36)=3.889; p=0.0563), but no effect of HFD nor an interaction PNS x HFD were found to be significant (F(1,36)=0.903, 0.757; p=0.3482, 0.3899, respectively for HFD and PNS x HFD).

----------FIGURE 1 about here----------

Interestingly, when expression levels of *Il-1β* in the dorsal hippocampus were related to immobility duration in the EPM (a test run in a companion paper on the same experimental animals, see (Panetta et al., 2017)), we

observed a significant strong negative association linking these two parameters only in the PNS-CD animals, overall suggesting that reduced levels of this cytokine can be related with reduced behavioral plasticity (see discussion; CTRL-CD: $F(1,8)=0.007$; $p=0.9367$; $R^2=0.01$; CTRL-HFD: $F(1,8)=3.461$; $p=0.1051$; $R^2=33.1$; PNS-CD: $F81,8)= 7.538$; $p=0.0287$; $R^2=51.8$; PNS-HFD: $F(1,9)= 1.739$; $p=0.2237$, $R^2=17.9$; see Figure 2).

----------FIGURE 2 about here----------

3.2 Neuronal plasticity marker - *Bdnf* mRNA expression

We first investigated the expression of *Bdnf*, a neurotrophin that is considered a prototype marker of plasticity. Within the dorsal hippocampus, overall PNS and postnatal HFD did not affect levels of *Bdnf* ($F(1, 36)= 3.678$; 1.964;$p= 0.0631$; 0.1696), main effect of PNS and HFD respectively), although we observed a significant interaction between PNS and postnatal HFD ($F(1, 36)=5.606$; $p=0.0234$). More in detail, PNS decreased *Bdnf* expression, while the postnatal administration of HFD was able to counteract this effect (PNS vs. CTRL, $p<0.05$; PNS vs. PNS-HFD, $p<0.05$). In the prefrontal cortex no effect of PNS, HFD or their interaction was observed ($F(1, 36)=0.200$, 1.377, 0.174; $p=0.6572$; 0.2482; 0.6794, respectively for PNS, HFD and PNS x HFD); (see Figure 3).

----------FIGURE 3 about here----------

Interestingly, levels of *Bdnf* in the dorsal hippocampus were negatively associated with the time spent immobile in the EPM (a test run in a companion paper on same experimental animals, see (Panetta et al., 2017)) only in the PNS-CD group (CTRL-CD: $F(1,9)=0.730$; $p=0.4261$; $R^2= 0.081$; CTRL-HFD: $F(1,9)= 0.090$; $p=0.7713$; $R^2=0.011$; PNS-CD: $F(1,8)=11.084$; $p=0.0127$; $R^2=0.612$; PNS-HFD: $F(1,9)=0.716$; $p=0.4221$; $R^2= 0.082$), supporting a role of this neurotrophin in the emotional phenotype (see Figure 4).

----------FIGURE 4 about here----------

3.3 Stress response mediators - *Nr3c1*, *Fkbp5* mRNA expression

Nr3c1 - In the dorsal hippocampus the postnatal HFD decreased the levels of *Nr3c1* when compared to controls and a similar nearly significant effect was observed for PNS (main effect: $F(1,36)=3.827$, 7.063, $p=0.0582$; 0.0117 respectively for PNS, HFD). Moreover, a significant PNS x HFD interaction was found showing that both PNS-CD and CTRL-HFD as well as the combination PNS-HFD were characterised by a decrease of GR mRNA levels ($F(1,36)=6.228$; $p=0.0173$ and PNS x HFD; post hoc comparisons $p<0.05$).

In the ventral hippocampus levels of *Nr3c1* were increased a result of postnatal HFD (main effect: $F(1,36)=10.405$; $p=0.0027$), while no effects of PNS nor of the PNS x HFD interaction were found ($F(1, 36)=0.011$, 0.258; $p= 0.9167$; 6146). In the prefrontal cortex the postnatal HFD overall increased the levels of *Nr3c1* (main effect of HFD: $F(1, 37)=12.453$; $p=0.0011$); no effects of PNS nor of the PNS x HFD interaction

were observed ((F(1, 37)=1.001, 0.702; p=0.3236; 0.4076). No effects of PNS, HFD nor of their interaction were found in the hypothalamus (F(1, 37)=0.157, 0.009, 1.067; p= 0.6943; 0.9257; 0.3084); (see Figure 5).

Fkbp5 - In the dorsal hippocampus the postnatal HFD increased the levels of *Fkbp5* (F(1,35)=10.331;p=0.0028), while no effect of PNS nor of the PNS x HFD interaction was observed (F(1,35)=1.502, 0.958; p= 0.2285; 0.3343). No effect of PNS, HFD or their interaction was found in the ventral hippocampus (F(1,36)=0.781, 3.222, 0.007; p=0.3828; 0.0811; 0.9359). In the prefrontal cortex, PNS increased the levels of *Fkbp5* (F(1,37)=5.244; p=0.0278), while no effect of HFD nor of the PNS x HFD interaction was observed (F(1,37)=1.219, 2.019; p=0.2767; 0.1637). A main effect of the postnatal HFD was also observed In the hypothalamus, the Postnatal HFD decreased levels of *Fkbp5* (F(1,37)=9.156; p=0.0045); no effect of PNS nor of the PNS x HFD interaction was observed (F(1,37)=0.094, 3.353; p= 0.7608; 0.0751); (see Figure 5).

----------**FIGURES 5 about here**----------

Interestingly, levels of *Fkbp5* in the dorsal hippocampus were positively related with increased time spent in the open arm of the EPM (a test run in a companion paper on same experimental animals, see (Panetta et al., 2017)) only in the CTRL-CD group (CTRL-CD: F(1,8)=26.617; p=0.0013; R^2= 0.792; CTRL-HFD: F(1,9)= 0.104; p=0.7713; R^2=0.013; PNS-CD: F(1,8)=0.253; p=0.6307; R^2=0.035; PNS-HFD: F(1,9)=0.503; p=0.6307; R^2= 0.4982). This result might suggest that *Fkbp5* is physiologically required to engage animals in the proper exploration of novel environments through a fine modulation of GR receptors (see Figure 6).

----------**FIGURES 6 about here**----------

3.4 Metabolic/mood regulator marker - *Ghs-R* mRNA expression

Ghs-R - In the dorsal hippocampus the postnatal HFD overall decreased levels of *Ghs-R* (F(1,33)=1,948; p=0.0007). By contrast, PNS increased *Ghs-R* levels (F(1,33)=5.192; p=0.0293), but no interaction between PNS x HFD was observed (F(1,33)=0.290; p= 0.5941). In the ventral hippocampus HFD overall increased the levels of *Ghs-R* (F(1,32)=6.278; p=0.0175), while no main effect of PNS was observed (F(1,32)=0.593; p=0.4468). However, *Ghs-R* was increased in the CTRL-HFD and in the PNS-CD groups (interaction between PNS and HFD: F(1,32)=14.778; p=0.0005, post hoc: CTRL vs. CTRL-HFD, p<0.01; CTRL vs. PNS, p<0.01). In the prefrontal cortex, PNS increased the levels of *Ghs-R* (F(1,29)=5.865; p=0.0219) and no effect of HFD was observed (F(1,29)=2.191; p=9.1496). However, in this brain area, *Ghs-R* was increased in the CTRL-HFD and in the PNS-CD groups (interaction between PNS and HFD: F(1,29)=6.870; p= 0.0138, post hoc: CTRL vs. CTRL-HFD, p<0.05; CTRL vs. PNS, p<0.01); (see Figure 7). As for the hypothalamus, neither PNS nor HFD affected levels of *Ghs-R* (F(1,34)=3.155, 3.033, 0.907; p=0.0846; 0.0906; 0.3477).

----------FIGURE **7** about here----------

4. DISCUSSION

In the present study we assessed the long-term effects of exposure to PNS (a first hit) on foetal brain programming with regard to neuronal plasticity and brain inflammation. Moreover, we used a metabolic challenge (HFD) at adulthood (second hit) to assess potential interaction effects with the PNS-induced phenotype. We have previously shown (Panetta et al., 2017) that although PNS resulted in a strong metabolic liability in male rats fed with HFD at adulthood it also dampened the negative effects driven by such a metabolic challenge on the emotional phenotype as assessed in the EPM. Here we extended such body of evidence by providing information on the possible mechanisms underlying this interaction. PNS-CD rats were characterised by reduced brain plasticity showing decreased expression levels of the neurotrophin *Bdnf* and of pro-inflammatory cytokines in a number of brain regions; moreover, HFD feeding, experienced at adulthood, induced an increase in pro-inflammatory cytokines in those animals that had experienced PNS. This suggests that PNS might impinge upon the same mechanisms underlying vulnerability to metabolic challenges at adulthood. Furthermore, HFD greatly affected the expression levels of the main effectors of HPA axis function (the glucocorticoid receptor - GR - gene *Nr3c1* and of the GR's co-chaperone *Fkbp5*) an effect possibly underlying the metabolic vulnerability previously observed in male rats.

In our study, we found that PNS animals were characterised by differential expression of mRNA levels of *Il-1β* and *Tnf-α* in almost all the brain regions investigated except for the hypothalamus, an area that, in our experimental condition, appeared to be resilient to inflammatory changes driven by both pre- and postnatal stressors. While *Tnf-α* was tightly modulated upon pre- and postnatal stressors, changes in *Il-1β* were observed only when considering the interaction with the second hit (HFD), suggesting that the former cytokine (*Tnf-α*) might be considered as a reliable marker of stress adaptation in response to a prenatal challenge. As for *Il-6*, no effect was observed upon PNS in neither of the brain areas considered, though HFD overall decreased its expression levels in the dorsal hippocampus. TNF-α, IL-1β and IL-6 are the main activators of the HPA and are in turn modulated (inhibited) by GC hormones. As described by O'Connors and colleagues these cytokines hold differential sensitivity for adrenal steroids with TNF-α being the most sensitive to such inhibition in the range of physiological levels, IL-1β being second and IL-6 being the most resistant (O'Connor et al., 2000).

There is general consensus that elevated inflammation can exacerbate or even give rise to depressive symptoms or may be associated to other psychiatric conditions (Dantzer et al., 2018, 2008). However, cytokines are also constitutively released in the healthy brain by resident myeloid cells to keep proper synaptic plasticity. As an example, the modulation of both IL-1β and TNF-α plays an important role in the processes of LTP and synaptic scaling (a form of homeostatic plasticity) (Rizzo et al., 2018; Salim et al., 2012). Here we found that lower mRNA levels of *Il-1β* in the dorsal hippocampus of PNS rats were associated to increased time spent immobile in the EPM (as previously assessed in (Panetta et al., 2017)). Moreover, and in line with our previous work (Panetta et al., 2017), PNS rats were characterised by decreased levels of the neurotrophin *Bdnf* (in the dorsal

hippocampus); this decrease was also associated to an increase in the time spent immobile in the EPM, suggesting that PNS might affect foetal brain programming by reducing neuronal plasticity. Interestingly, the interaction between PNS and adult HFD (PNS-HFD group) resulted in increased levels of *Il-1β* and *Tnf-α* mRNA. Alboni and colleagues have recently provided evidence for increased rather than decreased levels of pro-inflammatory cytokines in the brain of stressed mice treated with the antidepressant fluoxetine. Such an effect was associated to increased BDNF levels and stronger LTP in the hippocampus (Alboni et al., 2017, 2016), suggesting that brain plasticity may also be related to the activation of basal metabolism that in turn is positively associated to the ability to properly mount and control inflammatory responses. To this regard, we cannot exclude that prolonged exposure to the HFD or to a stronger metabolic insult, such as a western pattern diet (rich in fats and sugar), should lead to an overall excessive brain inflammation (not observed in this study) with main consequences on emotional/cognitive behaviour. Worth to notice, we have previously shown that upon PNS, male rats were characterized by increased corticosterone levels under basal conditions, this effect was associated with a decrease in reactive oxygen species (ROS) as well as a with decreased NF-kB signalling in the hippocampus suggesting a lower set-point under basal conditions in PNS male rats (Anacker et al., 2013). Here we observed a similar blunted activation upon PNS with HFD triggering a response only in PNS subjects (increased Il-1β and Tnf-α in the prefrontal cortex and Tnf-α in the dorsal and ventral hippocampus). To our knowledge this is one of the few instances in which a reduction in inflammatory mediators is described a result of PNS. Because cytokines have been shown to modulate hippocampal development and plasticity (Bourgognon and Cavanagh, 2020; Goshen et al., 2007) a decreased expression profile as a result of a "first hit" (PNS) might set the stage for an increased response to a "second hit" (metabolic challenge). Obesity is characterised by low-grade systemic and central inflammation and TNF-α and IL-1β appear to be key players in this condition and in the associated pathologies (Mighiu et al., 2012). Although the hypothalamus is a well-known area playing a main role in the crosstalk between brain and periphery in metabolic pathologies associated to obesity, other brain regions have been shown to be affected by high-fat diet- or obesity-induced inflammation such as the hippocampus, cortex, brainstem, or amygdala (Guillemot-Legris and Muccioli, 2017). There are data to suggest that the effects of a metabolic challenge may be different depending on the region of interest dealing with brain plasticity (hippocampus) or metabolic regulation and homeostasis (hypothalamus) (Rasgon and McEwen, 2016). Thus, our data also indicate that PNS might set the stage for a differential regulation of central inflammatory mediators in specific brain regions, an effect that can be unmasked only upon the occurrence of a second hit, such as the HFD at adult age.

Hyperactivity of the HPA axis and increased circulating GC have been implicated in the pathogenesis of mood and anxiety disorders suggesting an impairment in the ability of the HPA axis to self-regulate its function by shutting down the system (Binder, 2009; Holsboer, 2000; Pariante and Miller, 2001). Glucocorticoids have also an important function in metabolic regulations as they mobilize glucose to fuel the energy demands of the stress response and furthermore promote energy storage, feeding, and weight gain. Thus, it is possible to hypothesize that both psychological as well as metabolic stress might be able to affect HPA axis functionality (Balsevich et al., 2019). A growing body of evidence suggests that levels of *Fkbp5* mRNA GR co-chaperone

have been associated with higher levels of circulating cortisol and reduced negative feedback inhibition of the stress response associated with a depressive phenotype (Binder, 2009). We have previously shown that, following PNS, male rats were characterised by elevated GC levels when compared to controls (Anacker et al., 2013); in this study we show elevated levels of *Fkbp5* upon PNS in the prefrontal cortex and upon HFD administration in the dorsal hippocampus, suggesting that both psychological and metabolic stressors affect this mediator. When we looked at the hypothalamus, *Fkbp5* expression levels were reduced in the HFD group, possibly accounting for a compensatory mechanism. Interestingly, we also showed that levels of *Fkbp5* in the dorsal hippocampus were positively related to the time spent in the open arm of the EPM and that this positive correlation is lost as a result both of PNS as well as of HFD. This result might suggest that under physiological conditions *Fkbp5* is required to engage animals in the proper exploration of novel environments through a fine modulation of the GR receptors and that this balance can be greatly affected by both early life stressors as well as by metabolic challenges.

The prefrontal cortex is one of the most important cortical area among the network of regions being involved in the pathogenesis of depression that plays a main role in the ability to process positive and negative emotions (Harms et al., 2017; Kaya and McCabe, 2019). Thus, the observed increase in levels of *Fkbp5* in this brain area should not be surprising and may underlie a condition of potential increased vulnerability to stress.

Changes in *Nr3c1* and *Fkbp5* in the hippocampus, a result of HFD, indicate a cross-regulation between a metabolic challenge and *Nr3c1/Fkbp5* balance, strengthening the notion that indeed these molecules are involved in "sensing" environmental challenges, be these of psychological or metabolic nature. We also found that HFD increased the levels of *Nr3c1* in the prefrontal cortex; by contrast, changes in *Fkbp5* in this brain area were observed only as a result of PNS. These results possibly suggest a fine and complex multiple level regulation of the GR-negative feedback in this specific brain region highlighting the role of this brain area in mood disorders such as depression. Transcriptional control of GR relies in part upon the DNA methylation status at multiple alternative initiation sites that are tissue specific in fact, *Nr3c1* gene is characterised by an unusually complex promoter structure (Turner et al., 2010). This might possibly explain the unexpected decrease in the dorsal hippocampus observed as a result of HFD or the lack of effects observed in the hypothalamus (see also concluding remarks).

When we investigated levels of *Ghs-R* we found a specific decrease upon HFD in the dorsal hippocampus while an increase was observed in both the ventral hippocampus and the prefrontal cortex in CTRL-HFD and PNS-CD animals. It has become increasingly apparent that the dorsal and the ventral portions of the hippocampus are preferentially involved in different physiological functions the first playing a role in the regulation of cognitive processes (spatial memory) the second in the modulation of emotions - such that they have been defined the cold and the hot hippocampus respectively - (Fanselow and Dong, 2010). Chen and co-workers provided evidence that infusion of ghrelin in the dorsal hippocampus enhanced synaptic plasticity and spatial memory (Chen et al., 2020). By contrast, Kanoski and colleagues showed that ghrelin delivery to the ventral but not the dorsal hippocampus increased the ability of environmental food-related cues to stimulate meal initiation and to enhance motivation to obtain it by increasing food intake frequency (Kanoski et al.,

2013) confirming that this hippocampal sub-region might be of importance in feeding/appetitive rewarding behaviours. To this regard it is interesting to note that we have previously found an increase in caloric intake upon HFD (Panetta et al., 2017). Recently Guo and colleagues have shown that GHS-R knock-out mice were characterised by improved abilities to cope to social stressors, decreased emotionality and depressive-like behaviours (Guo et al., 2019). Thus, we can hypothesize that both the pre-natal and the postnatal stressors might act by increasing the ghrelin signal in two brain regions of main importance for the ability to process emotions (the ventral hippocampus and the prefrontal cortex) setting the stage for increased vulnerability to mood disorders.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We have here provided evidence that PNS can affect brain development (first hit) setting the stage for increased vulnerability to further insults during life (HFD, second hit). In particular, we observed important changes in the expression levels of GR, their chaperons and *Ghs-R* in response to both PNS and HFD, confirming that the there is an important overlap between pathways and effectors involved in the regulation of emotions and in food intake and metabolic balance. Further investigations are warranted to assess more in detail the role played by these molecules in setting the stage for co-morbidity between metabolic and psychiatric disorders. In addition, the role of additional co-regulators, such as endocannabinoids should be tested (Balsevich et al., 2019).

There are some limitations of the current study that should be mentioned. First, we examined here the expression level of selected genes of interest but did not complement this with the levels of the corresponding proteins, the functional end products of the genes. Secondly, we have used real-time PCR in combination with biopsy punching as a quantitative approach instead of in situ hybridization. Due to this approach we had to make a selection of brain areas and lose anatomical resolution. Thirdly, genomic and epigenomic regulation of glucocorticoids in the brain may affect mood and metabolism but is based upon the coordinated activity of many chaperone proteins, in addition to proper circadian and ultradian fluctuation of hormone release (Gray et al., 2017). Future studies should assess the interrelationship between psychological and metabolic stressors. In addition, given the observed changes of *Ghs-R* and *Nr3c1* in the dorsal hippocampus following a HFD, further research should be devoted to explore more in depth the effects of HFD and of PNS, and their interaction, on brain plasticity.

Conflict of Interest Statement

The authors have nothing to disclose.

Author's contribution

AB and CM analysed, interpreted data and wrote the manuscript; MM and AC collected all gene expression data; FC and MR designed the experiment and provided data interpretation.

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REFERENCES

Alboni, S., Poggini, S., Garofalo, S., Milior, G., El Hajj, H., Lecours, C., Girard, I., Gagnon, S., Boisjoly-Villeneuve, S., Brunello, N., Wolfer, D.P., Limatola, C., Tremblay, M.È., Maggi, L., Branchi, I., 2016. Fluoxetine treatment affects the inflammatory response and microglial function according to the quality of the living environment. Brain. Behav. Immun. 58, 261–271. https://doi.org/10.1016/j.bbi.2016.07.155

Alboni, S., Van DIjk, R.M., Poggini, S., Milior, G., Perrotta, M., Drenth, T., Brunello, N., Wolfer, D.P., Limatola, C., Amrein, I., Cirulli, F., Maggi, L., Branchi, I., 2017. Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. Mol. Psychiatry 22, 552–561. https://doi.org/10.1038/mp.2015.142

Anacker, C., Cattaneo, A., Luoni, A., Musaelyan, K., Zunszain, P.A., Milanesi, E., Rybka, J., Berry, A., Cirulli, F., Thuret, S., Price, J., Riva, M.A., Gennarelli, M., Pariante, C.M., 2013. Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. Neuropsychopharmacology 38, 872–883. https://doi.org/10.1038/npp.2012.253

Andersson, N.W., Li, Q., Mills, C.W., Ly, J., Nomura, Y., Chen, J., 2016. Influence of prenatal maternal stress on umbilical cord blood cytokine levels. Arch. Womens. Ment. Health 19, 761–767. https://doi.org/10.1007/s00737-016-0607-7

Balsevich, G., Abizaid, A., Chen, A., Karatsoreos, I.N., Schmidt, M. V., 2019. Stress and glucocorticoid modulation of feeding and metabolism. Neurobiol. Stress. https://doi.org/10.1016/j.ynstr.2019.100171

Barker, D.J.P., 1995. Intrauterine programming of adult disease. Mol. Med. Today. https://doi.org/10.1016/S1357-4310(95)90793-9

Barrientos, R.M., Sprunger, D.B., Campeau, S., Watkins, L.R., Rudy, J.W., Maier, S.F., 2004. BDNF mRNA expression in rat hippocampus following contextual learning is blocked by intrahippocampal IL-1β administration. J. Neuroimmunol. 155, 119–126. https://doi.org/10.1016/j.jneuroim.2004.06.009

Berry, A., Panetta, P., Luoni, A., Bellisario, V., Capoccia, S., Andrea Riva, M., Cirulli, F., 2015. Decreased Bdnf expression and reduced social behavior in periadolescent rats following prenatal stress. Dev. Psychobiol. 57, 365–373. https://doi.org/10.1002/dev.21297

Bilbo, S.D., Barrientos, R.M., Eads, A.S., Northcutt, A., Watkins, L.R., Rudy, J.W., Maier, S.F., 2008. Early-life infection leads to altered BDNF and IL-1β mRNA expression in rat hippocampus following learning in adulthood. Brain. Behav. Immun. 22, 451–455. https://doi.org/10.1016/j.bbi.2007.10.003

Binder, E.B., 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology. https://doi.org/10.1016/j.psyneuen.2009.05.021

Blum, I.D., Patterson, Z., Khazall, R., Lamont, E.W., Sleeman, M.W., Horvath, T.L., Abizaid, A., 2009. Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. Neuroscience 164, 351–359. https://doi.org/10.1016/j.neuroscience.2009.08.009

Boersma, G.J., Lee, R.S., Cordner, Z.A., Ewald, E.R., Purcell, R.H., Moghadam, A.A., Tamashiro, K.L., 2013. Prenatal stress decreases Bdnf expression and increases methylation of Bdnf exon IV in rats. Epigenetics 9, 437–447. https://doi.org/10.4161/epi.27558

Bourgognon, J.-M., Cavanagh, J., 2020. The role of cytokines in modulating learning and memory and brain plasticity. Brain Neurosci. Adv. 4, 239821282097980. https://doi.org/10.1177/2398212820979802

Bronson, S.L., Bale, T.L., 2014. Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment. Endocrinology 155, 2635–2646. https://doi.org/10.1210/en.2014-1040

551 Cattane, N., Räikkönen, K., Anniverno, R., Mencacci, C., Riva, M.A., Pariante, C.M., Cattaneo, A., 2020.
552 Depression, obesity and their comorbidity during pregnancy: effects on the offspring's mental and
553 physical health. Mol. Psychiatry. https://doi.org/10.1038/s41380-020-0813-6

554 Cattaneo, A., Macchi, F., Plazzotta, G., Veronica, B., Bocchio-Chiavetto, L., Riva, M.A., Pariante, C.M.,
555 2015. Inflammation and neuronal plasticity: A link between childhood trauma and depression
556 pathogenesis. Front. Cell. Neurosci. https://doi.org/10.3389/fncel.2015.00040

557 Chen, H.J., Antonson, A.M., Rajasekera, T.A., Patterson, J.M., Bailey, M.T., Gur, T.L., 2020. Prenatal stress
558 causes intrauterine inflammation and serotonergic dysfunction, and long-term behavioral deficits
559 through microbe- and CCL2-dependent mechanisms. Transl. Psychiatry 10, 1–12.
560 https://doi.org/10.1038/s41398-020-00876-5

561 Cirulli, F., 2017. Interactions between early life stress and metabolic stress in programming of mental and
562 metabolic health. Curr. Opin. Behav. Sci. https://doi.org/10.1016/j.cobeha.2016.12.009

563 Cirulli, F., Alleva, E., 2009. The NGF saga: From animal models of psychosocial stress to stress-related
564 psychopathology. Front. Neuroendocrinol. https://doi.org/10.1016/j.yfrne.2009.05.002

565 Cirulli, F., Musillo, C., Berry, A., 2020. Maternal Obesity as a Risk Factor for Brain Development and
566 Mental Health in the Offspring. Neuroscience. https://doi.org/10.1016/j.neuroscience.2020.01.023

567 Danese, A., Moffitt, T.E., Harrington, H.L., Milne, B.J., Polanczyk, G., Pariante, C.M., Poulton, R., Caspi,
568 A., 2009. Adverse childhood experiences and adult risk factors for age-related disease: Depression,
569 inflammation, and clustering of metabolic risk markers. Arch. Pediatr. Adolesc. Med. 163, 1135–1143.
570 https://doi.org/10.1001/archpediatrics.2009.214

571 Danese, A., Moffitt, T.E., Pariante, C.M., Ambler, A., Poulton, R., Caspi, A., 2008. Elevated inflammation
572 levels in depressed adults with a history of childhood maltreatment. Arch. Gen. Psychiatry 65, 409–
573 416. https://doi.org/10.1001/archpsyc.65.4.409

574 Dantzer, R., Cohen, S., Russo, S.J., Dinan, T.G., 2018. Resilience and immunity. Brain. Behav. Immun. 74,
575 28–42. https://doi.org/10.1016/j.bbi.2018.08.010

576 Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to
577 sickness and depression: When the immune system subjugates the brain. Nat. Rev. Neurosci.
578 https://doi.org/10.1038/nrn2297

579 Daskalakis, N.P., Bagot, R.C., Parker, K.J., Vinkers, C.H., de Kloet, E.R., 2013. The three-hit concept of
580 vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome.
581 Psychoneuroendocrinology 38, 1858–1873. https://doi.org/10.1016/j.psyneuen.2013.06.008

582 Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., Lanctôt, K.L., 2010. A Meta-
583 Analysis of Cytokines in Major Depression. Biol. Psychiatry 67, 446–457.
584 https://doi.org/10.1016/j.biopsych.2009.09.033

585 Fanselow, M.S., Dong, H.W., 2010. Are the Dorsal and Ventral Hippocampus Functionally Distinct
586 Structures? Neuron. https://doi.org/10.1016/j.neuron.2009.11.031

587 Fernandes, C., File, S.E., 1996. The influence of open arm ledges and maze experience in the elevated plus-
588 maze, in: Pharmacology Biochemistry and Behavior. Elsevier Inc., pp. 31–40.
589 https://doi.org/10.1016/0091-3057(95)02171-X

590 Goshen, I., Kreisel, T., Ounallah-Saad, H., Renbaum, P., Zalzstein, Y., Ben-Hur, T., Levy-Lahad, E.,
591 Yirmiya, R., 2007. A dual role for interleukin-1 in hippocampal-dependent memory processes.
592 Psychoneuroendocrinology 32, 1106–1115. https://doi.org/10.1016/j.psyneuen.2007.09.004

593 Gray, J.D., Kogan, J.F., Marrocco, J., McEwen, B.S., 2017. Genomic and epigenomic mechanisms of
594 glucocorticoids in the brain. Nat. Rev. Endocrinol. https://doi.org/10.1038/nrendo.2017.97

595 Guillemot-Legris, O., Muccioli, G.G., 2017. Obesity-Induced Neuroinflammation: Beyond the
596 Hypothalamus. Trends Neurosci. https://doi.org/10.1016/j.tins.2017.02.005

597 Guo, L., Niu, M., Yang, J., Li, L., Liu, S., Sun, Y., Zhou, Z., Zhou, Y., 2019. GHS-R1a Deficiency
598 Alleviates Depression-Related Behaviors After Chronic Social Defeat Stress. Front. Neurosci. 13.
599 https://doi.org/10.3389/fnins.2019.00364

600 Gur, T.L., Shay, L., Palkar, A.V., Fisher, S., Varaljay, V.A., Dowd, S., Bailey, M.T., 2017. Prenatal stress
601 affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult
602 female offspring. Brain. Behav. Immun. 64, 50–58. https://doi.org/10.1016/j.bbi.2016.12.021

603 Handa, R.J., Burgess, L.H., Kerr, J.E., O'keefe, J.A., 1994. Gonadal steroid hormone receptors and sex
604 differences in the hypothalamo-pituitary-adrenal axis. Horm. Behav. 28, 464–476.
605 https://doi.org/10.1006/hbeh.1994.1044

606 Hantsoo, L., Kornfield, S., Anguera, M.C., Epperson, C.N., 2019. Inflammation: A Proposed Intermediary
607 Between Maternal Stress and Offspring Neuropsychiatric Risk. Biol. Psychiatry.
608 https://doi.org/10.1016/j.biopsych.2018.08.018

609 Harms, M.B., Birn, R., Provencal, N., Wiechmann, T., Binder, E.B., Giakas, S.W., Roeber, B.J., Pollak,
610 S.D., 2017. Early life stress, FK506 binding protein 5 gene (FKBP5) methylation, and inhibition-
611 related prefrontal function: A prospective longitudinal study. Dev. Psychopathol. 29, 1895–1903.
612 https://doi.org/10.1017/S095457941700147X

613 Health Organization Regional Office for Europe, W., 2015. The European Mental Health Action Plan 2013-
614 2020.

615 Holsboer, F., 2000. The corticosteroid receptor hypothesis of depression. Neuropsychopharmacology 23,
616 477–501. https://doi.org/10.1016/S0893-133X(00)00159-7

617 Kanoski, S.E., Fortin, S.M., Ricks, K.M., Grill, H.J., 2013. Ghrelin signaling in the ventral hippocampus
618 stimulates learned and motivational aspects of feeding via PI3K-Akt signaling. Biol. Psychiatry 73,
619 915–923. https://doi.org/10.1016/j.biopsych.2012.07.002

620 Kaya, S., McCabe, C., 2019. What role does the prefrontal cortex play in the processing of negative and
621 positive stimuli in adolescent depression? Brain Sci. 9. https://doi.org/10.3390/brainsci9050104

622 Krontira, A.C., Cruceanu, C., Binder, E.B., 2020. Glucocorticoids as Mediators of Adverse Outcomes of
623 Prenatal Stress. Trends Neurosci. https://doi.org/10.1016/j.tins.2020.03.008

624 Liu, Y., Ho, R.C.M., Mak, A., 2012. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble
625 interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: A meta-
626 analysis and meta-regression. J. Affect. Disord. https://doi.org/10.1016/j.jad.2011.08.003

627 Lutter, M., Sakata, I., Osborne-Lawrence, S., Rovinsky, S.A., Anderson, J.G., Jung, S., Birnbaum, S.,
628 Yanagisawa, M., Elmquist, J.K., Nestler, E.J., Zigman, J.M., 2008. The orexigenic hormone ghrelin
629 defends against depressive symptoms of chronic stress. Nat. Neurosci. 11, 752–753.
630 https://doi.org/10.1038/nn.2139

631 Maccari, S., Piazza, P.V., Kabbaj, M., Barbazanges, A., Simon, H., Le Moal, M., 1995. Adoption reverses
632 the long-term impairment in glucocorticoid feedback induced by prenatal stress. J. Neurosci. 15, 110–
633 116. https://doi.org/10.1523/jneurosci.15-01-00110.1995

634 Marosi, K., Mattson, M.P., 2014. BDNF mediates adaptive brain and body responses to energetic challenges.
635 Trends Endocrinol. Metab. https://doi.org/10.1016/j.tem.2013.10.006

636 McCarthy, M.M., 2019. Stress during pregnancy: Fetal males pay the price. Proc. Natl. Acad. Sci. U. S. A.
637 https://doi.org/10.1073/pnas.1916909116

McEwen, B.S., 2000. Effects of adverse experiences for brain structure and function, in: Biological Psychiatry. Elsevier, pp. 721–731. https://doi.org/10.1016/S0006-3223(00)00964-1

Mighiu, P.I., Filippi, B.M., Lam, T.K.T., 2012. Linking inflammation to the brain-liver axis. Diabetes. https://doi.org/10.2337/db12-0330

Milaneschi, Y., Simmons, W.K., van Rossum, E.F.C., Penninx, B.W., 2019. Depression and obesity: evidence of shared biological mechanisms. Mol. Psychiatry. https://doi.org/10.1038/s41380-018-0017-5

Mueller, B.R., Bale, T.L., 2008. Sex-specific programming of offspring emotionality after stress early in pregnancy. J. Neurosci. 28, 9055–9065. https://doi.org/10.1523/JNEUROSCI.1424-08.2008

Nederhof, E., Schmidt, M. V., 2012. Mismatch or cumulative stress: Toward an integrated hypothesis of programming effects. Physiol. Behav. https://doi.org/10.1016/j.physbeh.2011.12.008

O'Connor, T.M., O'Halloran, D.J., Shanahan, F., 2000. The stress response and the hypothalamic-pituitary-adrenal axis: From molecule to melancholia. QJM - Mon. J. Assoc. Physicians. https://doi.org/10.1093/qjmed/93.6.323

Panetta, P., Berry, A., Bellisario, V., Capoccia, S., Raggi, C., Luoni, A., Longo, L., Riva, M.A., Cirulli, F., 2017. Long-term sex-dependent vulnerability to metabolic challenges in prenatally stressed rats. Front. Behav. Neurosci. 11. https://doi.org/10.3389/fnbeh.2017.00113

Pariante, C.M., Miller, A.H., 2001. Glucocorticoid receptors in major depression: Relevance to pathophysiology and treatment. Biol. Psychiatry. https://doi.org/10.1016/S0006-3223(00)01088-X

Patterson, Z.R., Ducharme, R., Anisman, H., Abizaid, A., 2010. Altered metabolic and neurochemical responses to chronic unpredictable stressors in ghrelin receptor-deficient mice. Eur. J. Neurosci. 32, 632–639. https://doi.org/10.1111/j.1460-9568.2010.07310.x

Rasgon, N.L., McEwen, B.S., 2016. Insulin resistance - A missing link no more. Mol. Psychiatry. https://doi.org/10.1038/mp.2016.162

Rizzo, F.R., Musella, A., De Vito, F., Fresegna, D., Bullitta, S., Vanni, V., Guadalupi, L., Stampanoni Bassi, M., Buttari, F., Mandolesi, G., Centonze, D., Gentile, A., 2018. Tumor Necrosis Factor and Interleukin-1β Modulate Synaptic Plasticity during Neuroinflammation. Neural Plast. https://doi.org/10.1155/2018/8430123

Salim, S., Chugh, G., Asghar, M., 2012. Inflammation in anxiety, in: Advances in Protein Chemistry and Structural Biology. Academic Press Inc., pp. 1–25. https://doi.org/10.1016/B978-0-12-398314-5.00001-5

Slopen, N., Koenen, K.C., Kubzansky, L.D., 2014. Cumulative adversity in childhood and emergent risk factors for long-term health. J. Pediatr. 164, 631-638.e2. https://doi.org/10.1016/j.jpeds.2013.11.003

Ślusarczyk, J., Trojan, E., Głombik, K., Budziszewska, B., Kubera, M., Lasoń, W., Popiołek-Barczyk, K., Mika, J., Wędzony, K., Basta-Kaim, A., 2015. Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells. Front. Cell. Neurosci. 9. https://doi.org/10.3389/fncel.2015.00082

Turner, J.D., Alt, S.R., Cao, L., Vernocchi, S., Trifonova, S., Battello, N., Muller, C.P., 2010. Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. Biochem. Pharmacol. 80, 1860–8. https://doi.org/10.1016/j.bcp.2010.06.037

Valkanova, V., Ebmeier, K.P., Allan, C.L., 2013. CRP, IL-6 and depression: A systematic review and meta-analysis of longitudinal studies. J. Affect. Disord. https://doi.org/10.1016/j.jad.2013.06.004

Verhagen, L.A.W., Egecioglu, E., Luijendijk, M.C.M., Hillebrand, J.J.G., Adan, R.A.H., Dickson, S.L.,

2011. Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity. Eur. Neuropsychopharmacol. 21, 384–392. https://doi.org/10.1016/j.euroneuro.2010.06.005

Weinstock, M., 2007. Gender differences in the effects of prenatal stress on brain development and behaviour. Neurochem. Res. https://doi.org/10.1007/s11064-007-9339-4

Zarouna, S., 2015. Mood disorders: A potential link between ghrelin and leptin on human body? World J. Exp. Med. 5, 103. https://doi.org/10.5493/wjem.v5.i2.103

FIGURE LEGENDS

GRAPHICAL ABSTRACT. Based on the "two hit model" of the developmental origin of diseases, PNS (first hit) affects foetal brain developmental trajectories; signalling pathways related to brain plasticity and function (*Bdnf*, *Tnf-α and Il-β*) are down-set potentially providing increased liability to later-life mood and metabolic disorders. A later-life challenge such as HFD feeding (second hit), experienced at adulthood, might impinge upon the same signalling pathways primed by PNS leading to enhanced responses to the metabolic challenge. The hippocampus and prefrontal cortex, being main targets of glucocorticoids, are finely modulated by PNS and readily respond to the HFD metabolic stress.

FIGURE 1. Overall subjects who experienced prenatal stress (PNS-CD) were characterised by decreased m-RNA levels of both *Il-1β* (panel A) and *Tnf-α* (panel B) in almost all the brain regions investigated; *Il-6* showed a decrease in its expression levels upon HFD in the dorsal hippocampus (panel C). *Il-1β* showed statistical significance only in the prefrontal cortex while *Tnf-α* was tightly modulated upon pre- and postnatal stressors. Data are presented graphically as means ± SEM box plot (observations outside the ranges are represented with dots outside the boxes). Post hoc comparisons: **$$p<0.01, main effect of diet (dorsal hippocampus, *Il-6*); post hoc comparisons: *p<0.05, interaction effect (dorsal hippocampus, *Tnf-α*: PNS-CD vs. CTRL-CD); **p<0.01, interaction effect (ventral hippocampus, *Tnf-α*: PNS-CD vs. PNS-HFD;** Prefrontal cortex, ***Tnf-α* and *Il1-β*: PNS-CD vs. PNS-HFD).** Number of subjects:8-10 within each experimental group.

FIGURE 2. Male rats exposed to prenatal stress (PNS) were characterised by an inverse relation between levels of *Il-1β* in the dorsal hippocampus and immobility duration as assessed in the elevated plus maze test (EPM). *p<0.05**.** Number of subjects: 6-10 within each experimental group.

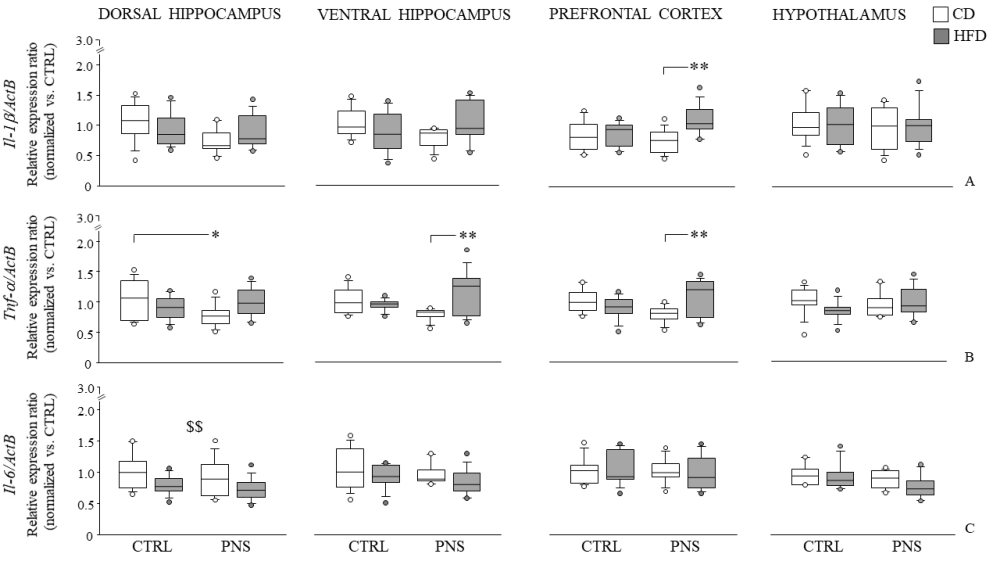
FIGURE 3. Prenatal stress decreased *Bdnf* m-RNA levels specifically in the dorsal hippocampus suggesting a reduced neuronal plasticity. This effect is reversed by HFD administration at adulthood. Data are presented graphically as means ± SEM box plot (observations outside the ranges are represented with dots outside the boxes). Post hoc comparisons *p<0.05, **interaction effect in the dorsal hippocampus, PNS-CD vs. CTR-CD and PNS-HFD**. Number of subjects is between 9-11 within each experimental group.

FIGURE 4. Male rats exposed to prenatal stress (PNS) were characterised by an inverse relation between levels of *Bdnf* in the dorsal hippocampus and immobility duration as assessed in the elevated plus maze test (EPM). Post hoc comparisons *p<0.05. Number of subjects: 9-10 within each experimental group.

FIGURE 5. Independently from the prenatal condition, post-natal HFD administration increases levels of *Nr3c1* in the ventral hippocampus and prefrontal cortex by contrast, in the dorsal hippocampus a significant interaction between PNS and HFD results in decreased levels of this gene both as a result of pre- and post-natal stressors. No change in *Nr3c1* was observed in the hypothalamus as a result of PNS nor of HFD (see panel A). As for *Fkbp5*, HFD independently from the prenatal condition, increased its expression levels in the dorsal hippocampus while a decrease was observed in the hypothalamus; no change was observed in the ventral hippocampus. PNS overall increased levels of Fkbp5 specifically in the prefrontal cortex. $$p<0.01, main effect of diet (*Nr3c1*: ventral hippocampus and prefrontal cortex; Fkbp5: dorsal hippocampus and hypothalamus; *Fkbp5*: dorsal hippocampus; hypothalamus); £p<0.05, main effect of PNS (*Fkbp5*: prefrontal cortex); post hoc comparisons: **p<0.01, interaction effect (*Nr3c1*: CTRL-CD vs. CTRL-HFD, PNS-CD and PNS-HFD); Number of subjects:8-10 within each experimental group.

FIGURE 6. Control subjects are characterized by a direct relationship between expression levels of *Fkbp5* in the dorsal hippocampus and the time spent in the open arms of the Elevated Plus Maze, overall suggesting a permissive role for explorative behavior of this GC-related genes under physiological conditions. Post hoc comparisons **p<0.01. Number of subjects: 6-9 within each experimental group.

FIGURE 7. Levels of *Ghs-R* were found to be specifically **decreased** upon HFD in the dorsal hippocampus while an increase was observed in both the ventral hippocampus and the prefrontal cortex in CTRL-HFD and PNS-CD animals. Main effect of HFD: **main effect of diet**, $$ p<0.01; post hoc comparisons **for the interaction effects**: ***p<0.05: prefrontal cortex, CTRL-CD vs. CTRL-HFD; **p<0.01: ventral hippocampus, CTRL-CD vs. CTRL-HFD and PNS-CD, prefrontal cortex, CTRL-CD vs. PNS-CD**. Number of subjects: 8-10 within each experimental group.



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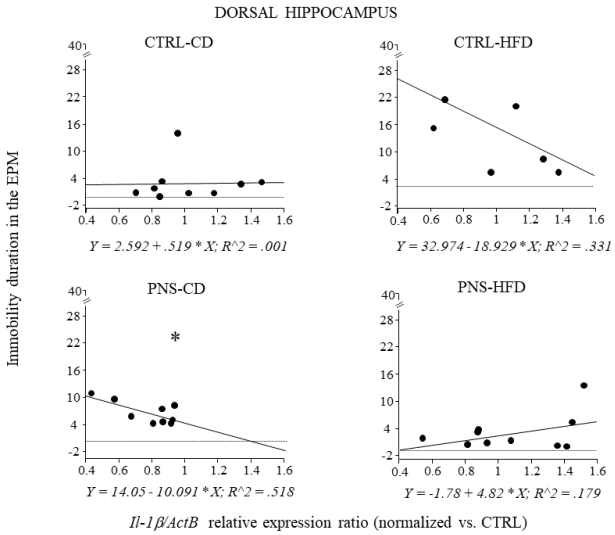

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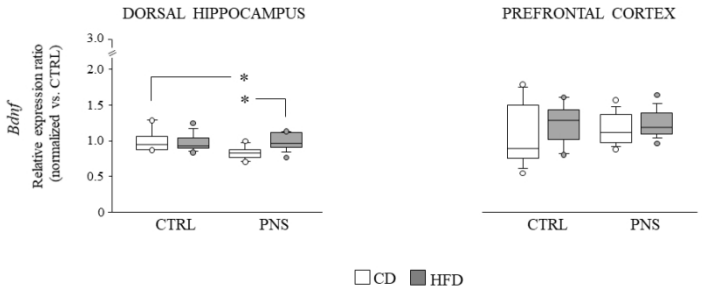

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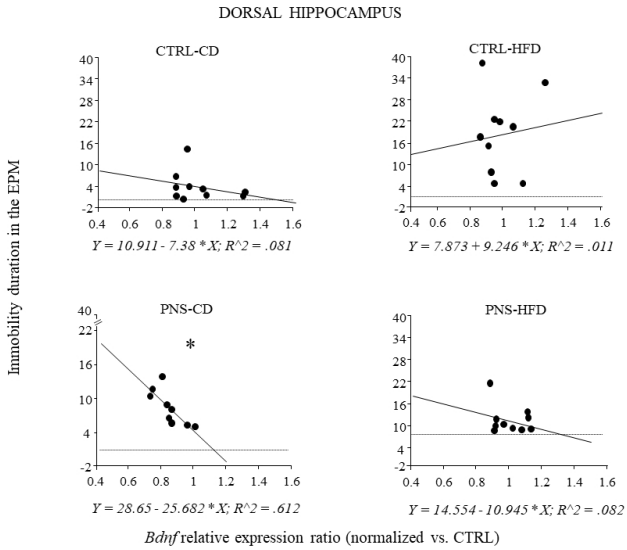

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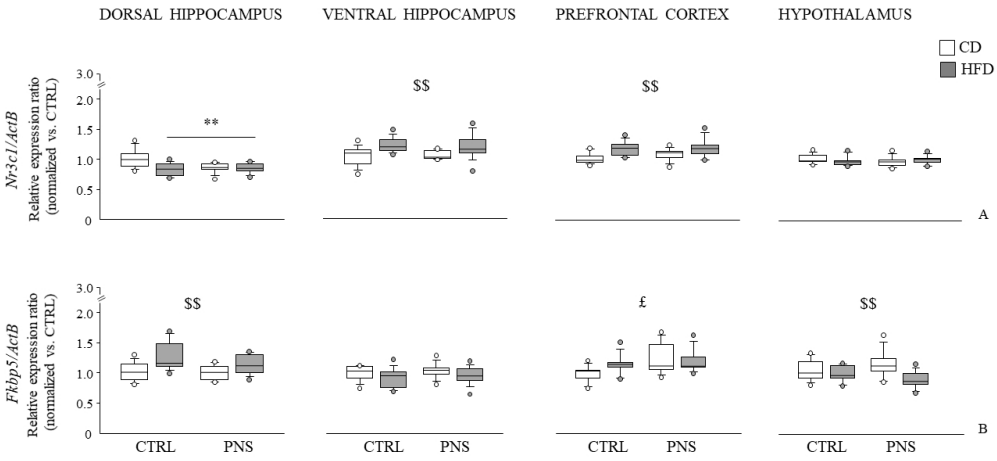

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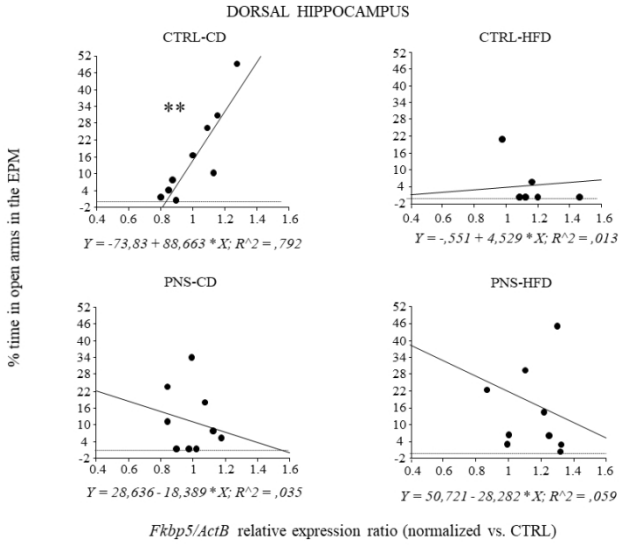

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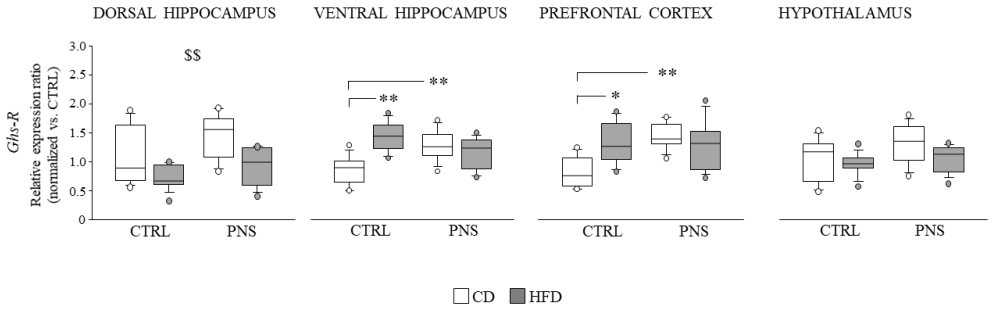

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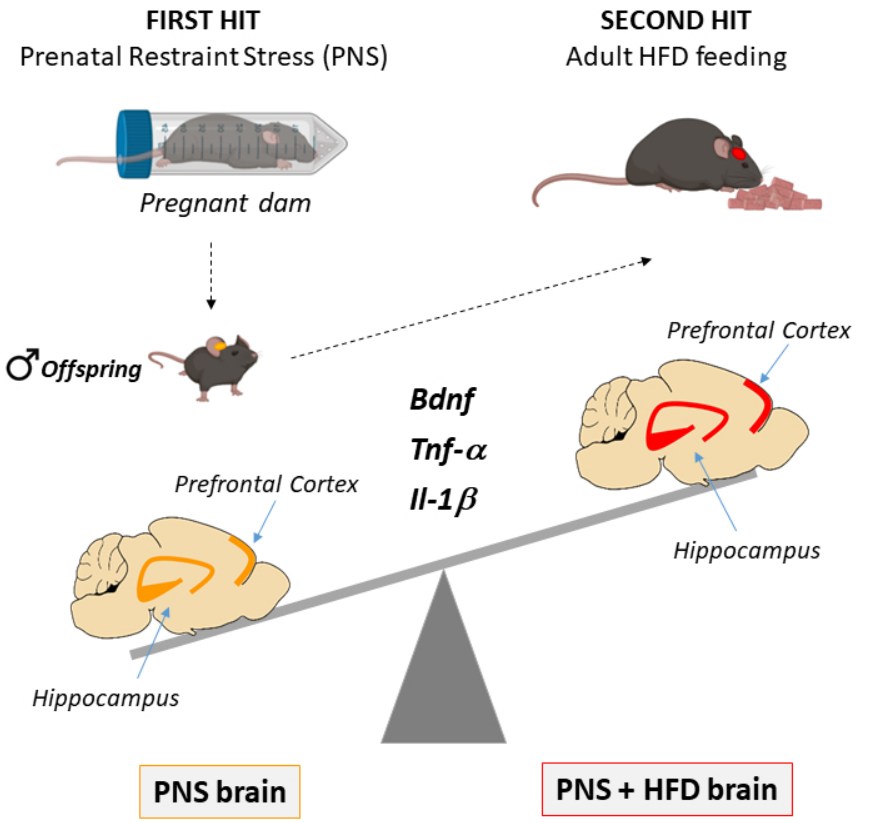

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