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Activity-based anorexia (ABA) model: effects on brain neuroinflammation, redox balance and neuroplasticity during the acute phase

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Abstract:	Several evidence suggest that immuno-inflammatory responses are involved in the pathogenesis of anorexia nervosa (AN). Herein we investigate the possible alteration of key mediators of inflammation, redox balance, and neuroplasticity in the brain of rats showing an anorexic-like phenotype. We modeled AN in adolescent female rats using the activity-based anorexia (ABA) paradigm and measured gene expression levels of targets of interest in the prefrontal cortex (PFC) and dorsal hippocampus (DH). We observed reduced mRNA levels of pro-inflammatory cytokines IL-1β and TNF-α, the inflammasome NLRP3, and the microglial marker CD11b in both PFC and DH of ABA animals. Conversely, the mRNA of IL-6, which acts as both a pro-inflammatory and anti-inflammatory cytokine, was increased. Moreover, we observed an overall upregulation of different antioxidant enzymes in PFC, while their profile was not affected or opposite in the DH except for MT1α. Interestingly, ABA animals showed elevated levels of the neuroplasticity marker BDNF in both PFC and DH. Our data indicate that ABA induction is associated with anatomical-specific cerebral alteration of mediators of neuroinflammation, oxidative balance and neuroplasticity. Although more research should be conducted, these results add important information about the role of these systems in the complex of AN etiopathogenesis.
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Dear Editor

Please find our manuscript entitled: "**Activity-based anorexia (ABA) model: effects on brain neuroinflammation, redox balance and neuroplasticity during the acute phase**", which we would like to submit for publication in Neurochemistry International, as Original Article.

The present study investigates the possible alteration of key mediators of inflammation, redox balance, and neuroplasticity in the brain of rats subjected to the activity-based anorexia model which currently represents the most well-known and validated animal model of Anorexia Nervosa.

All authors have exercised due care in ensuring the integrity of the work and do not have potential conflict of interest.
The work described has not been published previously and it is not under consideration for publication elsewhere.

Thank you in advance for your kind consideration.
Yours sincerely,

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Declaration of Interest Statement

The authors declare no conflicts of interest.

HIGHLIGHTS

-The ABA model is associated with altered neuroinflammation, oxidative stress and neuroplasticity.

-ABA animals show reduced levels of IL-1β, TNF-α, NLRP3, and CD11b in both PFC and DH.

-The mRNA levels of the cytokine IL-6 are increased in the PFC and DH of ABA rats.

-ABA animals present an overall upregulation of antioxidant enzymes in the PFC.

-Elevated BDNF levels were observed in both the PFC and DH in ABA rats.

Title

Activity-based anorexia (ABA) model: effects on brain neuroinflammation, redox balance and neuroplasticity during the acute phase

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ABSTRACT

Several evidence suggest that immuno-inflammatory responses are involved in the pathogenesis of anorexia nervosa (AN). Herein we investigate the possible alteration of key mediators of inflammation, redox balance, and neuroplasticity in the brain of rats showing an anorexic-like phenotype. We modeled AN in adolescent female rats using the activity-based anorexia (ABA) paradigm and measured gene expression levels of targets of interest in the prefrontal cortex (PFC) and dorsal hippocampus (DH). We observed reduced mRNA levels of pro-inflammatory cytokines IL-1β and TNF-α, the inflammasome NLRP3, and the microglial marker CD11b in both PFC and DH of ABA animals. Conversely, the mRNA of IL-6, which acts as both a pro-inflammatory and anti-inflammatory cytokine, was increased. Moreover, we observed an overall upregulation of different antioxidant enzymes in PFC, while their profile was not affected or opposite in the DH except for MT1α. Interestingly, ABA animals showed elevated levels of the neuroplasticity marker BDNF in both PFC and DH. Our data indicate that ABA induction is associated with anatomical-specific cerebral alteration of mediators of neuroinflammation, oxidative balance and neuroplasticity. Although more research should be conducted, these results add important information about the role of these systems in the complex of AN etiopathogenesis.

Keywords: Anorexia nervosa, activity-based anorexia, neuroinflammation, cytokines, oxidative stress, neuroplasticity.

Abbreviations:

AN, Anorexia Nervosa;
ABA, activity-based anorexia;
PFC, prefrontal cortex;
DH, dorsal hippocampus;
IL-1β, interleukin-1 beta;
IL-6, interleukin-6;
IL-15, interleukin-15;
TNF-α, tumor necrosis factor-alpha;
TGF-β, transforming growth factor-beta;
PBMCs, peripheral blood mononuclear cells;
BDNF, Brain-Derived Neurotrophic Factor;
NLRP3, NLR family Pyrin domain containing 3;
CD11B, CD11 Antigen-Like Family Member B;
MT1α, Metallothionein-1-alpha;
SRNX1, Sulfiredoxin-1;
CAT, Catalase;
GPX1, Glutathione Peroxidase-1;
GPX4, Glutathione Peroxidase-4;
Arc, activity-regulated cytoskeleton-associated protein.

1. Introduction

Inflammation is supposedly critical for the development of eating disorders, including anorexia nervosa (AN). Changes in immune-inflammatory states were described in both anorexic patients and in AN in vivo animal model (Butler et al., 2021). Specifically, clinical research reported increased serum levels of interleukin-1 beta (IL-1β), IL-6, IL-15, and tumor necrosis factor-alpha (TNF-α), along with significantly lower concentration of transforming growth factor-beta (TGF-β) in affected individuals (Solmi et al., 2015; Dalton et al., 2018). Correspondingly, an increased release of pro-inflammatory cytokines was described in peripheral blood mononuclear cells (PBMCs) isolated from AN patients, then normalized upon refeeding (Vaisman et al., 1991, Allende et al., 1998). Moreover, a recent proteomics study reported an aberrant profile of inflammatory-related proteins in the plasma of AN, characterized by both reduction and upregulation of different inflammatory mediators but limited to an active stage of AN (Nilsson et al., 2020).

At preclinical level, we recently reported impaired brain arachidonic acid-related metabolic pathways resulting in the overproduction of several pro- and anti-inflammatory eicosanoids in corticolimbic areas of animals subjected to induction and recovery from the activity-based anorexia (ABA) rodent model of AN (Collu et al., 2020). Moreover, ABA resulted in increased TNF-α and IL-1β mRNA levels in intestinal mucosa, together with IL-1β and IL-1 receptor type 1 (IL-1R1) in the hypothalamus, suggesting gut-brain axis involvement in the development of a pathological phenotype (Belmonte et al., 2016). In the dehydration-induced model, increased reactive microglial cells and expression of TNF-α, IL-6 and IL-1β were observed in the hippocampus and prefrontal cortex of young female rats (Ragu-Varman et al., 2019; Reyes-Ortega et al., 2020). Neuroinflammation was also described in the anx/anx mouse model, reporting selective microglial activation and overexpression inflammatory genes in the hypothalamus (Lachuer et al., 2005).

Additionally, deficits in oxidative balance might contribute to the progression and severity of AN, being these associated with several pathophysiological processes, including inflammation (Morgan et al., 2011). Meta-analyses show that AN is correlated with increased redox mediators in patients and weight restoration improves the oxidative state, although whether these processes represent causative or maintaining factors still needs to be addressed (Solmi et al., 2015; Solmi et al., 2016). A recent study shows increased oxidative state in patients' plasma, urine, and saliva (Kovalčíková et al., 2021). Similarly, ABA-exposed female rats show enhanced plasma oxidative stress with decreased levels of cysteine and glutathione in the medial prefrontal cortex, an effect remedied after weight restoration (Hurley et al., 2021).

Additionally, neuroinflammation is well-known to affect neuroplasticity. Specifically, reduced levels of the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) paralleled a chronic inflammatory state in many disorders (Lima Giacobbo et al., 2019). Accordingly, Keeler and colleagues recently found that reduced BDNF serum levels positively correlated with IL-8, eotaxin-3 and TNF-α, while negatively correlated with IL-16 in AN patients (Keeler et al., 2022). Moreover, BDNF was reportedly altered in the medial prefrontal cortex and amygdala of ABA-exposed rats, although discrepancies were observed between its peripheral and central modulation and between its transcriptional and translational profile (Ho et al., 2016; Mottarlini et al., 2022).

The current study aims to investigate immune system contribution in the acute/induction phase of the anorectic phenotype recapitulated in young adolescent female rats using the ABA paradigm. We assessed gene expression of pro- and anti-inflammatory markers (IL-6, IL-1β, TNF-α, NLRP3, CD11B and TGF- β) in the prefrontal cortex (PFC), as well as, in the dorsal hippocampus (DH), two key brain regions involved in the pathophysiology of AN and strongly interconnected with each other (Fuglset et al., 2016; Sigurdsson et al., 2016). Additionally, considering the crosstalk between neuroinflammation, redox balance, and neuroplasticity, we investigated if and how the observed inflammatory alterations were associated with changes in different antioxidant genes (GPX1, GPX4, MT1α, CAT) and BDNF transcripts (Total BDNF, BDNF long, BDNF IV, BDNF VI). The possible correlation of these markers with eating behavior and physical activity was evaluated

2.Methods and materials

2.1 Animals

Thirty-two adolescent (PND ~ 45) female Sprague-Dawley rats (Envigo, Italy) weighing 125-150g at the beginning of the study were used. Animals were housed, four per cage, in a climate-controlled room maintained on a reversed 12h/12h light/dark cycle (lights on at 12:00 a.m. and off at 12:00 p.m.) with 21 ± 2°C temperature and 60% humidity and fed with standard rat chow and ad libitum water available throughout the entire duration of the study. All procedures and experiments were performed in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments at the University of Cagliari (Sardinia, Italy) and the Italian Department of Health (452/2019-PR).

2.2 Experimental Design and ABA induction

The ABA induction was performed as previously described (Collu et al., 2020; Fuglset et al., 2016) (Fig. 1). Briefly, after one week of acclimatization to the animal facility, rats were individually housed in standard polycarbonate cages equipped (or not) with a running wheel (35 cm in diameter, 11 cm in width) associated to a manually operated lock and connected to a digital magnetic LCD revolution counter which continuously monitored whole revolutions (1-wheel revolution equals 1.1 m) (Ugo Basile, Varese, Italy). Animals were adapted to the new housing condition for 7 days with ad libitum food and running wheel access (when applicable). On the last day of the adaptation period, rats were divided into four experimental groups:
1.Control (CTRL) rats had 24h-free access to food, but no access to the running wheel;
2.Exercise (EXE) rats had 24h-free access to food and to the running wheel;
3.Food restriction (RESTR) rats were allowed food access 1.5 h/day and with no access to the running wheel;
4.ABA rats were allowed food access 1.5 h/day and 22.5 h/day free access to the running wheel.

All rats were maintained on their respective conditions throughout 6 days (ABA induction for ABA rats). ABA and RESTR groups had access to food at the onset of the 12 h dark cycle. Daily body weight, 1.5h food intake (ABA and RESTR groups), 24h food intake (CTRL and EXE groups) and running wheel activity (RWA) (EXE and ABA groups) were monitored daily 30 min before the start of the 12h dark cycle.
Diet: standard rat chow pellets: 3% kcal from fat, 61% kcal from carbohydrate, 16% kcal from protein, 0% moisture, containing 2.9 kcal/g, Safe, France.

2.3 Tissue collection

All animals were euthanized by decapitation at the end of the 12h light phase on day 6 , the brains were collected and cerebral areas of interest were dissected according to the Paxinos and Watson atlas (Paxinos, 2006) [i.e., PFC (Bregma: +3.72 to +2.52) and DH (Bregma: −2.16 to −4.08)] using a pre-cooled rat brain slicer with cold forceps and blades (Scherma et al., 2023). Dissected brain regions were disposed on an aluminum plate over dry ice until frozen and then stored at -80°C until following analysis.

2.4 Gene expression analysis

Total RNA was isolated from PFC and DH using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for quantitative retro-transcriptase real-time polymerase chain reaction (qRT-PCR) as previously reported (Spero et al., 2022) to assess the mRNA levels for inflammatory markers (i.e., TNF-α, IL-1β, NLRP3, IL-6, TGF-β, CD11B), oxidative balance mediators (i.e., MT1α, SRNX1, CAT, GPX1, GPX4) and brain-derived neurotrophic factor transcripts (i.e., total BDNF BDNF 3'UTR long, BDNF IV, BDNF VI) for both brain areas. Probes and primers were purchased from Eurofins Genetics and Life Technologies and are listed in Table 1.
Briefly, samples were loaded in triplicate in 96-wells plates as a multiplexed reaction with an internal control (β-Actin). Thermal cycling began with incubation at 50°C for the reverse transcription reaction, followed by 5 min at 95°C for the activation of the Taq Polymerase. Then, 39 cycles of PCR were run, starting from denaturation at 95°C for 10 s and annealing and extension of the strands at 60°C for 30 s. The threshold cycle for each individual PCR product was used to normalize the values of the target genes against the values of the housekeeping to yield the relative target gene expression.

2.5 Activation z-score calculation

To have information on the global effect of the paradigm on the antioxidant component, we calculated the overall activation score (z-activation) obtained by averaging the z-scores of each antioxidant gene for each experimental group. Increased score values reflected an increased activation of the antioxidant component. Specifically, the z-score was calculated for each experimental group according to the following formula, where X is the gene expression value of a specific sample, μ and σ are respectively the mean and the standard deviation for the animals lacking both food restriction and exercise (Control group):

$$z\ activation\ = \frac{\left(X-\frac{\mu}{\sigma}\right)CAT+\ \left(X-\frac{\mu}{\sigma}\right)MT1\alpha+\left(X-\frac{\mu}{\sigma}\right)GPX1+\left(X-\frac{\mu}{\sigma}\right)GPX4+\left(X-\frac{\mu}{\sigma}\right)SRXN1}{n°\ of\ antioxidant\ genes}$$

2.6 Statistical analysis

Body weight and running wheel activity (RWA) are presented as mean ± SEM and one-way analysis of variance (ANOVA) with group as a between-subjects factor or unpaired student's t-test, wherever appropriate, were performed as statistical analysis. Results of gene expression analyses were evaluated with two-way ANOVA, with RWA and Food restriction as independent variables and the specific gene examined as a dependent variable. For graphic clarity, data are presented as percentage versus CTRL (set at 100%) ± standard error of the mean (SEM). Tukey test was performed as post-hoc comparison analysis. Outliers that were more than two standard deviations away from the mean were excluded, as they were likely to be the result of technical errors. To evaluate the association between the anorectic phenotype and the alteration of gene expression, Pearson product-moment correlation coefficients (r) were calculated between average RWA or body weight of single animals on Day 6 and the corresponding mRNA levels of the genes of interest. The r coefficients are represented as correlation matrices generated with the Corrplot package (v. 4.2.2). All statistical analyses and graphs were designed using the software Graph Pad Prism 9 for Windows. Differences with $P \leq 0.05$ were considered significant.

3. Results

3.1 ABA induction

As previously reported (Collu et al 2020; Scherma et al., 2023), the exposure to the ABA paradigm, in which animals undergo free access to a running wheel in combination with a restricted feeding schedule, induced a marked change in running wheel activity (RWA) of ABA rats. Indeed, the average of RWA during the 6 days of the ABA induction phase was significantly higher as compared to the EXE group that only had access to the wheel without food restrictions ($P = 0.0221$; Fig. 2A). The ABA paradigm significantly affected body weight ($P < 0.0001$; Fig. 2B). Indeed, average of body weight in the ABA group was significantly lower as compared to all the other experimental groups ($P < 0.0001$) and RESTR animal's body weight was significantly lower than that of CTRL and EXE ($P < 0.0001$) groups. Moreover, the EXE animals gained some weight as compared to CTRL rats ($P < 0.01$). As a result of limited access to food, average of food intake of ABA group was significantly lower as compared to CTRL and EXE groups ($P < 0.0001$; Fig. 2C). On the other hand, no difference was found between the ABA and RESTR groups in terms of food consumption given that the latter was subjected to the same food restriction regimen as the ABA group. Moreover, EXE rats consumed significantly more food than CTRL rats ($P < 0.0001$) most likely due to exercise on the wheel.

3.2 Analysis of inflammatory mediators

In the PFC, the gene expression of the pro-inflammatory cytokine TNF-α (Fig. 3A) was significantly modulated by our experimental paradigm. As indicated by the significant RWA x Food Restriction interaction ($P < 0.0001$), mRNA levels were markedly increased in rats allowed only to exercise (EXE +45% vs. CTRL, $P < 0.01$), whereas a significant decrease was found in ABA animals when compared to the other experimental groups (-46% vs. CTRL, $P < 0.01$; -63% vs. EXE, $P < 0.01$; -45% vs. RESTR, $P < 0.05$). A similar profile was also observed for the pro-inflammatory cytokine IL-1β (Fig. 3B) whose expression increased in the EXE group (+36%, $P = 0.0511$) and was reduced in ABA animals (-41% vs. EXE, $P < 0.01$), as confirmed again by a significant RWA x Food Restriction interaction ($P = 0.0403$). The ABA paradigm also affected the expression of the inflammasome NLRP3 (Fig. 3C), whose activation is strictly connected to pro-inflammatory response mediated by IL-1β. Specifically, significantly reduced NLRP3 mRNA levels were found in the ABA group compared to both CTRL and EXE animals (-30% vs. CTRL and EXE, $P < 0.01$), although in this case the main effect was given by the Food Restriction variable ($P < 0.001$). Conversely, the expression of IL-6 (Fig. 3D) was modulated oppositely with a trend to a reduction in the EXE group (-29% vs. CTRL, $P > 0.05$) and an increase in the ABA group (+46% vs. CTRL, $P < 0.05$ and +105% vs. EXE, $P < 0.001$), effects once again supported by a significant ANOVA interaction between RWA and Food Restriction ($P = 0.0034$). We have also assessed the mRNA levels of the anti-inflammatory cytokine TGF-β (Fig. 3E) without finding any change. Moreover, to evaluate the potential involvement of microglia cells in the inflammatory response observed, we measured the gene expression of the marker of microglia recruitment and activation CD11B, finding reduced mRNA levels only in the ABA group (-62% vs. CTRL, -67% vs. EXE and -55% vs. RESTR, $P < 0.001$; Fig. 3F). A similar modulation was observed in the DH of ABA animals, finding an overall decrease in the inflammatory state. Specifically, ABA paradigm reduced the expression levels of TNF-α (-30% vs. EXE, $P = 0.051$; Fig. 3G), with a stronger effect of the Food Restriction factor ($P < 0,05$), IL-1β (-54% vs. CTRL, $P<0.01$, -47% vs. EXE, $P < 0.05$; Fig. 3H) and NLRP3 (-20% vs. EXE and RESTR, $P < 0.05$; Fig. 3I). Moreover, as already observed at cortical level, these effects were paralleled by increased IL-6 expression (+31% vs. CTRL, $P < 0.05$, +64% vs. EXE, $P < 0.001$; Fig. 3J), no changes for TGF-β (Fig. 3K) and reduced expression of CD11B (-48% vs. CTRL and -50% vs. RESTR, $P < 0.01$, -59% vs. EXE, $P < 0.001$; Fig. 3L). No significant modulation was found in this area in response to the individual manipulations.

3.3 Analysis of antioxidant mediators and z-activation

To evaluate if the induction of the ABA phenotype was associated with alteration in the oxidative state, we investigated the antioxidant component of the redox balance by analyzing the expression of different enzymes able to reduce the oxidative stress by specific mechanisms. Interestingly, the only changes were found in the ABA group without any modulation in response to the individual manipulations. In particular, in the PFC of ABA animals, we observed a marked increase in the expression of MT1α (+235% vs. CTRL and EXE, $P < 0.001$, and +172% vs. RESTR, $P < 0.01$; Fig. 4A) accompanied by upregulated levels of SRXN1 (+75% vs. CTRL, EXE and RESTR, $P < 0.0001$; Fig. 4B) and GPX1 (+31% vs. CTRL and EXE, $P < 0.05$; Fig. 4C), although in this case the observed effect was given only by the Food Restriction factor ($P < 0.01$). No differences occurred for GPX4 (Fig. 4D). Differently, we found a significant reduction of the CAT expression (-27% vs. CTRL, $P < 0.05$; Fig. 4E). To better clarify the antioxidant response of the animals we calculated the z-score, clustering the data from each antioxidant gene expression to obtain an estimation of the overall antioxidant status for each experimental group. As shown in Fig 4F, the antioxidant z-score in the PFC was significantly increased in the ABA group ($P < 0.01$ vs. CTRL and EXE, $P < 0.05$ vs. RESTR). A different antioxidant modulation was observed in the DH, where the impact of the ABA paradigm was only in part similar to what was found in the PFC. Specifically, although a significant increase of MT1α expression was still present in the ABA group (+167% vs. CTRL and EXE, $P < 0.05$; Fig 4G) specifically due to Food Restriction effect ($P < 0.01$), no significant modulation was found for SRXN1 (Fig. 4H) and GPX1 (Fig. 4I), while a reduction was observed for GPX4 (-34% vs. CTRL, $P < 0.01$; Fig. 4J). CAT expression was once again significantly reduced (-23% vs. CTRL, $P < 0.05$, and -27% vs. EXE and RESTR, $P < 0.01$; Fig. 4K). Finally, in this brain region the z-score did not show any significant modulation (Fig. 4L).

3.4 Analysis of BDNF expression

Based on the obtained results, the induction of ABA appears to be associated with a reduced inflammatory state in both PFC and DH, an effect paralleled by the upregulation of antioxidant mediators at cortical level. To better interpret these data, we investigated the impact of our experimental paradigm on the neurotrophin BDNF, a well-established neuroplastic marker whose expression and function have been associated with AN [18]. We first evaluated the total form of BDNF in the PFC (Fig. 5A) finding a marked increase in its gene expression in the ABA group (+71% vs. CTRL and +180% vs. RESTR, $P < 0.001$, +54% vs. EXE, $P < 0.01$) accompanied by a reduction in the RESTR group (-39% vs. CTRL, $P < 0.05$), as supported by a significant ANOVA RWA x Food Restriction interaction ($P < 0.0001$). Next, we assessed whether the modulation of BDNF was sustained by its major transcripts. Specifically, we measured the mRNA levels of BDNF long 3′UTR pool of transcripts, BDNF isoform IV and BDNF isoform VI which are among the most abundant and well-characterized transcripts in the brain regions examined. Similarly, to what observed for total BDNF, we found upregulated levels of the BDNF long 3'UTR (+49% vs. CTRL and +64% vs. RESTR, $P < 0.05$; Fig. 5B) and isoform IV (+60% vs. CTRL and +72% vs. RESTR, $P < 0.01$; +43% vs. EXE, $P < 0.05$; Fig. 5C). A comparable although less pronounced modulation was observed also for the isoform VI (Fig. 5D) with an increased expression in the ABA group that reaches the statistical significance only when compared to the RESTR group (+24% vs. RESTR, $P < 0.01$), that was indeed characterized by a significant reduction in the levels of this transcript (-23% vs. CTRL and EXE, $P < 0.01$).

A different modulation was found in the DH, suggestive of a stronger impact of the running wheel activity on the expression of the neurotrophin in this brain region. Indeed, in this case, the observed effect was specifically ascribable to the RWA variable in all the isoforms analyzed. Specifically, the total form of BDNF was increased in the ABA (+66% vs. CTRL, $P < 0.05$) and in the EXE group (+75% vs. CTRL, $P < 0.01$; RWA effect $P < 0,001$; Fig. 5E). A similar pattern was observed for the

pool of BDNF long 3'UTR isoforms (ABA: +58% vs. CTRL, $P < 0.01$; EXE: + 48% vs. CTRL, $P < 0.05$; RWA effect $P < 0,0001$; Fig. 5F) and for isoforms IV and VI. Specifically, BDNF IV was increased in both EXE (+ 52% vs. CTRL, $P = 0.08$) and ABA groups (+66% vs. CTRL, $P < 0.05$, +84% vs. RESTR, $P < 0.01$; RWA effect $P < 0,001$; Fig. 5G) although the modulation reached significance only in the latter, while BDNF VI (Fig. 5H) was significantly upregulated in the EXE (+29% vs. CTRL, $P < 0.05$) and in the ABA group (+31% vs. RESTR, $P < 0.05$; RWA effect $P < 0,0001$), in the latter only when compared to food restricted animals.

3.5 Correlation analysis

To explore the relationships between body weight loss or physical hyperactivity and the variation of markers of inflammation and oxidative stress in the PFC and DH, Pearson correlation coefficients were calculated and presented as correlation matrices (Fig. 6). Body weight measures in CTRL, EXE, RESTR and ABA animals at the end of the ABA induction significantly correlated with the levels of several markers in the PFC and DH. In the PFC, TNF-α ($r = 0.8109$, $p < 0.0001$), IL-1β ($r = 0.5088$, $p = 0.0048$), NLRP3 ($r = 0.4424$, $p = 0.0163$), IL-6 ($r = -0.5874$, $p = 0.0006$), CD11B ($r = 0.6214$, $p = 0.0003$), BDNF IV ($r = -0.4801$, $p = 0.0072$), MT1α ($r = -0.6592$, $p = 0.0001$), SRXN1 ($r = -0.5346$, $p = 0.0023$), GPX1 ($r = -0.4966$, $p = 0.0052$) showed a significant correlation with measures of body weight. In the DH, TNF-α ($r = 0.4404$, $p = 0.0149$), IL-1β ($r = 0.5134$, $p = 0.0037$), NLRP3 ($r = 0.4611$, $p = 0.0103$), IL-6 ($r = -0.5537$, $p = 0.0015$), CD11b ($r = 0.6088$, $p = 0.0005$), MT1α ($r = -0.6424$, $p = 0.0001$), SRXN1 ($r = -0.3809$, $p = 0.0378$), CAT ($r = 0.4199$, $p = 0.0209$) showed a significant correlation with body weight. When investigating the correlations between average RWA, in ABA and EXE groups, and the markers analyzed we observed significant correlations in the PFC for TNF-α ($r = -0.6876$, $p = 0.0094$), CD11B ($r = -0.5925$, $p = 0.0199$), MT1α ($r = 0.6625$, $p = 0.0071$). Differently, in the DH only MT1α significantly correlated with RWA ($r = 0.6490$, $p = 0.0089$). The detailed graphs showing Pearson's correlations for each gene are shown in Supplementary Fig. S1-S4**.**

4. Discussion

This study investigated the involvement of neuroinflammation, redox balance, and neuroplasticity in the early phase of AN by using the well-established ABA rodent model. To this end, we analyzed the transcriptional profile of inflammatory mediators, antioxidant enzymes and BDNF in the PFC and DH of adolescent female rats at the end of the induction phase of the ABA paradigm in which they showed an anorexic-like phenotype characterized by a progressive increase in physical activity and a massive body weight loss (Collu et al 2020; Scherma et al., 2023).

As already mentioned, an altered inflammatory state has been reported in AN patients (Vaisman et al.,1991; Allende et al., 1998; Nilsson et al., 2020). However, the results are often contrasting, possibly because of several individual variables affecting the clinical studies, such as the stage of the disease, body mass index, medications, smoking status, and age. For example, in contrast with a low-grade proinflammatory state found in adult AN patients with elevated levels of TNF-α, IL-1β or IL-6 (Solmi et al., 2015; Dalton et al 2018) a mixed inflammatory state with reduced IL-1β and IL-6 was found in hospitalized female adolescent patients' serum compared to age-matched healthy control subjects (Specht et al., 2022). Similarly, levels of IL-1β and IL-6 were significantly lower in young males and females with an early onset of eating disorders without previous treatments, suggesting immunosuppressive response in early illness stage (Ruiz Guerrero et al., 2022).

Our data clearly shows a significant decrease in pro-inflammatory markers such as IL-1β and TNF-α, NLRP3, and CD11B in both PFC and DH of the ABA group. Interestingly, the observed antithetical modulation of IL-6 might also contribute to the reduced neuroinflammation as it detains both a pro- and anti-inflammatory role (Scheller et al., 2011; Fuster et al., 2014; Del Giudice et al 2018). In addition to their involvement in inflammatory processes, it is well established that several cytokines, including IL-1β and TNFα, can suppress food intake in different ways (Buchanan et al., 2007). Moreover, they can induce the release of hormones that regulate eating behavior and energy homeostasis, such as leptin (Kiernan et al., 2021). On the other hand, leptin can modulate the immune response working as a pro-inflammatory cytokine and low leptin levels are an endocrinological feature of acute AN (Hebebrand et al., 1997). Thus, the changes observed could represent an adaptive response to the reduced caloric intake as well as of a decreased leptin signaling. In agreement, we previously demonstrated that ABA induction was associated with low levels of leptin (Scherma et al., 2017). Among the different cell populations potentially involved in the observed changes, glial cells may play a crucial role. It is noteworthy that both astrocytes and microglia possess a dual polarization phenotype - with either M1/A1 pro-inflammatory activity or M2/A2 anti-inflammatory activity (Fan and Huo, 2021). In particular, A2 polarization of astrocytes is known to reduce microglial activation and promote the expression of antioxidant enzymes (Kim et al., 2010). Thus, we can hypothesize a contribution of astrocytes to the modulations observed in our experimental setting, either by acting on microglia or by directly modulating the inflammatory state, an open question that could be addressed in further studies. Moreover, glial cells involvement in energy balance and food intake control has been outlined. Both microglia and astrocytes possess leptin receptors and, specifically, it has been reported that astrocyte activation can reduce hyperphagia meanwhile facilitating leptin-induced anorexia by acting on hypothalamic neurons involved in eating behavior regulation (Yang et al., 2015).

Additionally, our results suggest an involvement of the mechanisms regulating oxidative balance and neuroplasticity. We observed an overall antioxidant upregulation at cortical level that could be causative or consequent to the reduction of neuroinflammation, while in the DH we detected a different antioxidant modulation which only in part reflects what is seen in PFC. However, the gene expression of MT1α was strongly increased in both brain areas of ABA animals. MT1α detains antioxidant and metal detoxification functions, exerting its protective role by finely regulating the levels of heavy metals such as zinc (Thirumoorthy et al., 2011), which other than being essential for the functioning of several enzymes and transcription factors it acts as an anti-inflammatory and antioxidant agent (Andrews, 2000; Marriero et al., 2017). Accordingly, deficiency of MT1 and MT2

was associated with increased levels of pro-inflammatory cytokines and oxidative stress in animal models of multiple sclerosis (Dai et al., 2021) while overexpression of MT1 was shown to decrease inflammation and oxidative stress in transgenic mice (Molinero et al., 2003). In this context, increased MT1α levels could contribute to the reduced neuroinflammation observed. Importantly, metallothioneins are also involved in the regulation of eating behavior as apparently MT1 and MT2 genes dysfunction results in increased food intake and obesity in mice (Beattie et al., 1998). Further studies need to clarify if the opposite antioxidant score detected in PFC and in DH underlines a different role of these brain regions in AN etiopathogenesis. However, it is important to underline that clinical studies performed at peripheral level indicate an increased oxidative stress correlating with disease severity and reduced antioxidant capacity (Solmi et al., 2015; Kovalčíková et al., 2021; Wagner-Skacel et al., 2022). Interestingly, oral refeeding (Solmi et al., 2016) and antioxidant-inducer L-arginine supplementation (Vignini et al.,2010) seem to ameliorate some of these alterations. Additionally, studies on ABA-exposed rats reported transient redox derangements reverted after 10 days in an anatomical-dependent manner, supporting an area-specific modulation of the redox system during the anorexic phenotype (Bhasin et al., 2023).

The described expression pattern was paralleled by increased levels of BDNF in both brain regions. More specifically, we observed a significant increase in the expression of the total BDNF in the ABA animals in both brain areas. A similar pattern was found also for the different transcripts analyzed. Our data on BDNF are in line with our recent study reporting higher activity-regulated cytoskeleton-associated protein (Arc) and c-Fos positive cells in selected brain regions of ABA rats including PFC and DH. Arc and c-Fos are two markers commonly used for detecting neural plasticity and neural activation respectively (Scherma et al., 2023). It is important to underline that Arc expression is increased by BDNF and that the activity-dependent transcription and translation of BDNF relies on neuronal activity (El Hayek et al., 2019; Tao et al., 2002).

Seemingly, BDNF is involved in the regulation of feeding behavior, weight control and energy balance maintenance (An et al., 2015). Clinical studies reported reduced levels of BDNF in AN patients possibly being related to starvation, that were restored by rehabilitation (Keeler et al.,2022; Brandys et al., 2011; Trinh et al., 2023). Unfortunately, at preclinical level, only few studies pertain the impact of ABA on the neurotrophin. Food restriction and wheel running reportedly induced changes in BDNF mRNA in the mesolimbic system of 9-week-old female mice, although no changes were specific to the ABA condition (Ho et al., 2016). Recently, another study found increased BDNF protein levels paralleled however by reduced gene expression in the amygdala of ABA-exposed adolescent female rats (Mottarlini et al., 2022). Inflammation and BDNF are seemingly inversely connected as high levels of pro-inflammatory cytokines are often associated with a downregulation of BDNF (Calabrese et al., 2014) while its overexpression reportedly reduces neuroinflammation (Han et al., 2019; Bovolenta et al., 2010). Thus, BDNF modulation could participate in the mechanism engaged to endure the ABA effects. Interestingly, hippocampal BDNF was significantly upregulated by exercise alone, with a corresponding absence of inflammation. High-intensity exercise was indeed shown to protect the brain from autoimmune neuroinflammation (Zaychik et al., 2021). Moreover, treadmill exercise apparently suppressed hippocampal inflammation in rats (Park et al., 2021). Also, different studies reported increased BDNF levels after exercise (Gómez-Pinilla et al., 2002; El-Sayedet al., 2011) and it's indeed recognized that physical activity improves cognition and induces brain plasticity (Muscat et al., 2020).

Overall, our results indicate that neuroinflammation, redox balance and neuroplasticity are modulated by the ABA model of AN. We hypothesize that, initially, these systems can trigger an integrated compensatory mechanism aimed at restoring homeostasis. Later, reiteration of starvation and excessive exercise might eventually lead to the full-blown pathology and the establishment of an inflammatory state. We acknowledge that our study has some limitations because our molecular analyses are limited to the transcriptional level and the correlation between gene and protein is not always direct. Accordingly, we are aware that more in-depth studies are needed to better understand the mechanisms underlying the observed modulations, whether they persist at different stages of ABA

or during the recovery, and if they reflect protein changes and impairments in the function of the systems involved. Nevertheless, our study provides a detailed overview of the molecular systems involved in the early phase of the development of the anorectic phenotype, suggesting a critical role in the etiopathogenesis of the disease and their potential as targets for future therapeutic strategies.

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CRediT authorship contribution statement

Vittoria Spero: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation**. Maria Scherma:** Writing – review & editing, Conceptualization, Visualization, Validation, Investigation, Formal analysis, Data curation**. Sabrina D'Amelio:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation**. Roberto Collu:** Writing – original draft, Visualization, Formal analysis, Data curation**. Simona Dedoni:** Investigation, Formal analysis, Data curation**. Chiara Camoglio:** Investigation, Formal analysis, Data curation. **Carlotta Siddi:** Investigation, Formal analysis, Data curation**. Walter Fratta:** Writing – review & editing, Funding acquisition. **Raffaella Molteni:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Paola Fadda:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest
The authors declare no conflicts of interest

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Data availability
Data will be made available on request.

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Figure 1

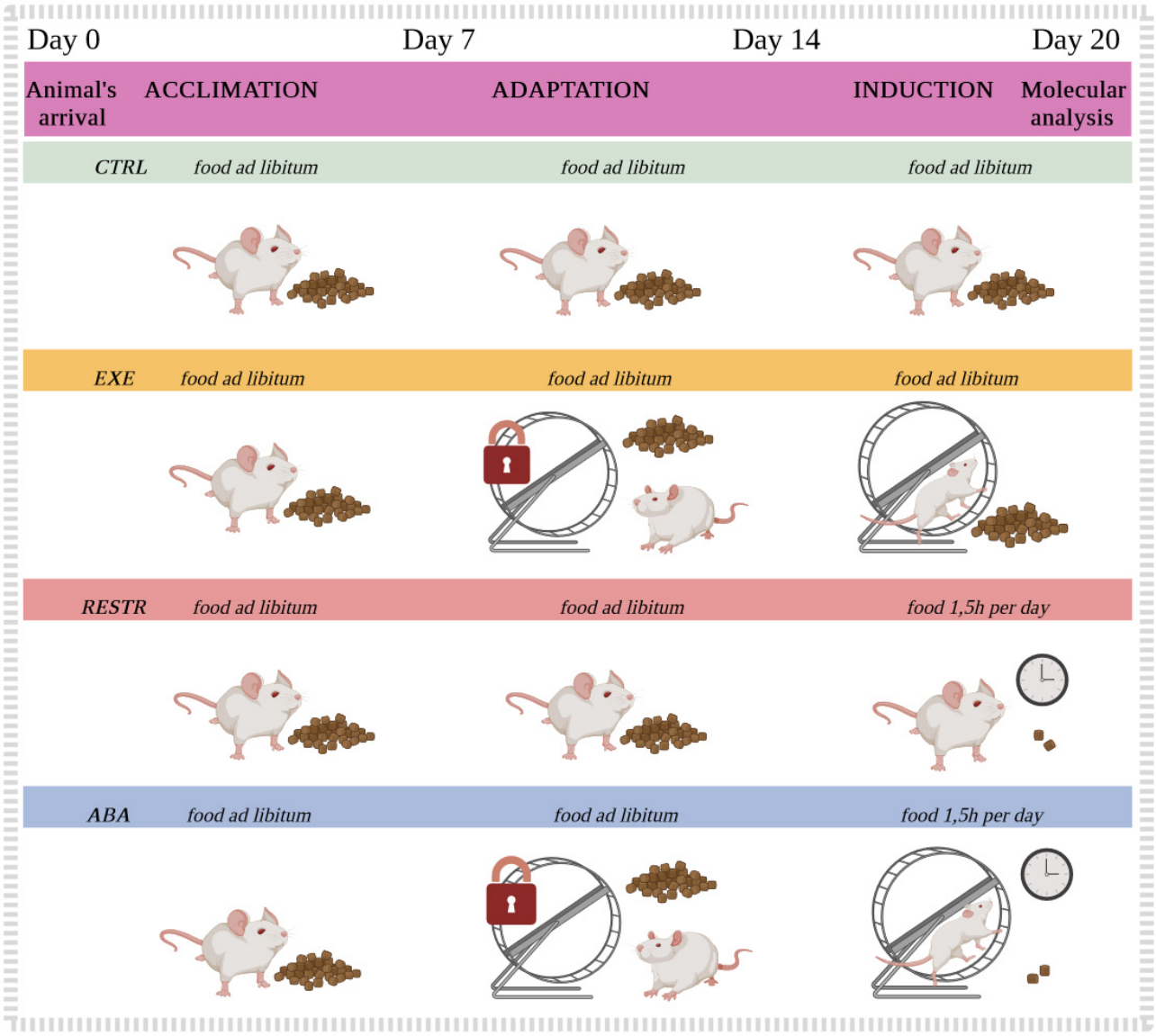

Fig. 1. Experimental design and timeline

Figure 2

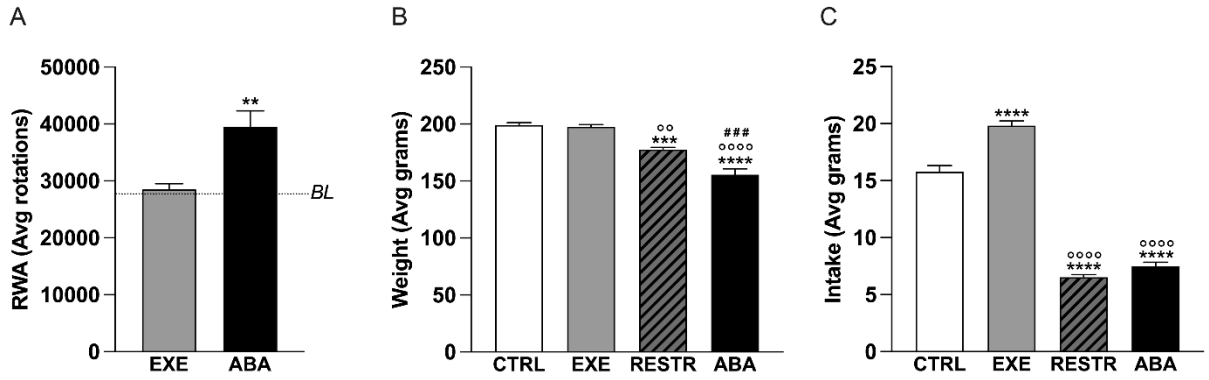

Fig.2.

(A) Average RWA (BL, baseline), (B) Average Body Weight (% of baseline, BL), and (C) Average food intake (Kcal/24h) in CTRL, EXE, RESTR and AB A groups during the ABA induction. Data are presented as the mean ± SEM (n = 7/8 rats per group). Statistical analysis was performed by one-way ANOVA followed by Bonferroni post hoc test or Unpaired T-test. (Average RWA * P< 0.05 ABA vs. EXE; Average Body Weight **P < 0.01 EXE vs. CTRL, **** P < 0.0001 ABA vs. CTRL, °°°° P < 0.0001 ABA vs EXE, #### P < 0.0001 ABA vs RESTR; Average Food Intake**** P < 0.0001 EXE vs. CTRL, **** P < 0.0001 REST vs. CTRL, °°°° P < 0.0001 REST vs. EXE,**** P < 0.0001 ABA vs. CTRL, °°°° P < 0.0001 ABA vs. EXE).

Figure 3

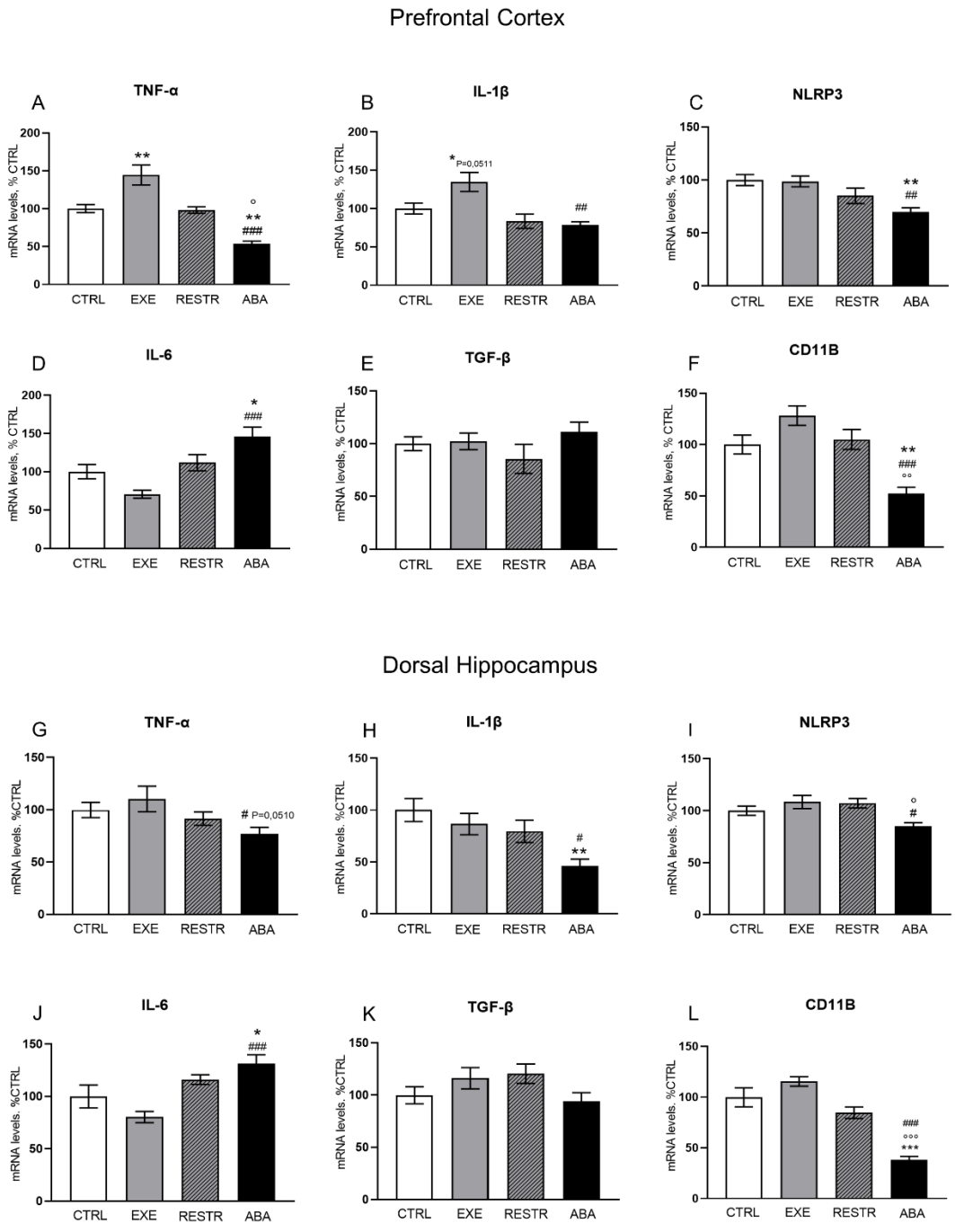

Fig.3. mRNA levels of inflammatory markers.
The mRNA levels of TNF-α (A, G), IL-1β (B, H), NLRP3 (C, I), IL-6 (D, J), TGF-β (E, K) and CD11B (F, I) measured in PFC and DH of CTRL, EXE, RESTR and ABA groups. Data are expressed as percentage (100%) of CTRL (n = 6/8 rats per group). Statistical analysis was performed by Two-Way ANOVA with Tukey post-hoc test. [PFC: * $P < 0.05$, ** $P < 0.01$ ABA vs. CTRL; ## $P < 0.01$ ### $P < 0.001$ vs EXE; ° $P < 0.05$ vs RESTR; DH: * $P < 0.05$, ** $P < 0.01$ vs CTRL; # $P < 0.05$ ### $P < 0.001$ vs EXE; ° $P < 0.05$ vs RESTR].

Figure 4

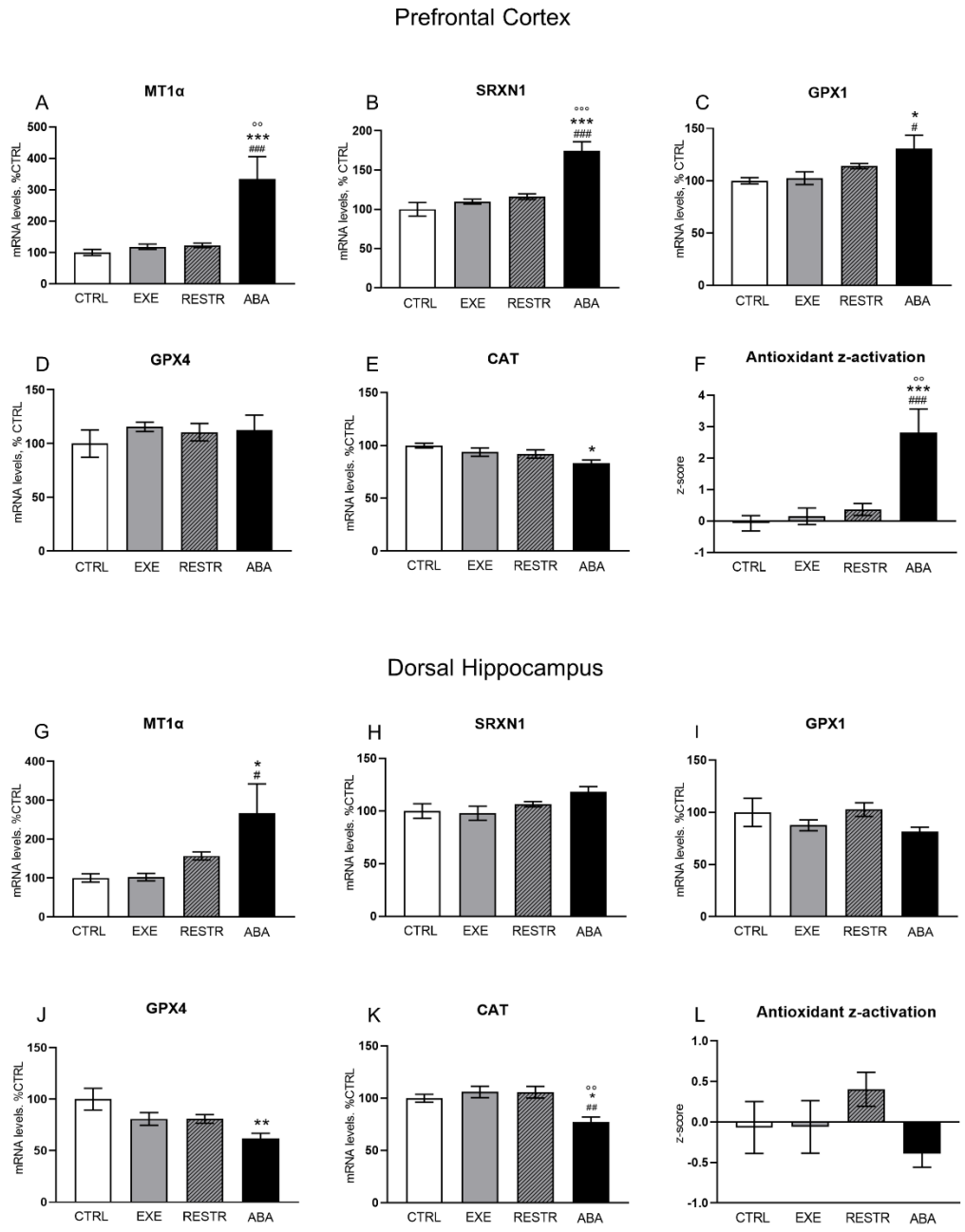

Fig.4. mRNA levels of antioxidant markers and z-activation.
mRNA levels of MT1α (A, G), SRXN1(B, H), GPX1 (C, I), GPX4 (D, J), CAT (E, K) and z-score of antioxidant genes (F, I) were analyzed in the PFC and DH of CTRL, EXE, RESTR and ABA groups. Data are expressed as percentage (100%) of CTRL (n = 6/8 rats per group). Statistical analysis was performed by Two-Way ANOVA with Tukey post-hoc test [PFC: * $P < 0.05$, *** $P < 0.001$ ABA vs. CTRL; # $P < 0.05$ ### $P < 0.001$ ABA vs. EXE; ° $P < 0.05$, °° $P < 0.01$ ABA vs. RESTR; DH: * $P < 0.05$, ** $P < 0.01$ ABA vs. CTRL; # $P < 0.05$ ## $P < 0.01$ ABA vs. EXE; °° $P < 0.01$ ABA vs. RESTR].

Figure 5

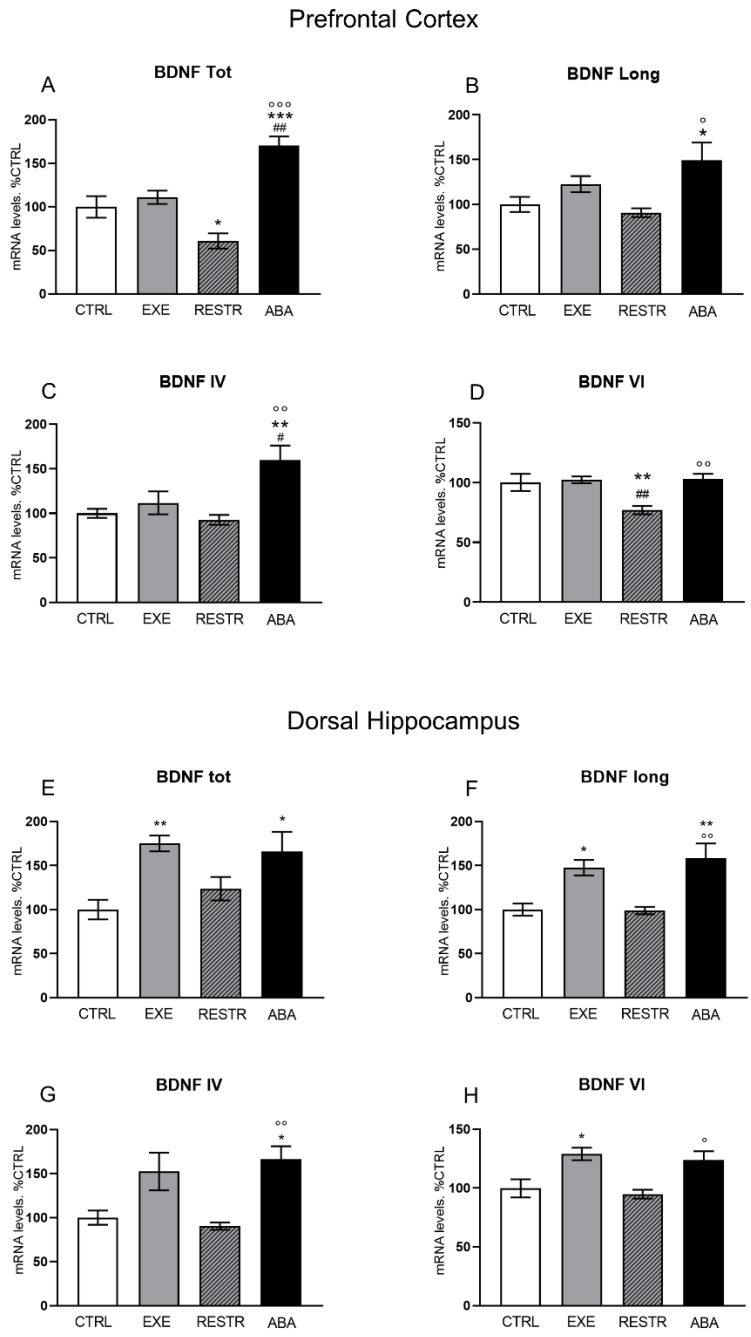

Fig. 5. Expression of BDNF transcripts.
mRNA levels of total BDNF (A, E), BDNF long (B, F), BDNF IV (C, G) and BDNF VI (D,H) assessed in the PFC and DH of CTRL, EXE, RESTR and ABA groups. Data are expressed as percentage (100%) of CTRL (n = 6/8 rats per group). Statistical analysis was performed by Two-Way ANOVA with Tukey post-hoc test. [PFC: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs CTRL; # $P < 0.05$ ## $P < 0.01$ vs EXE; ° $P < 0.05$, °° $P < 0.01$, °°° $P < 0.001$ vs RESTR; DH: * $P < 0.05$, ** $P < 0.01$ vs CTRL; ° $P < 0.05$, °° $P < 0.01$ vs RESTR].

Figure 6

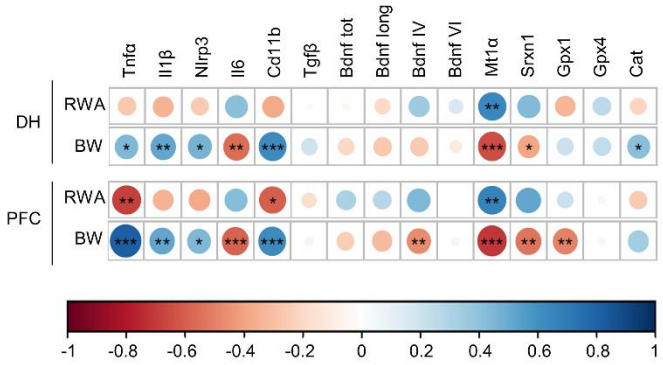

Fig.6. Correlation matrices with body weight or measures of physical activity.
Pearson's correlations coefficients were calculated between measures of body weight (BW) or running wheel activity (RWA) in CTRL, EXE, RESTR and ABA groups and the fold change variation levels of markers of inflammation and oxidative stress in DH and PFC. DH_RWA: MT1α (** p = 0.0089); DH_BW: TNF-α (* p = 0.0149), IL-1β (** p = 0.0037), NLRP3 (* p = 0.0103), IL-6 (** p = 0.0015), CD11b (*** p = 0.0005), MT1α (*** p = 0.0001), SRXN1 (* p = 0.0378), CAT (* p = 0.0209); PFC_RWA: TNF-α (** p = 0.0094), CD11B (* p = 0.0199), MT1α (** p = 0.0071) PFC_BW: TNF-α (***p < 0.0001), IL-1β (** p = 0.0048), NLRP3 (* p = 0.0163), IL-6 (*** p = 0.0006), CD11B (** p = 0.0003), BDNF IV (** p = 0.0072), MT1α (*** p = 0.0001), SRXN1 (** p = 0.0023), GPX1 (** p = 0.0052).

Supplementary Figure 1

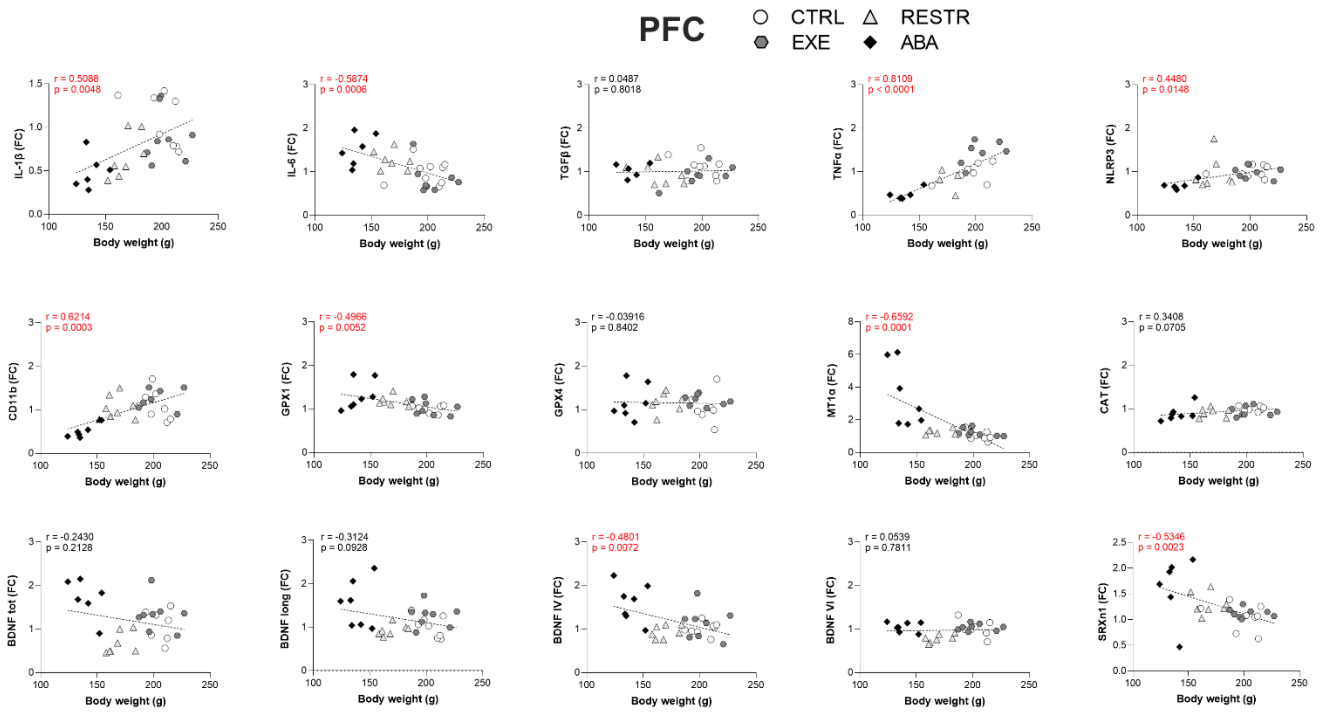

Fig. S1. Correlation analysis between markers of inflammation, or oxidative stress, or BDNF and Body Weight in the PFC of CTRL, EXE, RESTR and ABA groups. Data are expressed as body weight of single animals (expressed in grams) on Day 6 and the corresponding mRNA levels of the genes of interest.

Supplementary Figure 2

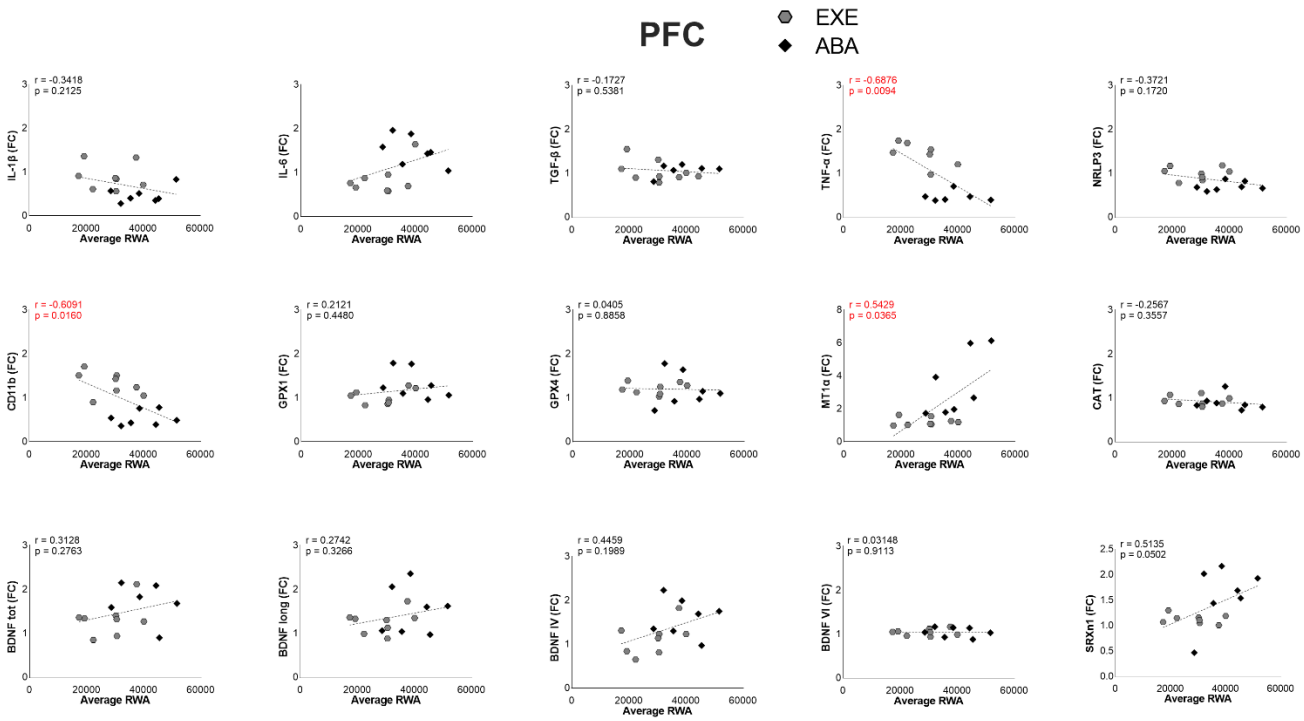

Fig.S2. Correlation analysis between markers of inflammation, or oxidative stress, or BDNF and Average RWA in the PFC of CTRL, EXE, RESTR and ABA groups. Data are expressed as average RWA of single animals and the corresponding mRNA levels of the genes of interest.

Supplementary Figure 3

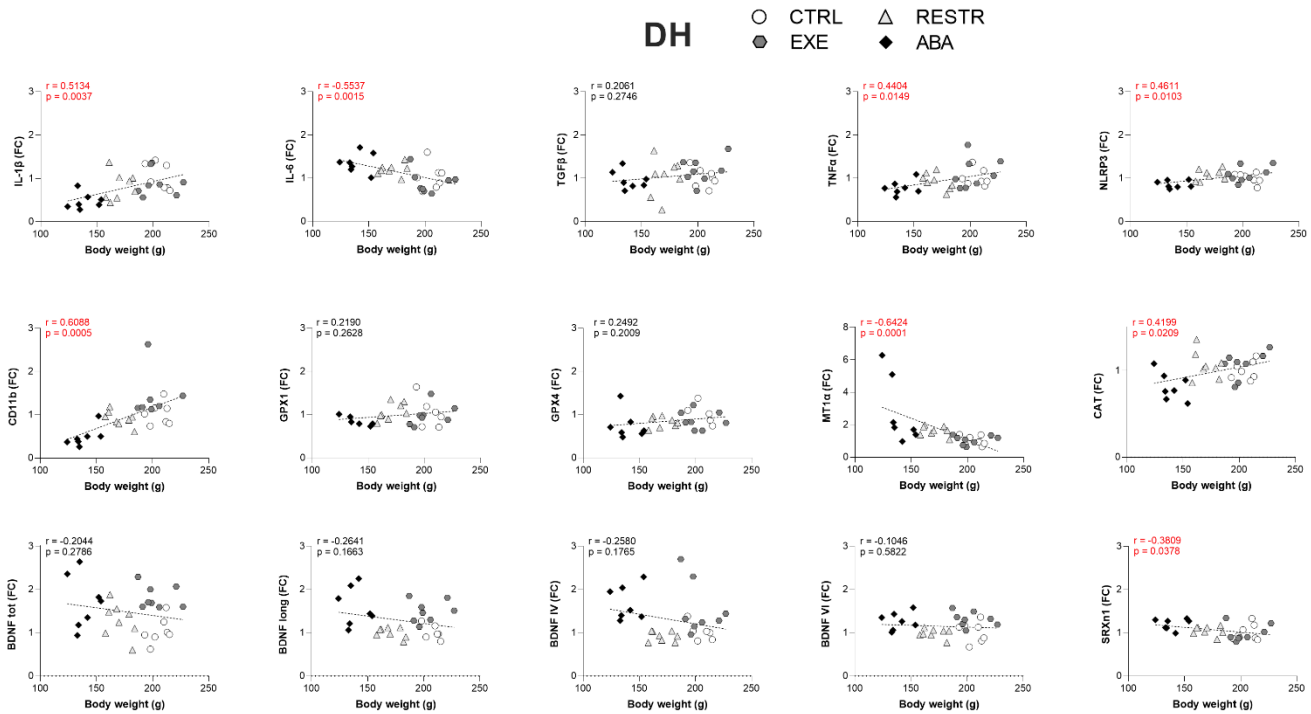

Fig.S3. Correlation analysis between markers of inflammation, or oxidative stress, or BDNF and Body Weight in the DH of CTRL, EXE, RESTR and ABA groups. Data are expressed as body weight of single animals (expressed in grams) on Day 6 and the corresponding mRNA levels of the genes of interest.

Supplementary Figure 4

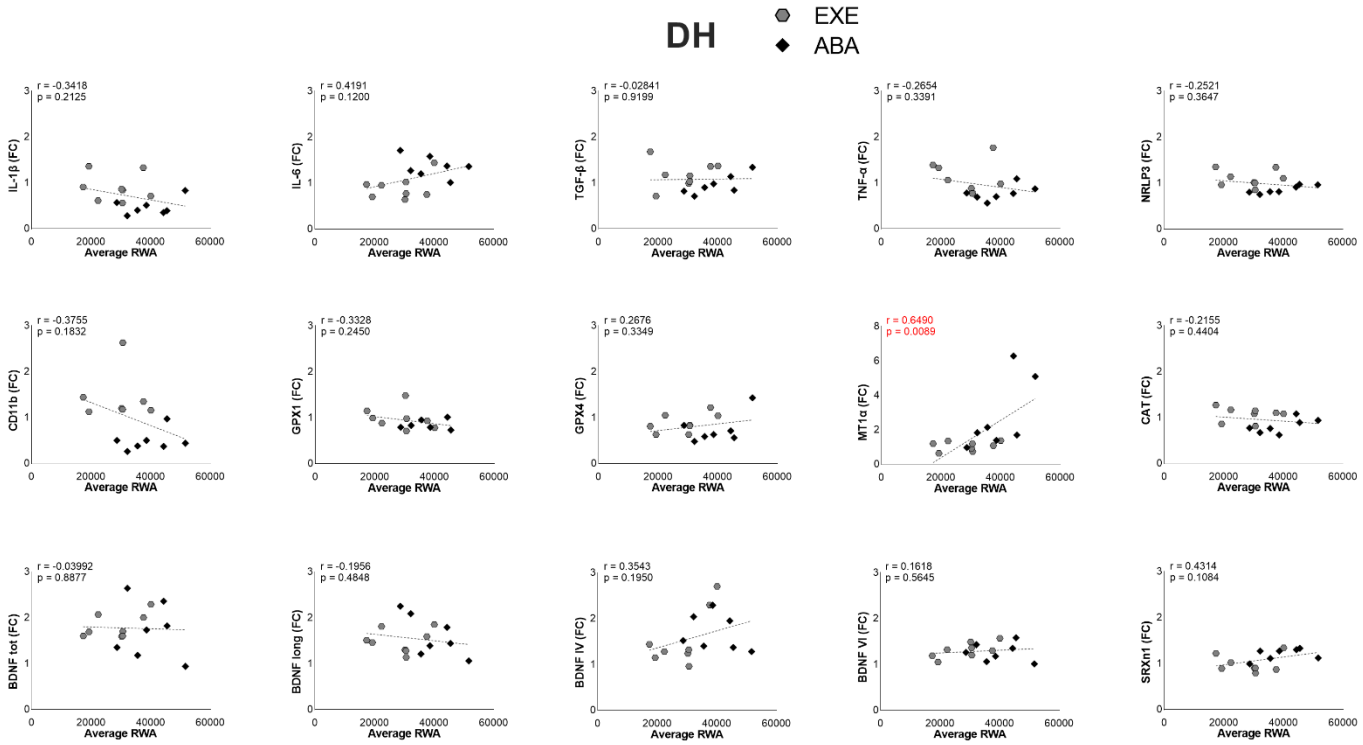

Fig.S4. Correlation analysis between markers of inflammation, or oxidative stress, or BDNF and Average RWA in the DH of CTRL, EXE, RESTR and ABA groups. Data are expressed as average RWA of single animals and the corresponding mRNA levels of the genes of interest.

Table 1

GENE	PRIMER	PROBE
β- Actin	Fwd: CACTTTCTACAATGAGCTGCG Rev: CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC
TGF-β	Fwd: CAACAATTCCTGGCGTTACC Rev: ACTGAAGCGAAAGCCCTGTA	TGAGTGGCTGTCTTTTGACG
BDNF total	Fwd: AAGTCTGCATTACATTCCTCGA Rev: GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
CD11b	Fwd: TTCCGGACTCACTTCACCTT Rev: TTCAGCTGCCTTATGGGTCT	TTCAAGAGAAACCCTGACCC
	APPLERA CODE	
GPX1	Rn00577994_g1	
GPX4	Rn00820816_g1	
CAT	Rn00560930_m1	
MT1α	Rn00821759_g1	
SRXN-1	Rn04337926_g1	
IL-1β	Rn99999009_m1	
TNF-α	Rn99999017_m1	
IL-6	Rn99999011_m1	
NLRP3	Rn04244620_m1	
BDNF long	Rn02531967_s1	
BDNF VI	Rn01484928_m1	
BDNF IV	Rn01484927_m1	