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Sex-dependent preventive effects of prenatal N-acetyl-cysteine on neuronal, emotional and metabolic dysfunctions following exposure to maternal high-fat diet in mice

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While a clear association between maternal obesity and an increased risk for neuropsychiatric disorders in the offspring has been described, the underlying mechanisms remain poorly understood. We hypothesised that a maternal high-fat diet (mHFD) would act as a stressor, increasing glucocorticoids, resulting in an altered redox balance and disrupted neuronal plasticity of the limbic system. Such enduring effects would impair the emotional and cognitive profile, neuroendocrine responses, and metabolic and redox homeostasis in the adult offspring. We utilised a mouse model and a translational cellular model employing human neurons derived from inducible Pluripotent Stem Cells (iPSCs) to evaluate the impact of mHFD on neurodevelopment and to test the protection afforded by the antioxidant N-acetyl-cysteine (NAC). Our approach combined behavioural and metabolic phenotyping, biochemical assays, morphological assessment, and targeted gene expression analysis. Results indicate that prenatal administration of NAC prevented anxiety-like and risk-taking behaviours, cognitive impairments and metabolic alterations in mHFD adult mouse offspring, particularly in females. These changes were accompanied by hippocampal downregulation of genes involved in neuronal plasticity, such as BDNF. Using human neurons in vitro, pre-treatment with NAC rescued the negative effects of glucocorticoids on neuronal plasticity via a BDNF-mediated mechanism. The protective effects of NAC over mHFD in females suggest that rebalancing the redox status could be exploited as an overall strategy to buffer the negative effects of early adversities on neurodevelopment.

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INTRODUCTION

Mental disorders have a profound social and economic impact, contributing to the global disease burden [1]. Current research is focused on identifying novel intervention targets. In this context, oxidative stress has emerged as a central factor in the pathophysiology of many psychiatric disorders, offering a promising avenue for new pharmacological approaches [2–4].

Adverse early life experiences have the potential to become embedded biological traces shaping brain developmental trajectories, eventually setting the stage for increased vulnerability to neurodevelopmental and psychiatric disorders [5]. Beyond the well-known effects of maternal psychological stress, other environmental challenges, such as unbalanced dietary habits, are emerging as risk factors for offspring mental health. In particular, maternal obesity has been directly linked to an increased risk of developing neuropsychiatric and mood disorders as well as cognitive disabilities (see [6, 7] and references therein), and has been associated with redox imbalance and excessive activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to high glucocorticoid levels and increased inflammation [8–12]. Overall, this combined response can result in severe short- and long-term negative outcomes in the offspring [6, 13–15]. We have previously demonstrated that maternal high-fat diet (mHFD) during pregnancy increases glucocorticoid levels at the maternal-fetal interface [16] also increasing pro-oxidant markers in maternal blood [17]. These alterations lead to sex-specific disruptions in brain antioxidant defenses and increased neuroinflammatory markers in female adolescent offspring [18]. This early exposure to excessive amount of glucocorticoids could also affect neuronal plasticity by reducing dendritic arborization [19–21], leading to a defective functioning of limbic structures, of relevance for adaptive behavioural and neuroendocrine regulations. At the cellular level, converging findings indicate a role for glucocorticoids in impairing neuronal plasticity via the modulation of BDNF-TrkB pathways, consistently observed in both hippocampal and dopaminergic (DA) neurons that innervate the hypothalamus, hippocampus and basal ganglia, all areas involved in the long-term effects of perinatal stress [21–24]. We have recently shown that maternal administration of N-acetyl-cysteine (NAC), a powerful scavenger of free radicals, prevents pro-oxidant and pro-inflammatory changes in adolescent mouse offspring exposed to mHFD [18].

In the present study, we aimed to investigate the enduring effects of mHFD in adult mouse offspring. We focused on

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cognitive, emotional, endocrine and metabolic derangements, as well as on their neuronal underpinnings, particularly those mechanistically mediated by glucocorticoids and oxidative damage. Moreover, we evaluated the protective effects of the antioxidant NAC during pregnancy. We combined behavioural, endocrine and metabolic phenotyping with targeted gene expression analysis to study neuroplasticity genes and excitatory/inhibitory balance in limbic structures involved in cognitive function, stress responsiveness and metabolic regulations in adult offspring. At the cellular level, we used an in vitro translational model of human midbrain DA neurons, as they are highly sensitive to both glucocorticoids and oxidative damage [25, 26]. Given the role of sex in determining the susceptibility to neuropsychiatric and metabolic disorders, an additional aim of this study was to assess whether the preventive effects of NAC differed between male and female offspring.

MATERALS AND METHODS

Study reporting conforms with the ARRIVE guidelines [27]. The experimental design is reported in Supplementary Fig. 1A.

Ethics approval

All experimental procedures were approved by the ethical body of the Istituto Superiore di Sanità for animal welfare, conducted in conformity with the European Directive 2010/63/EU and the Italian legislation on animal experimentation, D.Lgs. 26/2014 and authorized by the Italian Ministry of Health (license n° 893/2020-PR). Dermal biopsy from a 40-year-old healthy Caucasian female donor was obtained with informed consent and approval from the local ethics committee (CEIOC - Fatebenefratelli Hospital "San Giovanni di Dio," Brescia, Italy; approvals 44/2001 and 39/2005). Fibroblasts were used at passage 3 for hiPSC reprogramming [28].

Animal housing

Two-month-old C57BL/6 N mice, 82 females and 39 males (Charles River, Italy) were sex-matched housed 3/cage in Plexiglas cages (33 cm × 15 cm × 13 cm, Tecniplast), in an air-conditioned room (21 ± 1 °C, relative humidity 60 ± 10%) under a reversed 12/12 h light/dark cycle (lights on 7 p.m. – 7 a.m.). Fresh water and standard diet (SD, energy 3.3 kcal/g, fat 17%, carbohydrate 60% and protein 23%; Altromin-R, Italy) were available during the two weeks of habituation, then female breeders were randomly allocated into the experimental groups avoiding body weight bias [29].

Maternal high-fat diet administration

Females were fed either HFD ($n = 40$; energy 5.56 kcal/g, 58% fat, 16.4% protein, 25.5% carbohydrate; D12331) or control diet ($n = 42$; CD, 4.07 kcal/g, 10.5% fat, 16.4% protein, 73.1% carbohydrate; D12328), (Research Diets, USA, see Supplementary Fig. 1B and Supplementary Table 1), *ad libitum* for 10 weeks before breeding and throughout pregnancy, until gestational day (GD) 16 (for a total of 13 weeks). At GD 16 both HFD and CD were replaced with SD, to specifically investigate the contribution of the diets during the early-to-mid prenatal period [16].

Maternal antioxidant treatment with N-acetyl-cysteine

After five weeks on the diets, females received the antioxidant NAC (CD, $n = 23$; HFD, $n = 21$) or water as vehicle (CD, $n = 19$; HFD, $n = 19$). NAC (A7250; Sigma-Aldrich) was administered by drinking water to yield an average dose of 1 g/kg body weight [18]. Dosage was selected based on previous studies showing well-tolerance and suitability for chronic administration during pregnancy [18, 30]. Fresh solutions were prepared every three days to avoid NAC oxidation and solution consumption was monitored. NAC concentration was adjusted every two weeks based on body weight gain and consumption changes. NAC treatment was interrupted at GD 16, for a total of 8 weeks of administration, to assess the effects of antioxidants during the early-to-mid prenatal period.

Mating procedures and weaning of offspring

After 5 weeks on NAC and 10 weeks on diets, females were mated for 48 h. After mating, females were individually housed and body weight was monitored to confirm pregnancy and to ensure gestational length with a precision of ± 0.5 day. A weight gain of at least 3 g at GD 7 usually indicates that conception has occurred [18]. Litters with pups ≤3 were excluded. At PND 30, pups were weaned and housed 3 per cage. Following a split litter approach [31], starting from PND 90, one male and one female from each litter were chosen for the assessment of the behavioural or the metabolic phenotype.

Behavioural phenotype

Tests were conducted during the dark phase and video recorded. At the end of each session, apparatuses were cleaned with a 70% ethanol solution and wiped down with a paper towel. Behavioural analysis was performed blindly using The Observer 15XT (Noldus, The Netherlands) and AnyMaze (Stoelting, USA).

Emergence test. The apparatus was a Plexiglas arena (40 × 40 × 40 cm), virtually partitioned into a central zone (24 × 24 cm) and a peripheral zone, with a white floor (virtually divided into 25 equal-sized squares) enclosed by grey walls. A black plastic cup (Ø 15 cm) in one corner provided shelter from the brightly lit arena (600 lux) [32]. Mice started the test inside the shelter and the latency to emerge was evaluated as an index of behavioural disinhibition. Mice were free to explore for 20 min: distance, speed and time spent in the zones were measured.

Elevated plus maze. This test allowed evaluating the emotional profile and risk-assessment behaviours. The apparatus was made of Plexiglas (dim grey floor, transparent walls) and raised to a height of 60 cm above the floor level, with two open (30 × 5 cm) and two enclosed arms (30 × 5 × 15 cm) extending from a common central platform (5 × 5 cm). Mice were individually placed in the center of the maze, facing one open arm, and free to explore for 5 min (100 lux) [33, 34]. The time spent in open vs closed arms was assessed as a measure of emotionality; the frequency of head dipping was used to measure risk assessment.

Morris water maze. A circular pool (Ø 88 cm × 33 cm) filled with water at 24–26 °C, virtually divided into 4 quadrants (Target, Opposite, Right, Left) was used [35]. Visual cues were placed on the walls of the room (200 lux). The latency to reach the platform during the acquisition phase and the time spent in the target quadrant during the probe were analyzed. For further details on the procedure see Supplementary materials.

Forced swim test. This test was used to evaluate the coping strategies in response to an acute stress [36]. Mice were placed in a glass tank (30 cm h × 20 cm diameter), filled with water (26 ± 1 °C), for a single 6-min session. Time spent performing passive strategy (floating) and active strategy (swimming and struggling) was assessed during the last 4 min of the test [18, 37].

Metabolic phenotype through indirect calorimetry

Indirect calorimetry was assessed using the PhenoMaster system (TSE Systems, Germany) through the measurement of the respiratory exchange ratio (RER), described as the ratio between CO_2 produced (VCO_2) and the O_2 consumed during respiration (VO_2) [38]. This parameter varies depending on the macronutrients that are oxidized and preferably used for fuel utilization, ranging from 1.0 (pure carbohydrate utilization) to 0.7 (pure lipid utilization) [39]. Animals were individually placed in the cages

for 24 h of habituation and a further 48 h of metabolic parameters recording.

Blood sampling and tissue collection

Blood samples were collected by tail nick at 3 different time points: at baseline (0 min), 30 and 180 min after the Forced swim test. All samples were collected in potassium EDTA coated tubes (Sarstedt, Germany), centrifuged at 3000 rpm for 15 min at +4 °C to collect plasma.

At the end of the behavioural and metabolic characterisation, offspring was sacrificed by cervical dislocation and trunk blood and brain were collected for molecular analysis. Hippocampi were dissected out from the left hemispheres and immediately frozen at −80 °C. Blood samples were collected in heparinized coated tubes (Sarstedt, Germany), kept on ice and centrifuged at 3000 rpm for 15 min at +4 °C to collect plasma, and stored at −80 °C.

Corticosterone and leptin concentrations in plasma

Corticosterone and leptin concentrations were detected using commercially available ELISA kits (ADI-900-097 and ADI-900-019A, respectively, ENZO LifeTech; USA). Briefly, plasma samples (6 µl for corticosterone and 14 µl for leptin) were analyzed in duplicate and only data derived from duplicates with < 20% coefficient of variation were included in the analysis. The sensitivity of the assay was 27.0 pg/ml for corticosterone and 25.4 pg/ml for leptin. Light absorbance was read with a light absorption microplate reader (AMR-100, Hangzhou Allsheng Instruments, China) at 405 nm (corticosterone) or 450 nm (leptin). Data were elaborated by sigmoidal 4-parameter logistic curve fit.

RNA extraction and gene expression analysis

Total RNA was extracted from left hippocampi following the previously described protocol [18]. All samples were run in a 384-well plate in triplicates with both ß-Actin and GAPDH as internal controls (housekeeping gene), an average of both housekeeping genes was further used for data analysis. Primers and probes for Bdnf (total and exon IV), Gclc, InsR, LepR, NPY, Nrf-2, PVB, Slc1a3, SST, Vgat, Vglut1 were purchased from Thermo Fisher Scientific or Eurofins Genomics, and their ID's or sequences are shown in Supplementary Table 2. The efficiency-corrected model was used for qRT-PCR analysis [40]. Data are presented as fold change % compared to the CD-Vehicle group (set at 100%).

Differentiation of human iPSCs into midbrain DA neurons

Human iPSC (hiPSC) clone F3 [28] was differentiated into floorplate (FP)-derived midbrain dopaminergic (DA) neurons using dual-SMAD inhibition and FP induction protocols [41, 42]. On day 21, cells were replated on Polyornithine/Fibronectin/Laminin pre-coated coverslides and co-cultured with mouse primary cortical astrocytes [28]. Pharmacological treatments started on day 50. At this stage, DA neurons co-expressed tyrosine hydroxylase (TH) and MAP2 and accounted for approximately 30% of the total MAP2 population [28], with the remainder being GABAergic (20–25%) and glutamatergic (35–40%) [28, 43].

In-vitro pharmacological treatments of midbrain DA neurons

The first experiment investigated the effects of NAC on DA neurons that were pre-exposed to hydrocortisone (50 µM) for 5 days. Four concentrations of NAC (0.02, 0.2, 2, and 20 mM) were applied for 1 h, after which the medium was replaced with fresh Neurobasal/B27. 72 h after the final treatment (Day 7) cultures were fixed with 4% paraformaldehyde for immunocytochemistry [21].

The second experiment aimed to determine whether the effects of NAC involved the BDNF-TrkB signaling pathway. After being exposed to cortisol (50 µM) for 5 days, DA neurons were pre-treated with either: K252a (200 nM), TrkB-Fc Chimera (5 µg/ml), PP2 (10 µM), LY-294002 (10 µM), or Rapamycin (20 nM) for 20 min followed by NAC (2 mM) for 1 h. As for the first experiment, cultures were fixed with 4% paraformaldehyde 72 h after the final treatment. All treatments were tested in duplicate, and experiments were repeated three times. See Supplementary for methodological details.

Human midbrain DA neurons immunocytochemistry and computer-assisted morphological analysis

DA neurons were immunostained with an anti-TH rabbit polyclonal antibody (Santa Cruz Biotechnology) as previously described [21]. The samples were visualized using an Olympus IX51 microscope (Olympus Italia Srl, Italy). Morphometric measurements were performed by a blinded examiner on digitized images using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The morphological indicators of structural plasticity in DA neurons included: (i) the maximal dendrite length, (ii) the number of primary dendrites, (iii) the soma area [21]. Two coverslips per treatment group per experimental replicate were analyzed, providing measurements from at least 50 neurons per group.

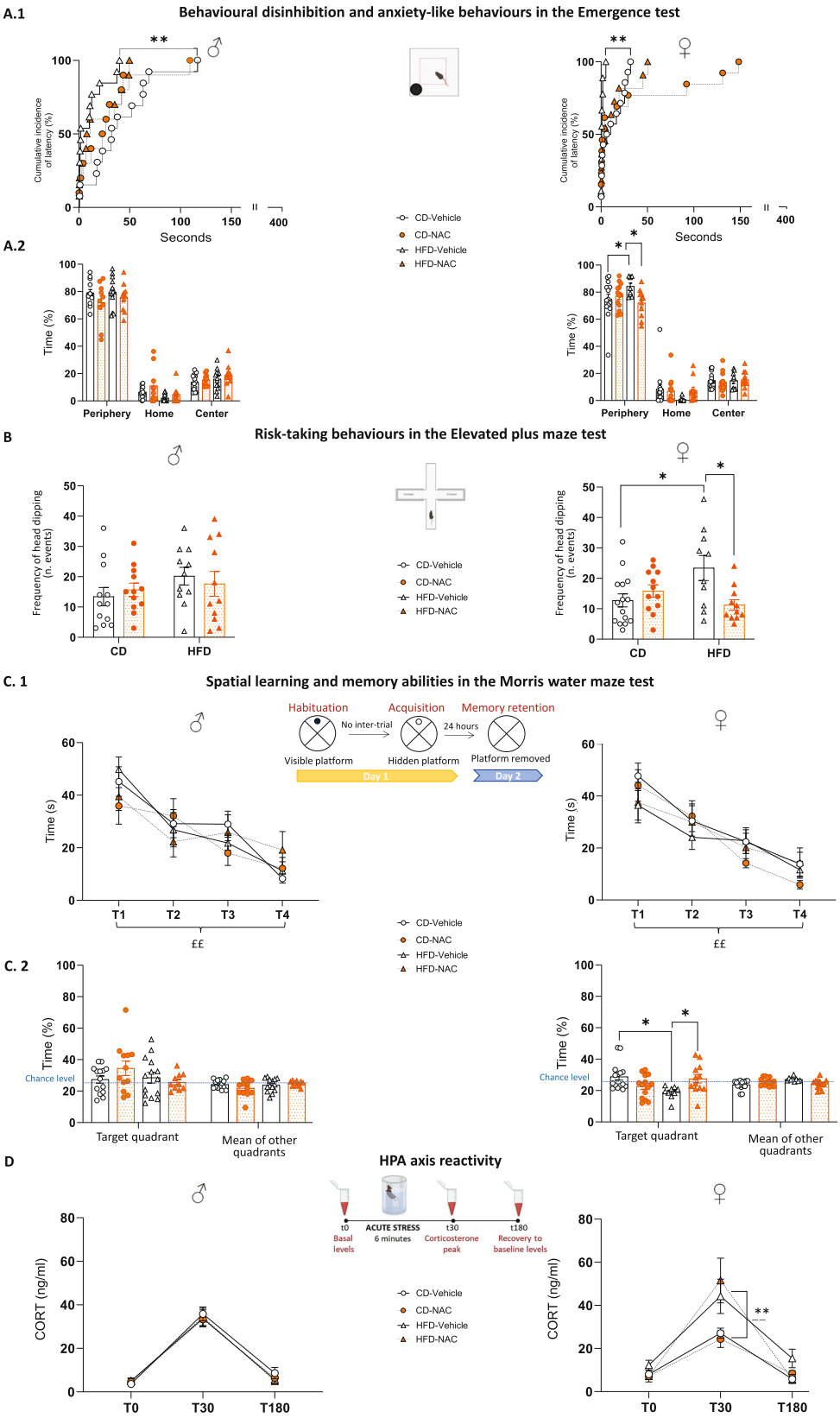
Statistical analysis

The sample size was calculated through the G*Power 3.1 software (www.gpower.hhu.de). Statistical analysis was performed using GraphPad Prism version 10.4 (GraphPad Software, USA). Bartlett's test was used to test the homogeneity of variances among groups. Total corticosterone secretion was calculated using the area under the curve with respect to ground (AUC) formula [44] and was expressed in arbitrary units. Two-way ANOVA was used with prenatal diet (HFD/CD) and prenatal treatment (NAC/Vehicle) as between-subject factors (e.g., AUC, gene expression, iPSC study); two-way repeated measures ANOVA was used with zone or time as a within-subjects factor (e.g., Emergence test, Morris water maze, corticosterone curve, metabolic cages); Šídák's test or Bonferroni's test were used for two-way ANOVA post-hoc comparisons and Tukey's test for two-way repeated measures ANOVA post-hoc comparisons. Data were expressed as mean ± standard error of the mean (SEM). Log-rank (Mantel-Cox) test with Bonferroni correction were used for latencies. This test compares time-to-event between groups without assuming a normal distribution and accounts for performance failures within the cut-off time [45, 46]. Behavioural and molecular data were analyzed using a principal component analysis (PCA); only animals with a complete data-set were included in the analysis. PC scores were analyzed by a two-way ANOVA. A level of probability set at $p < 0.05$ was considered statistically significant. Grubb's test, using 5% significance level critical values, was used to detect outliers [47]. Two-Way ANOVA followed by a Bonferroni's test for multiple comparisons was used for studies in human dopaminergic neurons.

RESULTS

HFD-induced behavioural phenotype and preventive effects of NAC treatment

Prenatal NAC prevented anxiety-like and risk-taking behaviours specifically in female offspring. In the Emergence test, mHFD significantly decreased the latency to emerge from the shelter, regardless of sex (males: $\chi^2 = 8.932$, $p = 0.0028$; females: $\chi^2 = 7.680$, $p = 0.0056$; Fig. 1A.1). Furthermore, mHFD increased speed and distance travelled in females ($F_{1,43} = 4.864$, $p = 0.0328$; $F_{1,43} = 4.920$, $p = 0.0319$; Supplementary Table 3) as well as time spent in the peripheral zone, an effect prevented by NAC ($F_{3,129} = 3.044$, $p = 0.0313$; Fig. 1A.2). In the Elevated plus maze mHFD females showed an increased frequency of head dipping, while prenatal NAC prevented this effect ($F_{1,44} = 8.856$, $p = 0.0047$; Fig. 1B). The analysis of the time spent in the arms of the Elevated plus maze as well as the coping strategies in the Forced swim test revealed no differences (Supplementary Table 4).



Prenatal NAC prevented mHFD-induced impairments in memory retention in adult female offspring. In the Morris water maze test, during the acquisition phase, all mice improved their performance over time (males $F_{3,138} = 25.95$, $p < 0.0001$; females $F_{3,141} = 20.17$; $p < 0.0001$, Fig. 1C.1). In the probe trial, mHFD females showed an impairment in memory retention indicated by reduced time spent in the target quadrant. Interestingly, prenatal NAC effectively prevented this impairment ($F_{1,46} = 10.71$, $p = 0.0020$; Fig. 1C.2). No changes in male offspring were observed.

Fig. 1 HFD-induced behavioural phenotype and preventive effects of NAC treatment. A.1 In the Emergence test, exposure to mHFD reduced the latency to emerge from the shelter in both males and females (males: $\chi^2 = 8.932$, $p = 0.0028$; females: $\chi^2 = 7.680$, $p = 0.0056$; post-hoc: $^{**}p < 0.01$ HFD-Veh vs CD-Veh;). Log-rank (Mantel-Cox) test with Bonferroni correction, $n = 9$–14 mice per group. **A.2** Furthermore, mHFD increased time spent in the peripheral zone specifically in females, while NAC prevented this effect ($F_{3,129} = 3.044$, $p = 0.0313$; post-hoc: $^{*}p < 0.05$ HFD-Veh vs CD-Veh; and HFD-NAC vs HFD-Veh). Two-way repeated measures ANOVA with Tukey's multiple comparisons test, $n = 9$–14 mice per group. **B** In the Elevated plus maze test, NAC prevented the increased frequency of head dipping induced by mHFD, specifically in female offspring ($F_{1,44} = 8.856$, $p = 0.0047$; post-hoc: $^{*}p < 0.05$ HFD-Veh vs CD-Veh; and HFD-NAC vs HFD-Veh). Two-way ANOVA with Sidak's multiple comparisons test, $n = 10$–15 mice per group. **C.1** Evaluation of spatial learning in the acquisition phase revealed that all mice improved their performance over time (males $F_{3,138} = 25.95$, $p < 0.0001$; females $F_{3,141} = 20.17$; $p < 0.0001$; $^{\text{£}\text{£}}p < 0.01$ main effect of time). Two-way repeated measures ANOVA, $n = 10$–15 mice per group. **C.2** Prenatal NAC prevented impairments in memory retention in the probe phase, specifically in mHFD female offspring ($F_{1,46} = 10.71$, $p = 0.0020$; post-hoc: $^{*}p < 0.05$ HFD-Veh vs CD-Veh; $^{*}p < 0.05$ HFD-NAC vs HFD-Veh). Two-way repeated measures ANOVA with Tukey's multiple comparisons test, $n = 10$–15 mice per group. **D** In mHFD females, an enhanced peak of corticosterone release 30 min after stress exposure was observed ($F_{2,86} = 8.445$, $p = 0.0004$, post-hoc: $^{**}p < 0.001$ T30 HFD vs CD). Two-way repeated measures ANOVA with Tukey's multiple comparisons test, $n = 7$–14 mice per group. The data are presented as the mean ± SEM.

Prenatal NAC did not prevent the heightened sensitivity of the HPA axis to acute stress in female offspring exposed to mHFD. An enhanced sex-dependent HPA axis reactivity was found, with increased AUC in mHFD female offspring, ($F_{1,43} = 12.69$; $p = 0.0009$; Supplementary Fig. 2). In detail, over time, we observed an exacerbated corticosterone peak release 30 min after stress in mHFD females ($F_{2,86} = 8.445$, $p = 0.0004$; Fig. 1D). No changes in male offspring were observed.

Prenatal NAC enhanced hippocampal antioxidant defences and prevented mHFD-induced impairments in neuroplasticity markers and excitatory/inhibitory balance

Analysis of antioxidant defenses: We observed a significant effect of treatment on *Nrf-2* expression in both male and female mice ($F_{1,37} = 9.221$, $p = 0.0044$ and $F_{1,41} = 21.61$; $p < 0.0001$, respectively), with a notable interaction "diet × treatment" restricted to females ($F_{1,41} = 4.291$; $p = 0.0446$). Prenatal NAC administration significantly increased *Nrf-2* mRNA levels in males, independently from the prenatal diet (Fig. 2A), an effect that was more pronounced in mHFD females compared to their counterparts ($p = 0.0002$). For *Gclc*, we detected a significant interaction "diet × treatment" in males ($F_{1,32} = 7.552$; $p = 0.0098$; Fig. 2A), with NAC reducing its expression in control mice ($p < 0.05$). In contrast, females exhibited both a significant effect of treatment and an interaction "diet × treatment" ($F_{1,40} = 12.82$; $p = 0.0009$ and $F_{1,40} = 12.02$; $p = 0.0013$ respectively, Fig. 2A), characterised by a marked upregulation of *Gclc* mRNA in HFD-fed mice treated with NAC compared to vehicle-treated controls ($p = 0.0001$).

Analysis of neuroplasticity and excitatory/inhibitory balance: HFD produced a significant and sex-specific down-regulation of *Bdnf* mRNA levels, particularly for Bdnf exon IV in female animals. NAC restored *Bdnf* reduction selectively in mHFD-NAC females (Total Bdnf: $F_{1,45} = 4.819$, $p = 0.0334$; Bdnf exon IV: $F_{1,40} = 9.908$; $p = 0.0031$; Fig. 2B), while NAC alone reduced the expression of the neuroplastic marker in males (Total Bdnf: $F_{1,37} = 11.32$, $p = 0.0018$; Bdnf exon IV: $F_{1,32} = 9.497$; $p = 0.0042$).

We also examined the expression of markers involved in hippocampal functionality in response to a specific stimulus. First, we examined the *Vglut1/Vgat* ratio as a main index of excitatory/inhibitory (E/I) balance. Exposure to mHFD significantly increased such ratio, regardless of sex, while prenatal NAC restored the E/I balance (males $F_{1,33} = 12.69$, $p = 0.0009$; females $F_{1,44} = 18.66$; $p < 0.0001$; Fig. 2C; Supplementary Fig. 3A for independent assessment of *Vglu1* and *Vgat*). Subsequently, we investigated the expression of *SST*, *PVB* and *NPY* labelling distinct populations of GABAergic neurons. mHFD reduced *SST* in both sexes, an effect prevented by NAC (males: $F_{1,30} = 22.37$, $p < 0.0001$; females: $F_{1,38} = 9.806$; $p = 0.0033$; Fig. 2C). As for *NPY*, mHFD reduced its expression in males ($F_{1,32} = 12.86$, $p = 0.0011$), while NAC increased *NPY* expression in combination with mHFD in females ($F_{1,38} = 4.712$; $p = 0.0363$; Fig. 2C). NAC alone resulted in a paradoxical decrease of *SST* and *NPY* in control males. When considering *PVB*, mHFD did not have main effects, while NAC treatment increased it in female offspring ($F_{1,45} = 5.518$; $p = 0.0233$, Fig. 2C). No significant changes were found for *Slc1a3*, a subtype of glutamate transporter present on glial cells (Supplementary Fig. 3A).

Markers of brain metabolic homeostasis: *InsR* and *LepR* were increased in male offspring following prenatal NAC (InsR: $F_{1,38} = 12.90$; $p = 0.0009$; LepR: $F_{1,37} = 9.140$; $p = 0.0045$; Supplementary Fig. 3B), while only mHFD-NAC females showed increased LepR expression ($F_{1,43} = 5.220$; $p = 0.0273$; Supplementary Fig. 3B).

Principal component analysis. The PCA generated six components with eigenvalues > 1 that explained 70% of the total variance. According to a parallel analysis that allows to select the number of PCs to be included by determining the point at which the PCs are indistinguishable from those generated by simulated noise, three main PCs were identified (Supplementary Table 5). In detail, PC1 was positively associated with the mRNA expression of Vglu1, Vgat, NPY, SST, Gclc, Slc1a3, Bdnf (exon IV). The score plot clearly indicated the diet as a main clustering factor with mHFD mice grouped in the negative quadrant and CD mice grouped on the positive quadrant of PC1 (Fig. 3A). Interestingly, prenatal NAC shifted HFD mice (mHFD-NAC) in the positive quadrant, where the control group is found (Fig. 3A). PC2 was positively associated with risk assessment behaviours in the Elevated plus maze, as well as *PVB, Nrf-2* and *InsR* mRNA levels, all linked to oxidative stress, anxiety and metabolic function. PC3 was, by contrast, negatively associated with relevant phenotypes linked to emotionality.

Results from ANOVA performed on PC scores revealed, in the PC1, a reduced score due to mHFD exposure in both sexes that was effectively restored by prenatal NAC selectively in females (males: $F_{1,38} = 13.71$; $p = 0.0007$; females: $F_{1,45} = 14.18$; $p = 0.0005$; Fig. 3B). A reduction in males was also observed as a result of NAC alone (Fig. 3B). As for the PC2, mHFD specifically increased the score in females ($F_{1,45} = 9.771$, $p = 0.0031$), while CD-NAC males only showed an increased score ($F_{1,38} = 5.199$, $p = 0.0283$; Supplementary Fig. 4A). Finally, in the PC3, overall prenatal NAC increased the score in both sexes (males: $F_{1,38} = 5.427$; $p = 0.0252$; females: $F_{1,45} = 8.427$; $p = 0.0057$), while mHFD reduced such parameter only in males ($F_{1,38} = 5.483$; $p = 0.0245$, Supplementary Fig. 4B).

Prenatal NAC prevented the mHFD-driven shift toward fatty acids oxidation in female offspring. We found that mHFD females preferably used fat as fuel, while NAC restored carbohydrates utilization ($F_{47,1081} = 2.002$; $p < 0.0001$; Fig. 4). A similar fat preference was observed in CD-NAC females (Fig. 4). No changes were observed in male offspring. Notably, both males and females showed no differences in body weight (Supplementary Fig. 5A). Circulant leptin was increased in females upon prenatal NAC

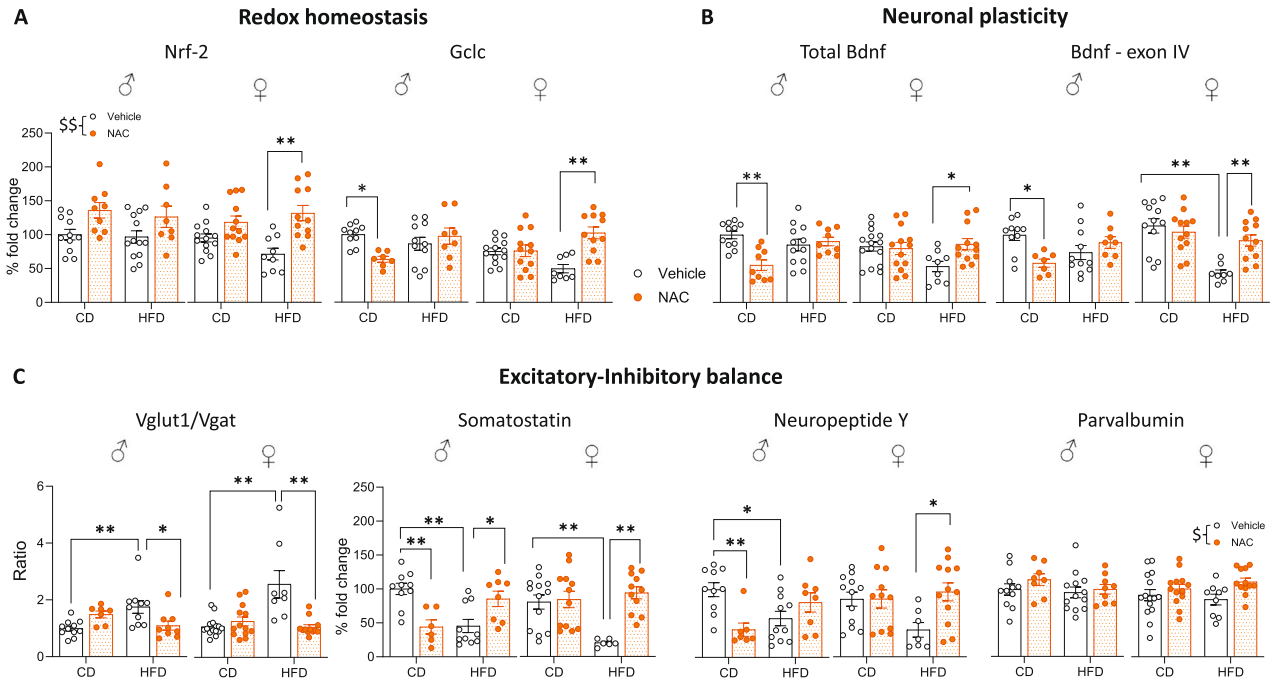


Fig. 2 Prenatal NAC prevented mHFD-induced reduction of hippocampal antioxidant defenses as well as markers of neuroplasticity and excitatory/inhibitory imbalance. A NAC was able to enhance antioxidant defenses (*Nrf-2* and *Gclc*) in mHFD females (Nrf-2: $F_{1,41} = 4.291$; $p = 0.0446$; post-hoc: $^{**}p < 0.01$ HFD-NAC vs HFD-Vehicle; Gclc: $F_{1,40} = 12.02$; $p = 0.0013$; post-hoc: $^{**}p < 0.01$ HFD-NAC vs HFD-Vehicle). Prenatal NAC overall increased *Nrf-2* expression, ($F_{1,37} = 9.221$; $p = 0.0044$; $^{\$\$}p < 0.01$ main effect of treatment), while NAC alone decreased *Gclc* in male offspring ($F_{1,32} = 7.552$; $p = 0.0098$; post-hoc: $^{*}p < 0.05$ CD-NAC vs CD-Vehicle). **B** mHFD reduced hippocampal levels of *Bdnf* exon IV in female offspring, while prenatal NAC was able to prevent this alteration (Total Bdnf: $F_{1,45} = 4.819$, $p = 0.0334$; post-hoc: $^{*}p < 0.05$ HFD-NAC vs HFD-Vehicle; Bdnf exon IV: $F_{1,40} = 9.908$; $p = 0.0031$; post-hoc: $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; and HFD-NAC vs HFD-Vehicle). NAC alone reduced the expression of the neuroplastic marker in males (Total Bdnf: $F_{1,37} = 11.32$, $p = 0.0018$; post-hoc: $^{**}p < 0.001$ CD-NAC vs CD-Vehicle; Bdnf exon IV: $F_{1,32} = 9.497$; $p = 0.0042$; post-hoc: $^{*}p < 0.05$ CD-NAC vs CD-Vehicle). **C** Both male and female offspring showed increased *Vglut1/Vgat* ratio and reduced *Somatostatin* mRNA expression, alterations that were effectively prevented by NAC (males $F_{1,33} = 12.69$, $p = 0.0009$; post-hoc: $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; $^{*}p < 0.05$ HFD-NAC vs HFD-Vehicle; females $F_{1,44} = 18.66$; $p < 0.0001$; post-hoc: $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; HFD-NAC vs HFD-Vehicle). *Somatostatin* was reduced by mHFD in both sexes, an effect that was effectively prevented by NAC (males: $F_{1,30} = 22.37$, $p < 0.0001$; post-hoc: $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; $^{*}p < 0.05$ HFD-NAC vs HFD-Vehicle; females: $F_{1,38} = 9.806$; $p = 0.0033$; post-hoc: $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; HFD-NAC vs HFD-Vehicle). *NPY* was reduced in mHFD males ($F_{1,32} = 12.86$, $p = 0.0011$; post-hoc: $^{*}p < 0.05$ HFD-Vehicle vs CD-Vehicle), while it was increased in females exposed to mHFD in combination with NAC ($F_{1,38} = 4.712$; $p = 0.0363$; post-hoc: $^{*}p < 0.05$ HFD-NAC vs HFD-Vehicle). NAC alone resulted in a paradoxical decrease of *Somatostatin* and *NPY* in control males ($^{**}p < 0.01$ CD-NAC vs CD-Vehicle) as well as in an increased expression of *Parvalbumin* in females ($F_{1,45} = 5.518$; $p = 0.0233$; $^{\$}p < 0.05$ main effect of treatment). Two-way ANOVA with Sidak's multiple comparisons test, $n = 6$–15 mice per group. The data are presented as the mean ± SEM.

($F_{1,34} = 7.223$; $p = 0.0111$), while in male offspring a trend of NAC in buffering mHFD-induced increase was found ($F_{1,32} = 4.834$; $p = 0.0352$; Supplementary Fig. 5B).

Pre-exposure to NAC antagonized the inhibitory effects of glucocorticoids on neuronal plasticity in human midbrain DA neurons differentiated from iPSC. Since prenatal NAC did not affect overall neuroendocrine activity in mHFD-exposed mice, we tested whether NAC might exert its protective effects directly on glucocorticoid target responsivity. For these reasons, we tested, *in vitro*, the effects on NAC pre-exposure using a published translational protocol that allows quantification of neuronal plasticity impairment produced by sub-chronic glucocorticoid exposure in human iPSC-derived midbrain DA neurons [21].

In the first in vitro experiment, a dose response curve of NAC (0.02–20 mM) was studied in human midbrain DA neurons previously exposed to 50 µM human cortisol (HC) or to vehicle for 5 days (Fig. 5A). Previously, we showed that various doses of sub-chronic HC significantly reduce the dendritic arborization and soma size of human midbrain DA neurons with reliable effects at 50 µM [21]. The NAC results showed a significant inverted U profile for the three neuronal plasticity parameters, i.e., maximal dendritic length (HC main effect $F_{1990} = 347.2$; $p < 0.0001$, NAC main effect $F_{4990} = 99.3$; $p < 0.0001$, Interaction $F_{4990} = 26.7$; $p < 0.0003$), primary dendrite numbers (HC main effect $F_{1990} = 20.6$; $p < 0.0001$, NAC main effect $F_{4990} = 19.6$; $p < 0.0001$, Interaction $F_{4990} = 2.9$; $p = 0.021$), and soma area (HC main effect $F_{1490} = 28.1$; $p < 0.0001$, NAC main effect $F_{4490} = 26.6$; $p < 0.0001$, Interaction $F_{4490} = 3.4$; $p = 0.009$). Post-hoc analysis indicated that NAC at the concentration of 0.2 and 2 mM effectively restored the impairment produced by HC (Fig. 5B–D and Supplementary Fig. 6A), while NAC 0.02 mM was ineffective. Interestingly, the higher dose of 20 mM produced impairment in both HC and vehicle group for maximal dendritic length, primary dendritic dendrites and soma areas (Fig. 5B–D and Supplementary Fig. 6A). In these experiments, ketamine 0.5 µm was used as an active internal control at the concentration that was already known to counteract the sub-chronic HC effects on neuronal plasticity [21]. The HC-counteracting effects of NAC 0.2 and 2 mM were similar to those of ketamine on all neural plasticity parameters. In the second experiment, we investigate the role of the BDNF-mTOR pathway in mediating the restoring properties of NAC against cortisol-induced impairment of neuronal plasticity in human midbrain DA neurons. At the end of the 5-day HC exposure period, the human midbrain DA neurons were pre-treated with a single dose of BDNF pathway inhibitors (BDNFi) already tested in the same paradigm [43] for 20 min, followed by NAC 2 mM for 1 h. Six BDNFi were targeting, respectively, the molecule itself (TrkB-Fc chimera), the BDNF-TrkB receptor functions (K252a), and the intracellular pathways leading to

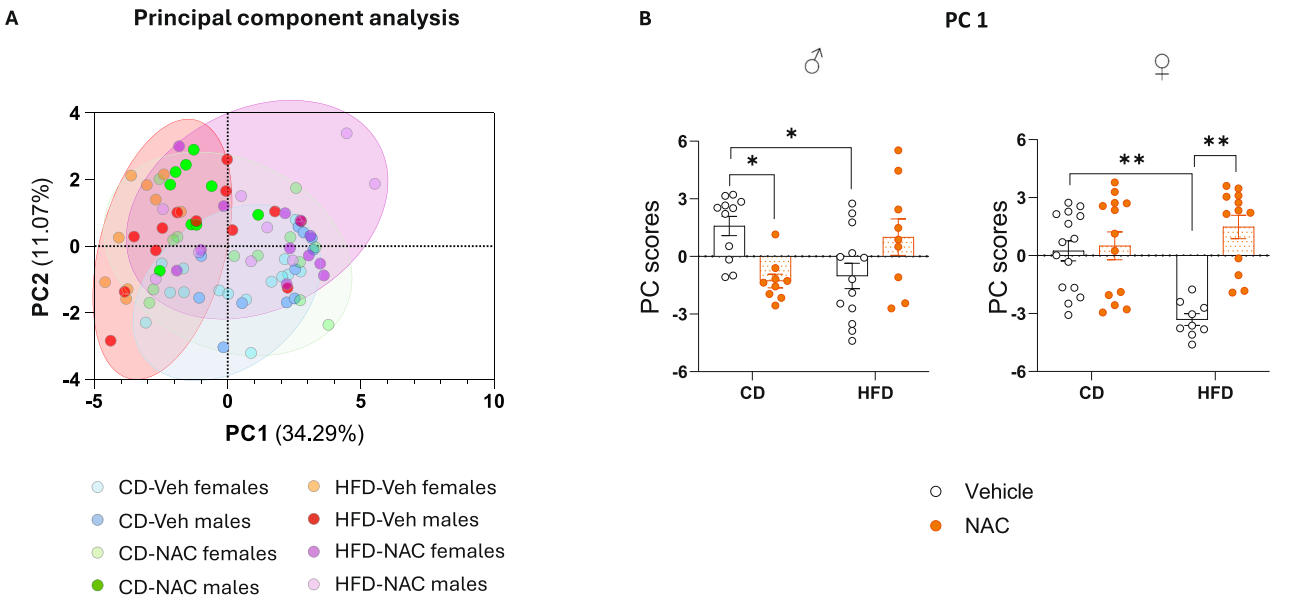


Fig. 3 Principal component analysis. A The score plot of PC1 and PC2 shows subjects clustering: the blue circle groups CD-Vehicle mice; the green circle groups CD-NAC mice; the red circle groups HFD-Vehicle mice; the purple circle groups HFD-NAC mice, regardless of sex. **B** Analysis of PC1 scores revealed that mHFD reduced its values in both sexes, while prenatal NAC prevented such reduction, specifically in female offspring (males: $F_{1,38} = 13.71$; $p = 0.0007$; post-hoc: $^{*}p < 0.05$ HFD-Vehicle vs CD-Vehicle, $^{*}p < 0.05$ CD-NAC vs CD-Vehicle; females: $F_{1,45} = 14.18$; $p = 0.0005$; $^{**}p < 0.01$ HFD-Vehicle vs CD-Vehicle; $^{**}p < 0.01$ HFD-NAC vs HFD-Vehicle). Two-way ANOVA with Sidak's multiple comparisons test, $n = 9$–15 mice per group. The data are presented as the mean ± SEM.

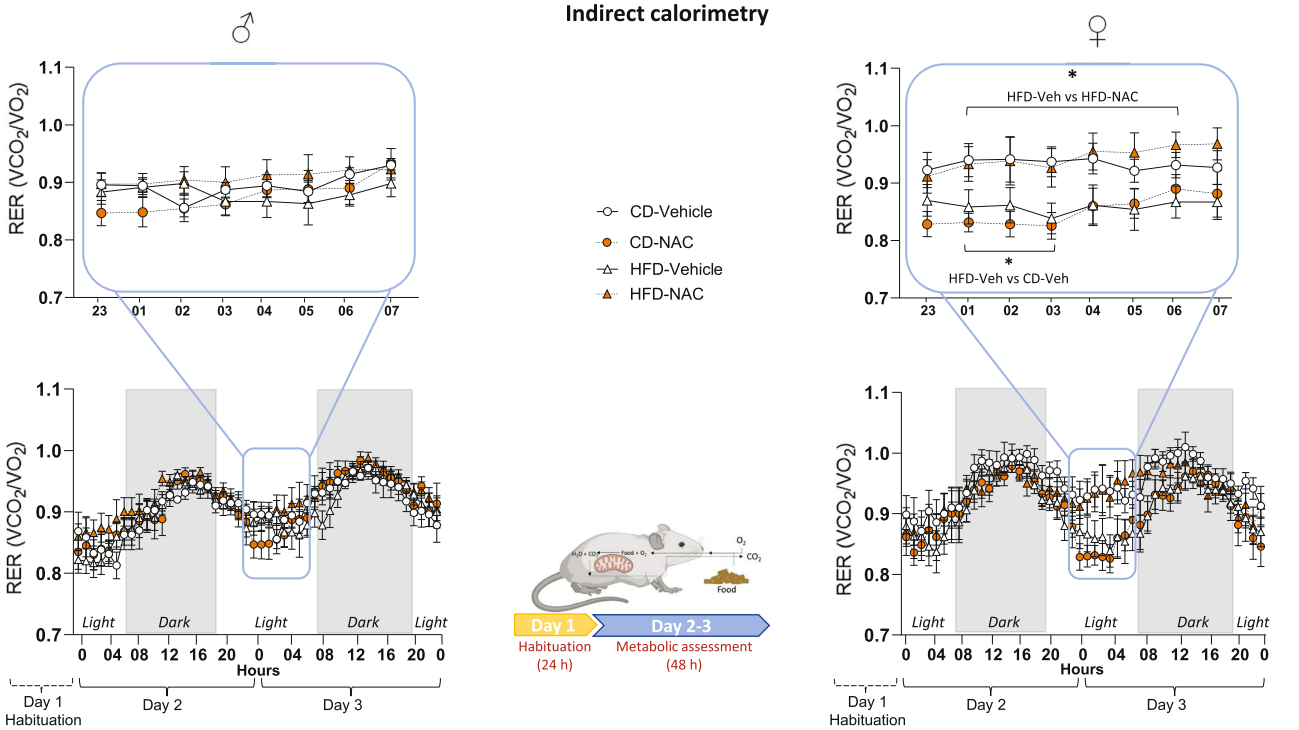
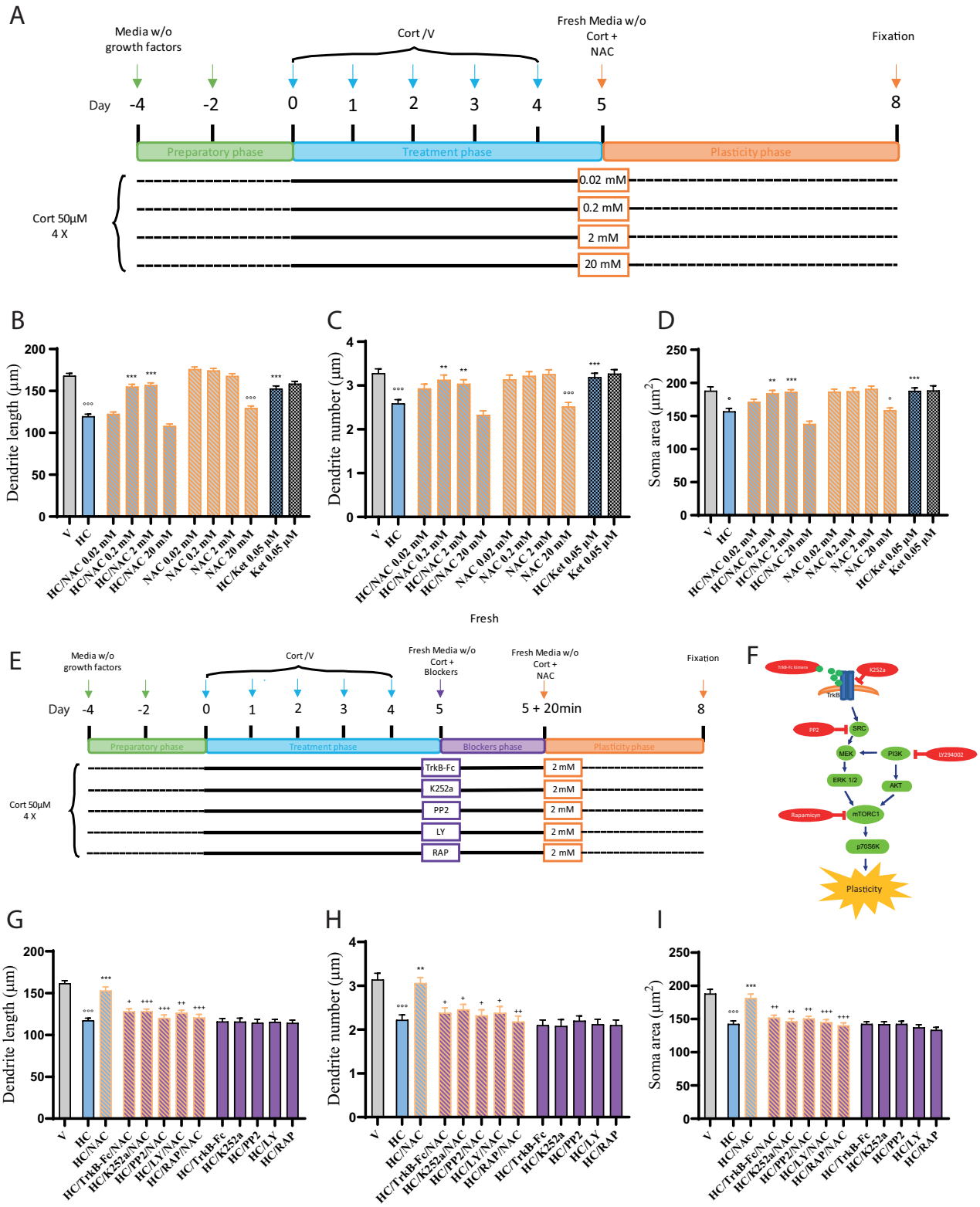


Fig. 4 Prenatal NAC prevented the mHFD-driven shift toward fatty acids oxidation in female offspring. Female offspring exposed to mHFD preferably used fatty acids as substrate to produce energy (RER ~ 0.8), instead of carbohydrates, while prenatal NAC effectively rescued such alterations (RER ~ 1.0) ($F_{47,1081} = 2.002$; $p < 0.0001$; post-hoc: $^{*}p < 0.05$ HFD-Vehicle vs CD-Vehicle from 01 to 03 of Day 2; HFD-Vehicle vs HFD-NAC from 01 to 06 of Day 2). Two-way repeated measures ANOVA with Tukey's multiple comparisons test, $n = 6$–7 mice per group. The data are presented as the mean ± SEM.

Akt-mTOR phosphorylation cascade activation (i.e., PP2, LY294002 and Rapamycin), as represented in Fig. 5E, F. All BDNFi blocked the neuroplasticity-enhancing effects of NAC on all neuroplasticity parameters when human DA neurons were exposed to HC (Fig. 5G–I and Supplementary Fig. 6B), i.e., maximal dendritic length (HC/NAC main effect $F_{1588} = 45.3$, $p < 0.000$; BDNFi main effect $F_{5588} = 6.7$, $p < 0.0001$; Interaction $F_{5588} = 4.8$, $p = 0.0003$), primary dendrite numbers (HC/NAC main effect $F_{1588} = 20.7$, $p < 0.0001$;



BDNFi main effect $F_{5588} = 3.9$, $p = 0.002$; Interaction $F_{5588} = 2.4$, $p = 0.034$), and soma area (HC/NAC main effect $F_{1585} = 30.1$, $p < 0.0001$; BDNFi main effect $F_{5585} = 8.4$, $p = 0.0001$; Interaction $F_{5585} = 4.7$, $p = 0.0003$). These findings indicate a critical role for BDNF in mediating the restorative effects of NAC on neuronal plasticity in human midbrain DA neurons from a female donor, supporting observations made in mouse offspring.

DISCUSSION

This study strengthens the notion that, like maternal stress, prenatal exposure to metabolic stressors may increase susceptibility to mental disorders. The data highlight a significant sexual dimorphism in the effects of a mHFD on neurobehavioural outcomes in the offspring, aligning with recent findings that indicate greater vulnerability in females [18, 48]. In addition, the

Fig. 5 Study of the effects of NAC on human midbrain DA neurons pre-treated with cortisol and analysis of the involvement of the BDNF-TrkB pathway. A Experimental design of dose response curve of NAC restoring properties of the impaired neuroplasticity produced by cortisol. **B**–**D** Morphometric quantification of **B** maximal dendrite length, **C** primary dentrite number and **D** soma area performed on human DA neurons previously exposed to cortisol (50 μM) or vehicle for 5 days, followed by exposure to various concentrations of NAC (0.02, 0.2, 2, or 20 mM) or vehicle; low dose of ketamine (0.05 μM) was used as internal active control. **E** Experimental design to study the role of BDNF pathway in mediating the restoring properties of NAC. **F** Cartoon representing the BDNF-mTORC1 pathway and the sites of action of the inhibitors used to study it. **G**–**I** Inhibition of NAC restoring properties of impaired structural plasticity produced by cortisol following pretreatment (20 min) with a TrkB-Fc Chimera (5 μg/ml), the TrkB phosphorylation inhibitor K252a (200 nM), the Src phosphorylation inhibitor PP2 (10 μM), the PI3 Kinase inhibitor LY294002 (10 μM) and mTORC1 blocker rapamycin (20 nM). Data are expressed as mean ± SEM ***$p < 0.001$, **$p < 0.01$, *$p < 0.5$ vs HC; $p < 0.001$, $p < 0.01$, $p < 0.05$ vs. vehicle, post hoc Bonferroni's.

present results show that the adverse long-term consequences of mHFD can be prevented by the concurrent administration of prenatal antioxidants, such as NAC.

Several evidence indicate that NAC may exert its antioxidant and anti-inflammatory properties both on the mother and the fetus. On the one hand, we have shown that NAC can buffer oxidative stress in maternal blood [17], thus alleviating the burden of mHFD in the intrauterine *milieu*. On the other hand, it has also been shown that NAC can cross both the placenta and the fetal blood-brain barrier, reaching the developing brain and exerting neuroprotective action [49].

In this study, mice prenatally exposed to mHFD showed a behavioural phenotype characterised by increased anxiety-like behaviour, impulsivity and cognitive impairment. In particular, in the Emergence test, all mice were characterised by increased exploration and risky behaviours. Female offspring exposed to mHFD exhibited an increased frequency of head dipping in the Elevated plus maze and displayed anxiety-like behaviours, increasing the time spent in the periphery of the arena in the Emergence test. This behavioural phenotype has been described in adult mice following prenatal exposure to mild stressors, which underlies the involvement of the DA system [50, 51], underscoring the powerful and long-lasting effects of the nutritional stressor. Importantly, prenatal NAC treatment was able to counteract these changes, reducing the frequency of head dipping in the Elevated plus maze test and the time spent in the periphery of the Emergence test. This combination of behavioural disinhibition and high emotional reactivity also aligns with the increased HPA axis reactivity observed in mHFD females in response to acute stress. Consistent sex-dependent effects have been found in children from mothers with elevated BMI [52, 53]. Sex differences in the behavioural phenotype also extend to the cognitive domain, prenatal NAC treatment preventing mHFD-induced impairments in spatial memory retention in female offspring. This effect was mirrored by the reduction of hippocampal *Bdnf* expression levels observed specifically in female offspring, normalized by prenatal NAC. This piece of data confirms and expands our previous findings showing a similar decrease in *Bdnf* hippocampal expression in adolescent females exposed to mHFD [18], and suggests that the *Bdnf* signalling pathway is a critical driver of neuronal plasticity in the context of metabolic stressors.

Importantly, in this study, we were able to show that the protective effects of NAC on BDNF expression are mechanistically linked to the blockade of glucocorticoids resulting from in-utero exposure to the mHFD. This is especially relevant for fetal neurodevelopment and critical for the progression into depressive disorders later in life [19, 20, 54, 55]. In the present study, we used a translational human cellular model based on the differentiation of iPSC from female donors into midbrain DA neurons known to be sensitive to cortisol [21, 23, 28]. We showed that NAC can antagonise, in a dose-dependent manner, the neural plasticity deficits produced by cortisol in human midbrain DA neurons differentiated in vitro at a stage compatible with the perinatal phenotype. Using appropriate pharmacological tools, we demonstrate that NAC effects are dependent upon the integrity of the BDNF-mTOR phosphorylation pathway. This cascade is a critical molecular driver for neuronal survival and plasticity in several brain structures, including the cortex and hippocampus, and it is known to be attenuated in depression and activated by ketamine, a clinically active antidepressant acting via glutamatergic neurotransmission [43, 56, 57].

We found that exposure to mHFD can also lead to an E/I imbalance at the hippocampal level, as shown by the increased *Vglut1/Vgat* ratio, and to a significant reduction of *SST* and *NPY* mRNA levels. We suggest that sex-specific mechanisms may underlie the E/I imbalance in mHFD exposed mice. Indeed, the increased E/I ratio observed in males is primarily driven by a reduction in *Vgat* mRNA levels, while in females, reductions in both *Vglut1* and *Vgat* expression were observed indicating differential susceptibility of the glutamatergic and GABAergic systems in the two sexes. Disrupted E/I balance has been also observed across a broad range of neuropsychiatric disorders, including depression, anxiety and schizophrenia [58–60]. Notably, prenatal NAC restored these neurotransmitter-related alterations, possibly providing cysteine for the activation of the cystine/glutamate antiporter located on astrocytes, which modulates both glutamate and GABA levels [61–63]. These preventive effects of NAC are likely to be associated with *Nrf-2* mediated cell signaling as we found increased hippocampal *Nrf-2* and *Gclc* levels in mHFD-NAC females compared to the mHFD vehicle group, as also suggested in a mouse model of LPS-induced toxicity [64].

While both male and female offspring exhibited molecular alterations in the hippocampus, our data suggest that females display greater phenotypic vulnerability to mHFD exposure, consistent with our previous findings in adolescent animals [18]. Notably, females showed more pronounced changes across multiple molecular markers, including a specific reduction in *Bdnf* expression that was not observed in males. This distinct molecular profile implies that the behavioural deficits observed exclusively in females may arise from the cumulative, and possibly synergistic, disruption of multiple pathways.

To characterise more thoroughly the greater vulnerability of female offspring exposed to mHFD, we performed an overall statistical analysis (PCA) which revealed strong preventive effects of maternal antioxidant NAC specifically in females. Conversely, the modulatory effects in males were less consistent, showing variability in response to NAC treatment. This finding emphasizes that the protective effects of maternal NAC are notably stronger in females. This may be attributed to sex-specific mechanisms that influence biological differences in the regulation of redox homeostasis between males and females [65] highlighting the importance of considering sex as a key factor when assessing the efficacy of prenatal antioxidants.

As the effects of a prenatal HFD are expected to affect the metabolic programming of the offspring, we characterised indirect calorimetry to assess substrate utilization for energy consumption in metabolic cages. Also in this case, females appeared more affected than males. In particular, mHFD exacerbated the dropping of RER values during the light phase in female offspring, NAC restoring the physiological oxidation of carbohydrates. It is possible to hypothesize that mHFD, by

inducing redox imbalance during early developmental stages, may predispose mitochondria towards a maladaptive respiratory efficiency, as shown in response to chronic stress [66], ultimately affecting oxidative phosphorylation of macronutrients. In addition, it has been shown that glucocorticoids, which are excessively released in our mHFD females, enhance mitochondrial fatty acid oxidation, thereby facilitating the switch from carbohydrates to fatty acids as the primary energy substrate [67].

Although the effects of NAC were always positive when administered in combination with a condition of high oxidative stress (i.e., mHFD), we observed some negative effects in "healthy" mouse controls. This phenomenon can be partially explained by results from the in vitro model on DA neurons differentiated from human iPSC showing that, in the absence of stress, high NAC concentrations produced negative effects, inhibiting dendritic arborization.

Overall, exposure to mHFD resulted in distinct sex-dependent effects in the adult offspring, with female mice exhibiting more pronounced consequences. These effects included behavioural and hippocampal dysfunctions coupled with neuroendocrine, metabolic and redox dysregulations. Combined with previous evidence showing immune dysregulations in adolescent female mice [18], our data suggest that mHFD produces immune-metabolic-neuroplasticity dysregulations reminiscent of a clinical subtype of major depression described in female patients [17, 68]. Overall, the specific rescuing effects exerted by prenatal NAC treatment on these dysregulations highlight oxidative stress as a potential future target for psychiatric disorders with an immune-metabolic-neuroplasticity component, such as major depression or bipolar disorders.

DATA AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data are available from authors upon request.

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AUTHOR CONTRIBUTIONS

CM contributed to planning the experimental design, supervised animal experiments, and drafted the initial version of the paper; MS performed in vivo experiments and statistical analysis; KCC, VB and BC performed ex vivo analyses; JB performed in vitro analyses; MAR contributed to draft the experimental design, supervised molecular analysis and contributed to writing the paper; GC contributed to draft the experimental design, supervised cellular analysis and contributed to writing the paper; AB contributed to the work plan and the paper draft; FC acquired the funds, conceptualized the work and contributed to writing the paper. All authors approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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