Sex-Specific Effects of Prenatal Stress on Bdnf Expression in Response to an Acute Challenge in Rats: a Role for Gadd45β

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Abstract

Exposure to early adversities represents a major risk factor for psychiatric disorders. We have previously shown that exposure to prenatal stress (PNS) in rats alters the developmental expression of brain-derived neurotrophic factor (*Bdnf*) with a specific temporal profile. However, exposure to early-life stress is known to alter the ability to cope with challenging events later in life, which may contribute to the enhanced vulnerability to stress-related disorders. Since *Bdnf* is also an important player for activity-dependent plasticity, we investigated whether the exposure to PNS in rats could alter

Bdnf responsiveness to an acute challenge at adulthood. We found that exposure to PNS produces significant changes in *Bdnf* responsiveness with brain region- and gender-specific selectivity. Indeed, exposure to an acute stress upregulates *Bdnf* expression in the prefrontal cortex, but not in the hippocampus, of control animals. Moreover, such modulatory activity is selectively impaired in PNS female rats, an effect that was associated with changes in the modulation of the DNA demethylase *Gadd45β*. Our results suggest that exposure to ~~prenatal stress~~PNS may reprogram gene transcription through epigenetic mechanisms reducing the ability to cope under adverse conditions, a trait that is disrupted in psychiatric diseases.

Keywords

Acute stress
Bdnf
Gadd45β
Prenatal restraint stress
Gender effect

Introduction

Exposure to early adverse life events, which might occur already in the uterine environment, produces a complex, interrelated series of consequences that result in long-lasting changes of brain function and behaviour, thus predisposing to adult psychiatric disorders [1, 2]. Indeed, a growing body of evidence suggests that exposure to early adversities leads to changes in a number of physiological systems involved in mental health, including the hypothalamic-pituitary-adrenal (HPA) axis [3], as well as different regulators of neuronal plasticity [4–7], which are also well-established targets for pharmacological intervention [8]. Indeed, rats exposed to stress in the perinatal period show, at adulthood, increased peripheral blood levels of corticosterone [9, 10] and a significant reduction of brain-derived neurotrophic factor (BDNF) expression, a key player for neurodevelopment and neuronal plasticity [11–15]. Such changes may lead to structural modifications in the brain and reduce the ability to adapt or respond to environmental challenges [16, 17]. Indeed, since early-life adversities may reshape the trajectories of physiological responses to stressful life events, we decided to investigate whether the inability to cope with an acute challenging event at adulthood may be triggered by alterations in gene transcription following an acute stressor. More specifically,

we analysed the expression of the neurotrophin *Bdnf*, whose modulation under acute conditions represents a coping mechanism to protect neuronal integrity, and ~~participates~~whose participation in the encoding of salient environmental cues ~~with~~bears cognitive implications [18]. As an example, it has been demonstrated that contextual fear learning is associated with a significant upregulation of *Bdnf* expression that is mediated by corticosteroids and may result in focused attention and memory improvements [16, 19]. Moreover, we investigated a number of genes that may respond to the activation of the glucocorticoid system in order to establish the possible relationship with the altered responsiveness to an acute stress. Last, we addressed the potential contribution of epigenetic mechanisms that may be responsible for the long-lasting nature of transcriptional changes as a consequence of early-life adversities [7, 20].

Materials and Methods

Animals and Stress Procedures

Nulliparous adult female (body weight 230–260 g) and male (400 g) Sprague Dawley rats were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, they were pair-housed with a same-sex conspecific with food and water available ad libitum (21 ± 1 °C, 60 ± 10 % relative humidity, reversed 12/12-h light/dark cycle).

After 10 days of habituation in the facility, rats were mated for 24 h and individually housed immediately thereafter. Pregnant females were randomly assigned to control (Ctrl) and prenatal stress (PNS) conditions. PNS consisted of restraining pregnant dams in a transparent Plexiglas cylinder (7.5 cm diameter, 19 cm length) under bright light for 45 min three times daily during the last week of gestation [12, 21]. PNS sessions were separated by 2–3-h intervals and conducted at varying periods of the day to reduce habituation. Control rats were left undisturbed. The offspring of both Ctrl and PNS dams were used in the experiment.

On postnatal day (PND) 1, pups from 7 Ctrl and 9 PNS dams were culled to 5 males and 5 females. Weaning occurred on PND 21 and rats of the same sex and experimental group were housed in groups of 3 per cage. One female and one male from different litters (in order to avoid litter effects) pertaining to Ctrl and PNS groups were sacrificed by decapitation on PND 1 (Ctrl $n = 5$; PNS $n = 7$; for each sex), PND 7 (Ctrl $n = 5$; PNS $n = 7$; for each sex), PND 21 (Ctrl $n = 5$;

PNS $n = 7$; for each sex), PND 40 (Ctrl $n = 5$; PNS $n = 8$; for each sex) and PND 62 (see below), in order to create a time profile of the changes under investigation. On PND 62, rats pertaining to the Ctrl and PNS groups were divided in two. Of each, one half (one male and one female from each litter) was sacrificed (sham group), while the other half underwent an acute forced swim stress (AS group) (Ctrl/sham $n = 7$; PNS/sham $n = 8$; Ctrl/AS $n = 8$; PNS/AS $n = 8$; for each sex). Briefly, in a quiet room, with a dim light, each AS subject was gently placed into a cylindrical plastic container (height = 59 cm; diameter = 25 cm) filled up with water (26 ± 1 °C) to a level of 36 cm. The session lasted 5 min. All AS animals were sacrificed 1 h after the end of the acute stress procedure in order to investigate rapid transcriptional changes in response to the acute challenge [22].

All animal experiments were conducted according to the authorization from the Health Ministry n. 295/2012-A (20/12/2012), in full accordance with the Italian legislation on animal experimentation (Decreto Legislativo 116/92) and adherent to EU recommendation (EEC Council Directive 86/609). All efforts were made to minimize animal suffering and to reduce the total number of animals used, while maintaining statistically valid group numbers.

RNA Preparation for qRT-PCR and Analysis of mRNA Levels

After each sacrifice, hippocampi and prefrontal cortices were dissected, immediately frozen on dry ice and stored for later analyses. Dissections were performed according to the atlas of Paxinos and Watson [23]. In detail, the prefrontal cortex (PFC) was dissected from 2-mm-thick slices (PFC defined as Cg1, Cg3, and IL subregions corresponding to plates 6–9), whereas the hippocampus (including both the ventral and dorsal parts) was dissected from the whole brain.

Total RNA was isolated by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy), according to the manufacturer's instructions and quantified by spectrophotometric analysis. An aliquot of each sample was treated with DNase to avoid DNA contamination.

RNA was analysed by a TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in

triplicate as multiplexed reactions with a normalizing internal control (*36b4*). We choose *36b4* as an internal standard for gene expression analyses since its expression was not affected by developmental changes and it was not altered by prenatal stress or acute stress at adulthood.

Probe and primer sequences of long 3′-UTR *Bdnf* (assay id: Rn02531967_s1), *Bdnf* exon IV (assay id: Rn01484927_m1), *Bdnf* exon VI (assay id: Rn01484928_m1) and *Gadd45β* (Fwd primer: GCTGCGACAATGACATTGACATC; Rev primer: CTCGTTTGTGCCTAGAGTCTCT) were purchased from Life Technologies (Monza, Italy) and are available on request, while the other TaqMan gene expression assays were purchased from Eurofins Genomics (Vimodrone, Italy) and are summarized in Table 1 . Thermal cycling was initiated with an incubation at 50 °C for 10 min (RNA retrotranscription) and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process and then for 30 s at 60 °C for the annealing and extension reaction. Relative target gene expression was calculated according to the $2^{(-\Delta\Delta C(T))}$ method.

Table 1

Sequences of forward and reverse primers and probes used in qRT-PCR analysis and pu

Gene	Forward primer	Reverse primer
Bdnf exon I	GGGAGACGAGATTTTAAGACACTG	GTCATCACTCTTCTCACCTG
Bdnf exon IIa	ACAGAGCCAGCGGATTTGT	GTCATCACTCTTCTCACCTG
Bdnf exon IIb	AGTTGGCTTCCTAGCGGTGTA	GTCATCACTCTTCTCACCTG
Bdnf exon IXa	GGTGTCCCCAAGAAAGTAAGG	GTCATCACTCTTCTCACCTG
Dusp1	TGTGCCTGACAGTGCAGAAT	ATCTTTCCGGGAAGCATGG
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAA
FoxO1	GAGTGGATGGTGAAGAGTGTG	GGACAGATTGTGGCGAATT
Gilz	CGGTCTATCAACTGCACAATTTC	CTTCACTAGATCCATGGCCT

Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGA
Total *Bdnf*	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACA
36b4	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC

Protein Extraction and Western Blot Analysis

Western blot analysis was used to investigate BDNF protein levels in the PFC (which was dissected as described in "RNA Preparation for qRT-PCR and Analysis of mRNA Levels" section) from animals that underwent gestational stress and acute stress exposure at adulthood. Protein analysis was performed on the crude membrane fraction (P2). Brain samples from the different experimental groups were manually homogenized in a glass-glass potter in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 1 mM HEPES, 0.1 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), in the presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors. The homogenate was clarified at 1000*g* for 10 min. The resulting supernatant was then centrifuged at 9000*g* for 15 min. The supernatant was discarded while the pellet (P2), corresponding to the crude membrane fraction, was resuspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol, 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. This fraction is enriched in synaptic proteins as previously demonstrated [24]. Total protein content was measured according to the Bradford protein assay procedure (Bio-Rad Laboratories), using bovine serum albumin as the calibration standard.

Equal amounts of protein (10 μg) were run under reducing conditions on Any Kd Criterion TGX precast gels (Bio-Rad Laboratories) and then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. Unspecific binding sites were blocked for 2 h in 10 % nonfat dry milk in Tris-buffered saline, and membranes were then incubated with the primary antibody which recognizes the mature form of the neurotrophin (mBDNF 14 kDa; 1:500, Santa Cruz Biotechnology) in blocking solution at 4 °C overnight. Membranes were washed for 1 h with Tris-buffered saline and incubated for 1 h at room temperature with a peroxidase-conjugated antirabbit IgG (1:1000 for mBDNF) in 5 % nonfat dry milk in Tris-buffered saline, and immunocomplexes were visualized by chemiluminescence using the Western Lightning *Plus* ECL (PerkinElmer) and the Chemidoc MP imaging system (Bio-

Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa after blocking the membranes with 10 % nonfat dry milk and probing them with a polyclonal antibody (1:10,000, Sigma-Aldrich) followed by a 1:10,000 dilution of peroxidase-conjugated antimouse IgG (Sigma-Aldrich). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used. β-Actin was employed as an internal standard because its expression is not regulated by the experimental paradigm used.

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Correlation Analysis

Pearson product-moment correlations (*r*) between levels of *Gadd45β* messenger RNA (mRNA) and the mRNA levels of total *Bdnf* were performed to establish the correlation between the expression levels of these genes in single animals.

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Statistical Analysis

Changes produced by prenatal exposure to stress and ~~development~~age/acute stress were analysed using a two-way ANalysis Of VAriance (ANOVA), followed by Fisher's LSD post hoc comparisons.

SPSS for Mac OS X (release 22.0.0.0) was used to statistically analyse the data. A probability level of $p < 0.05$ was taken as significant in every test.

Results

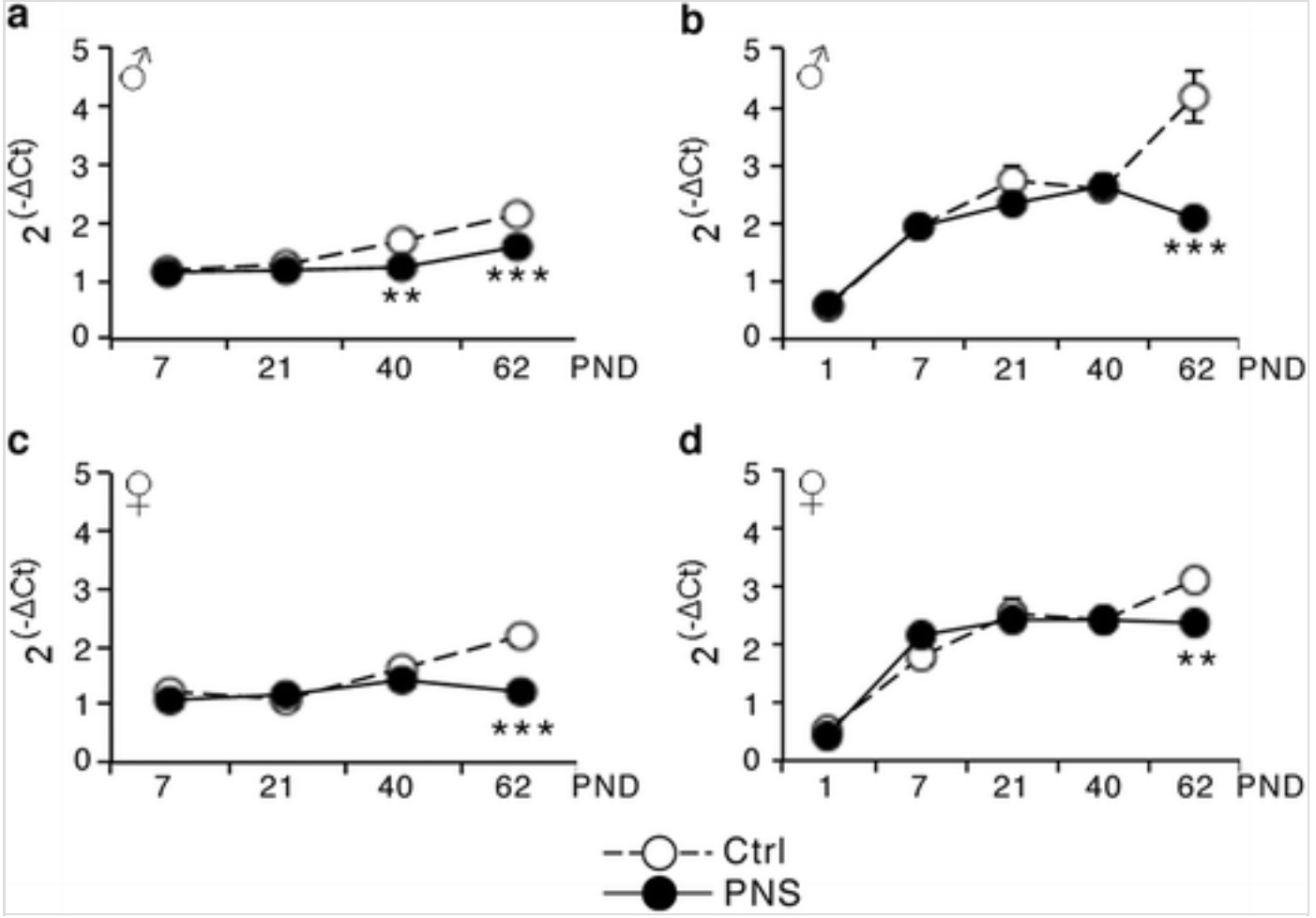
Prenatal Stress Alters Glucocorticoid Receptor Expression

Since one of the long-lasting alteration produced by early-life stress is a disruption of the HPA axis function [7], we first carried out a time course analysis of glucocorticoid receptor (GR) mRNA levels (namely *Nr3c1*) in the hippocampus and in the PFC of rats exposed to gestational stress (PNS), as compared to controls (Ctrl). We found gender- and brain region-specific differences between Ctrl and PNS animals. Within the hippocampus of Ctrl male rats, *Nr3c1* mRNA levels rose progressively until early adulthood (PND 62) (Fig. 1a, age effect: $p < 0.001$), differently from what occurred in PNS rats, an observation that is confirmed by the statistically significant effect of ~~prenatal stress~~PNS ($p < 0.001$) and a significant interaction age × PNS ($p < 0.05$). Indeed, when compared to Ctrl, PNS rats showed a less pronounced increase of *Nr3c1*

expression, which ~~results~~resulted in lower mRNA levels starting from PND 40 (PND 40: $p < 0.01$; PND 62: $p < 0.001$) (Fig. 1a). A similar effect was also found in the hippocampus of female rats (Fig. 1c), with a significant effect of age ($p < 0.001$) and of PNS ($p < 0.001$), as well as a significant interaction age × PNS ($p < 0.001$), although a statistically significant difference in *Nr3c1* expression between Ctrl and PNS rats was only found at PND 62 ($p < 0.001$). The expression levels of *Nr3c1* in the PFC (Fig. 1b, d) showed a marked increase during the postnatal development in male and female Ctrl rats (age effect: $p < 0.001$ for both genders). In addition, male rats displayed a significant effect of prenatal stress exposure (PNS effect: $p < 0.001$) and a significant interaction age × PNS ($p < 0.001$) (Fig. 1b). Indeed, while *Nr3c1* mRNA levels ~~rise~~rose between PND 40 and PND 62 in Ctrl rats, this effect ~~does~~did not occur in PNS animals, resulting in a statistically significant difference at PND 62 ($p < 0.001$), but not at earlier stages of the development (Fig. 1b). In the PFC of female rats, we did not find a significant effect of PNS ($p = 0.197$), but a statistically significant age × PNS interaction ($p < 0.05$). Once again, we found a rise of ~~of~~ *Nr3c1* expression after PND 40 in Ctrl but not in PNS rats, leading to significantly lower *Nr3c1* mRNA levels in PNS rats at PND 62 ($p < 0.01$) (Fig. 1d).

Fig. 1

Postnatal developmental changes of the glucocorticoid receptor (*Nr3c1*) mRNA levels in control and prenatally stressed rats. Postnatal developmental analyses of the mRNA levels for *Nr3c1* were carried out in the hippocampus (**a**, **c**) and in the prefrontal cortex (**b**, **d**) of male (**a**, **b**) and female (**c**, **d**) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The data, expressed as fold change (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene), are the mean ± sem of 5–8 animals per group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Ctrl of the same postnatal age (two-way ANOVA followed by post hoc test)



Prenatal Stress Alters the Modulation of Bdnf Expression Under an Acute Challenge at Adulthood

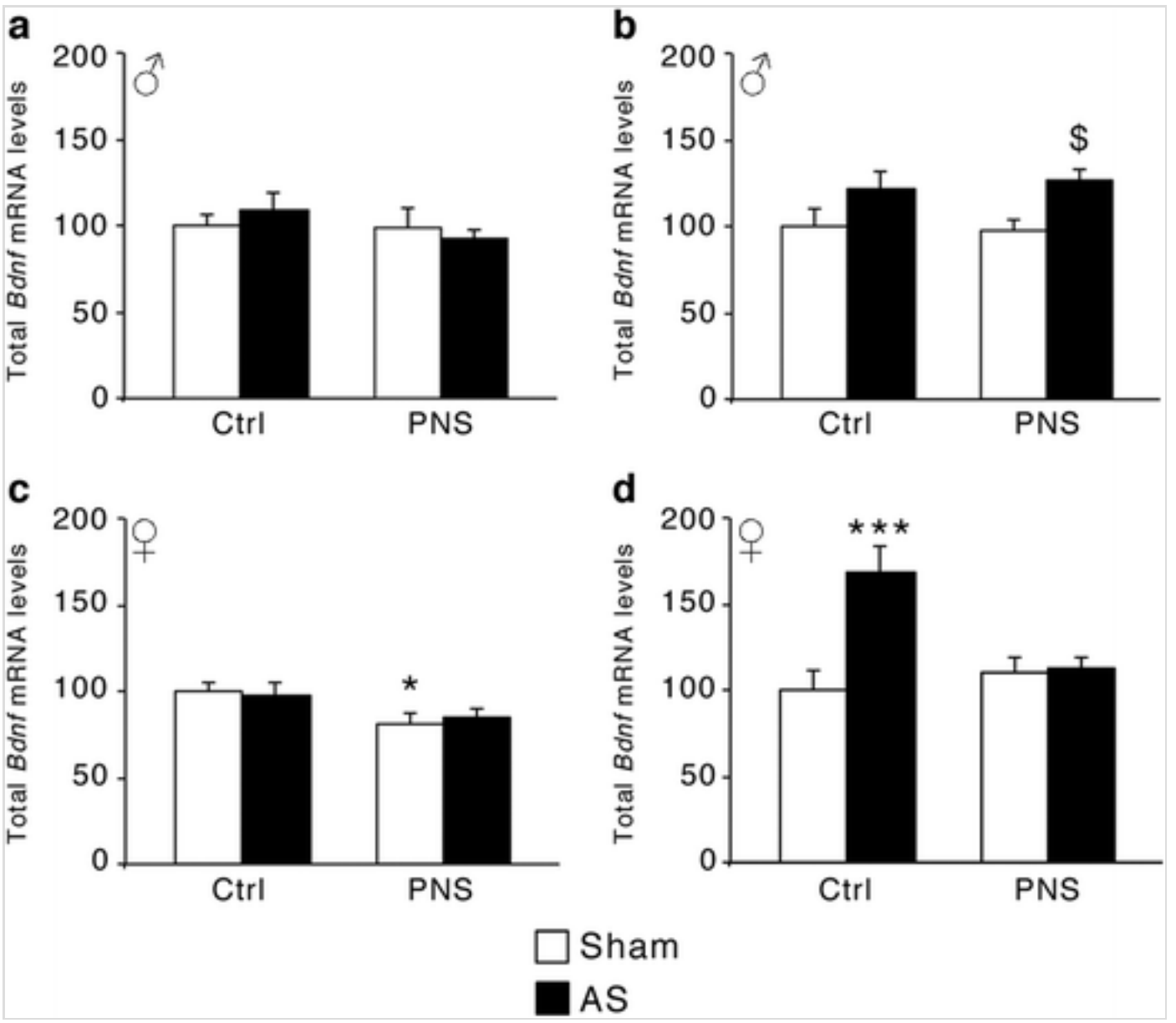
Thus, seeing the long-lasting changes in the expression of *Nr3c1*, we hypothesized that PNS rats may show an altered ability to respond to stress at adulthood. One way to address this issue is to investigate the rapid modulation of genes that may contribute to the ability to respond under challenging conditions [25, 26]. First, we ~~choose~~chose to investigate the expression of the neurotrophin *Bdnf*, which undergoes rapid changes following acute manipulations and may therefore be considered an index of adaptive plasticity under challenging conditions [27–29]. Hence, we measured *Bdnf* mRNA levels in adult Ctrl and PNS rats under resting conditions (sham), as well as after an acute forced swim stress (AS).

As shown in Fig. 2, the exposure to PNS reduced the hippocampal expression of total *Bdnf* in female (PNS effect: $F_{1,29} = 7.233$, $p < 0.05$; Fig. 2c), but not in male (PNS effect: $F_{1,28} = 1.016$, $p > 0.05$; Fig. 2a)~~,~~ rats, without affecting the stress responsiveness of both genders. In the PFC of male rats (Fig. 2b), we found a significant effect of AS ($F_{1,30} = 10.116$, $p < 0.01$), but no PNS effect ($F_{1,30} = 0.029$, $p = 0.866$) or PNS × AS interaction ($F_{1,30} = 0.175$, $p = 0.68$). Indeed, total *Bdnf* mRNA expression was upregulated in response to the acute stress in Ctrl as well as in PNS animals (+22 % vs. Ctrl/sham, $p = 0.07$, and +30 % vs. PNS/sham, $p < 0.05$, respectively). Conversely, within the PFC of

female rats (Fig. 2d), we found a significant effect of PNS ($F_{1,30} = 4.464$, $p < 0.05$) and of AS ($F_{1,30} = 10.736$, $p < 0.01$), as well as a significant PNS × AS interaction ($F_{1,30} = 9.859$, $p < 0.01$). Interestingly, the exposure to the acute challenge increased total *Bdnf* mRNA levels in Ctrl animals (+69 % vs. Ctrl/sham, $p < 0.001$), without affecting its expression in PNS female rats (+2 % vs. PNS/sham, $p > 0.05$).

Fig. 2

Modulation of total *Bdnf* mRNA levels following prenatal stress under basal conditions or after exposure to an acute stress at adulthood. The mRNA levels of total *Bdnf* were analysed in the hippocampus (**a**, **c**) and in the prefrontal cortex (**b**, **d**) of Ctrl or PNS adult male (**a**, **b**) and female (**c**, **d**) rats under resting conditions (sham) or following exposure to an acute forced swim stress (AS). The data, expressed as a percentage of Ctrl/sham animals (set at 100 %), are the mean ± sem of 6–8 animals per group. *$p < 0.05$ and ***$p < 0.001$ vs. Ctrl/sham and $^{\$}p < 0.05$ vs. PNS/sham (two-way ANOVA followed by post hoc test)

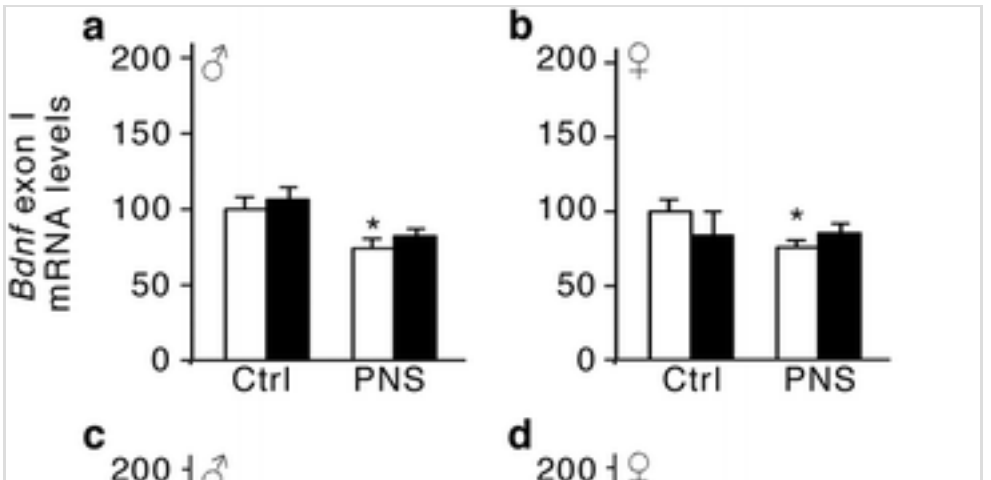


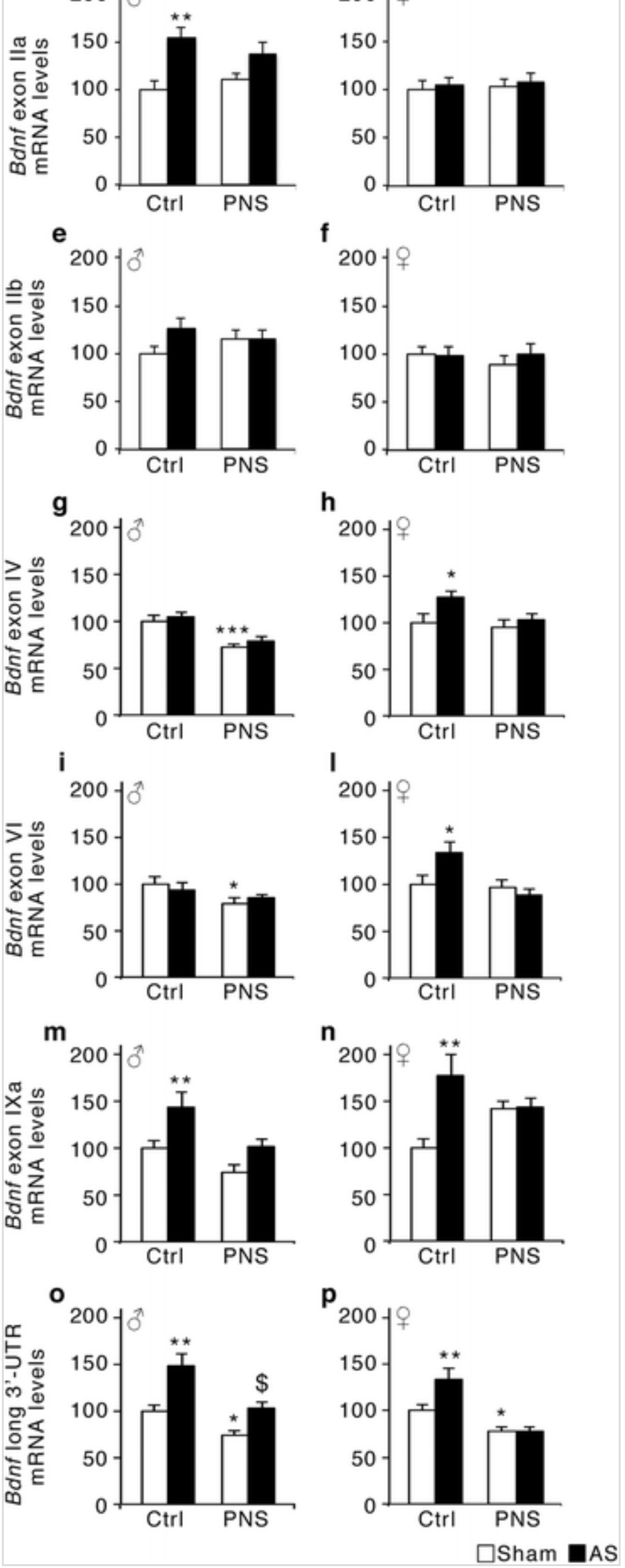
Based on these results, we decided to investigate in more detail the modulation of *Bdnf* in the PFC, by measuring the mRNA levels of its main transcripts

(namely exon I, exon IIa, exon IIb, exon IV, exon VI, exon IXa), as well as of the pool of *Bdnf* transcripts with the long 3′-UTR, which holds important implications for synaptic trafficking and localization of the neurotrophin [30, 31]. In male rats, we found that exposure to PNS had a significant effect on the expression of different *Bdnf* exons (exon I: $F_{1,29} = 12.907$, $p < 0.01$, Fig. 3a; exon IV: $F_{1,30} = 30.436$, $p < 0.001$, Fig. 3g; exon VI: $F_{1,30} = 5.113$, $p < 0.05$, Fig. 3i; exon IXa: $F_{1,31} = 10.715$, $p < 0.01$, Fig. 3m; long 3′-UTR: $F_{1,29} = 17.264$, $p < 0.001$, Fig. 3o), which are reduced to different extents as a consequence of the prenatal manipulation. Conversely, a significant effect of the acute stress was only found for *Bdnf* exon IIa and exon IXa and for the pool of transcripts with the long 3′-UTR ($F_{1,30} = 16.826$, $p < 0.001$, Fig. 3c; $F_{1,31} = 12.519$, $p < 0.01$, Fig. 3m; $F_{1,29} = 20.502$, $p < 0.001$, Fig. 3o, respectively), with no significant interaction between PNS and AS in all cases. Indeed, in line with the changes of total *Bdnf* levels (Fig. 2b), exposure to acute stress increased the mRNA levels of exon IIa (+54 % vs. Ctrl/sham, $p < 0.01$, and +21 % vs. PNS/sham, $p = 0.056$; Fig. 3c), of exon IXa (+44 % vs. Ctrl/sham, $p < 0.01$, and +35 % vs. PNS/sham, $p = 0.07$; Fig. 3m) and of *Bdnf* long 3′-UTR (+48 % vs. Ctrl/sham, $p < 0.01$, and +39 % vs. PNS/sham, $p < 0.05$; Fig. 3o), independently from the prenatal manipulation.

Fig. 3

Modulation of *Bdnf* transcript expression following prenatal stress under basal conditions or after exposure to an acute stress at adulthood. The mRNA levels of *Bdnf* exon I (**a**, **b**), exon IIa (**c**, **d**), exon IIb (**e**, **f**), exon IV (**g**, **h**), exon VI (**i**, **l**) and exon IXa (**m**, **n**) and of the pool of transcripts with the long 3′-UTR (**o**, **p**) were analysed in the prefrontal cortex of Ctrl or PNS adult male (*on the left*) and female (*on the right*) rats under resting conditions (sham) or following exposure to an acute forced swim stress (AS). The data, expressed as a percentage of Ctrl/sham animals (set at 100 %), are the mean ± sem of 5–8 animals per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Ctrl/sham and $^{\$}p < 0.05$ vs. PNS/sham (two-way ANOVA followed by post hoc test)




Bdnf exon IIa mRNA levels
Bdnf exon IIb mRNA levels
Bdnf exon IV mRNA levels
Bdnf exon VI mRNA levels
Bdnf exon IXa mRNA levels
Bdnf long 3'-UTR mRNA levels
♂
♀
Ctrl
PNS
Sham
AS

In female rats, exposure to PNS produced a significant effect on the expression of *Bdnf* exon I ($F_{1,27} = 7.337$, $p < 0.05$; Fig. 3b) and *Bdnf* exon VI ($F_{1,30} = 8.265$, $p < 0.01$; Fig. 3l) and of the pool of *Bdnf* transcripts with the long 3′-UTR ($F_{1,26} = 29.518$, $p < 0.001$; Fig. 3p). Interestingly, with the exception of *Bdnf* exon I (AS effect: $F_{1,27} = 0.408$, $p = 0.53$; PNS × AS effect: $F_{1,27} = 0.827$, $p = 0.37$) (Fig. 3b), exon IIa (AS effect: $F_{1,30} = 0.366$, $p = 0.55$; PNS × AS effect: $F_{1,30} = 0.001$, $p = 0.97$) (Fig. 3d) and exon IIb (AS effect: $F_{1,30} = 0.232$, $p = 0.63$; PNS × AS effect: $F_{1,30} = 0.431$, $p = 0.52$) (Fig. 3f), the different *Bdnf* transcripts were also regulated by the acute swim stress, an effect that was highly dependent upon the prenatal experience. Indeed, *Bdnf* exon IV mRNA levels were upregulated after AS in Ctrl rats (+27 % vs. Ctrl/sham, $p < 0.05$), but not in PNS animals (+8 % vs. PNS/sham, $p > 0.05$), although this difference was not sustained by the statistical interaction between AS and PNS ($F_{1,29} = 1.310$, $p > 0.05$) (Fig. 3h). A similar pattern was also found for *Bdnf* exon VI, whose expression was significantly increased by AS in Ctrl (+34 % vs. Ctrl/sham, $p < 0.05$), but not in PNS female rats (−9 % vs. PNS/sham, $p > 0.05$) (Fig. 3l), with a significant interaction of AS × PNS ($F_{1,30} = 5.973$, $p < 0.05$). *Bdnf* exon IXa also displayed a significant interaction between PNS and AS ($F_{1,29} = 6.895$, $p < 0.05$), together with a significant effect of AS ($F_{1,29} = 8.774$, $p < 0.01$) (Fig. 3n). Once again, we found that the acute challenge upregulated *Bdnf* exon IXa mRNA levels in Ctrl female rats (+78 % vs. Ctrl/sham, $p < 0.01$), but not in PNS animals (+1 % vs. PNS/sham, $p > 0.05$). Last, the pool of *Bdnf* transcripts with the long 3′-UTR was also elevated in the PFC of Ctrl female rats following exposure to AS (+34 % vs. Ctrl/sham, $p < 0.01$), whereas no changes were found in PNS animals (Fig. 3p), and such differential modulation was again supported by the statistical significance of PNS × AS interaction ($F_{1,26} = 5.476$, $p < 0.05$).

Prenatal Stress Alters the Modulation of BDNF Protein Following an Acute Challenge at Adulthood

Seeing the differential modulation of *Bdnf* mRNA in the PFC of PNS rats as compared to Ctrl animals, we investigated if exposure to the AS could affect the levels of BDNF protein. We specifically investigated the levels of mature neurotrophin (mBDNF) in the crude synaptosomal fraction of PFC where its analysis may reflect the pool of “synaptic” BDNF and possibly changes related to local translation as well as neurotrophin release. In line with the significant changes of *Bdnf* mRNA levels found in PNS rats, we found that the levels of

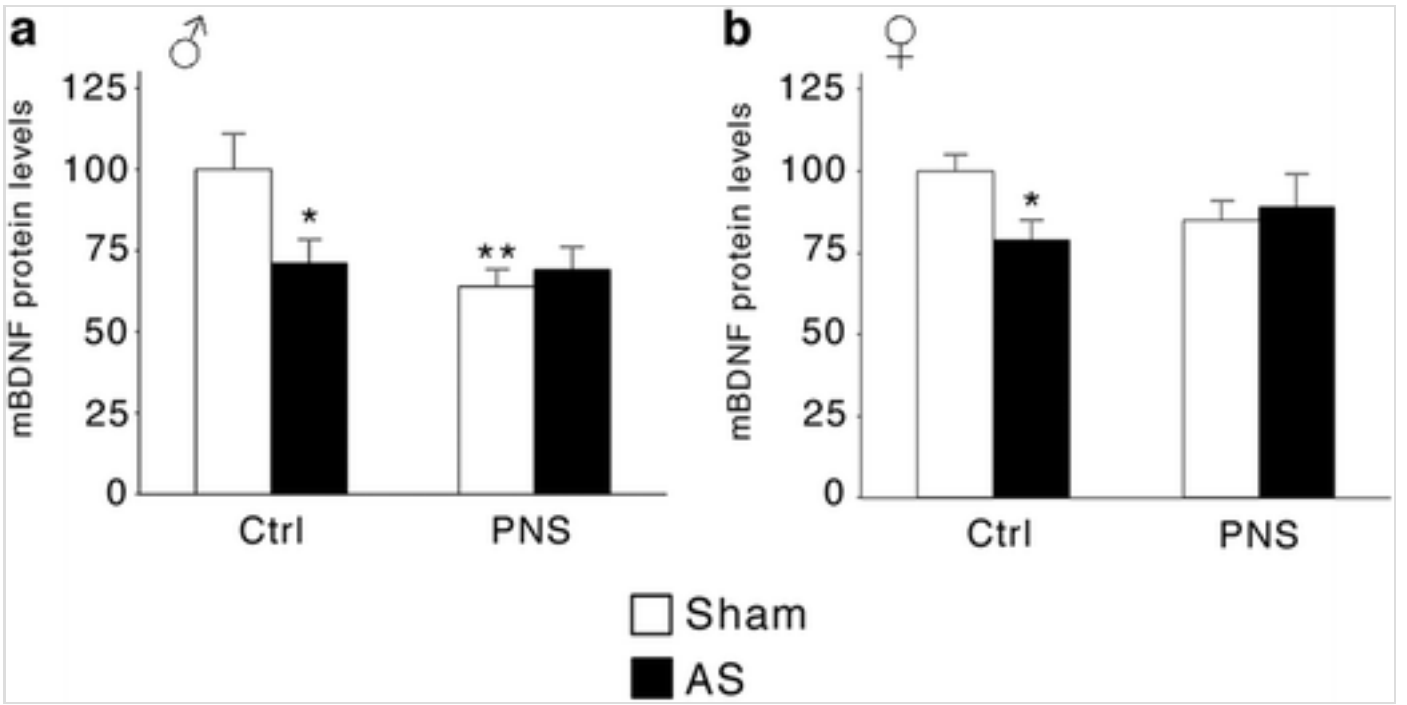
mBDNF were significantly decreased in the crude synaptosomal fraction of PFC from male but not female rats that were exposed to PNS ($F_{1,24} = 5.735$, $p < 0.05$, and $F_{1,24} = 0.411$, $p = 0.53$, respectively) (Fig. 4) . Indeed, PNS male rats showed a reduction of mBDNF while females did not (−36 % vs. Ctrl/sham, $p < 0.01$, and −15 % vs. Ctrl/sham, $p > 0.05$, respectively). Exposure to the AS produced a significant decrease of mBDNF in Ctrl animals from both genders (−29 % vs. Ctrl/sham, $p < 0.05$, and −21 % vs. Ctrl/sham, $p < 0.05$, respectively, in males and females), whereas no significant changes were found in PNS male and female rats when exposed to the acute challenge (Fig. 4).

Transcriptional Activity of GR-Responsive Genes in Prenatally Stressed Rats Exposed to an Acute Challenge at Adulthood

Seeing the role of GR in regulating the expression of *Bdnf* [32, 33], we analysed the expression of different representative GR-responsive genes in order to establish if altered *Bdnf* responsiveness was the consequence of altered GR transcriptional activity. Specifically, we measured the mRNA levels of dual specificity phosphatase 1 (*Dusp1*), FK506 binding protein 5 (*Fkbp5*), forkhead box O1 (*FoxO1*) and the glucocorticoid-induced leucine zipper (*Gilz*) in the PFC of Ctrl or PNS rats, under resting conditions or following an acute stress. As shown in Fig. 45, we observed a significant effect of AS on the expression of *Dusp1* selectively in male rats ($F_{1,29} = 6.809$, $p < 0.05$; Fig. 45a), but not in females ($F_{1,30} = 0.174$, $p > 0.05$; Fig. 45e). Indeed, AS determined a trend toward a significant increase both in Ctrl and PNS male rats (+26 % $p = 0.072$ and +22 % $p = 0.083$, respectively). Moreover, no significant PNS effect, or PNS × AS interaction, was observed on the expression of *Dusp1*. While *Fkbp5* expression was not altered in male rats (Fig. 45b), we found a significant reduction in PNS females ($F_{1,30} = 8.566$, $p < 0.01$; Fig. 45f), although its mRNA levels were not modulated by the AS ($F_{1,30} = 1.037$, $p > 0.05$). *FoxO1* mRNA expression was not modulated in our experimental conditions in both male and female rats (Fig. 45c, g), whereas we found a significant effect of PNS exposure on the expression of *Gilz* in female rats ($F_{1,29} = 4.650$, $p < 0.05$) and a significant AS × PNS interaction ($F_{1,29} = 5.282$, $p < 0.05$) (Fig. 45h). Indeed, PNS female rats exposed to AS showed a significant increase of *Gilz* mRNA levels (+44 % vs. PNS/sham, $p < 0.01$), an effect that was not present in Ctrl rats exposed to AS (−7 % vs. Ctrl/sham, $p > 0.05$; Fig. 45h). We also found a similar increase of *Gilz* mRNA levels in male PNS rats, although it did not reach the statistical significance (+29 % vs. PNS/sham, $p > 0.05$; Fig. 45d).

Fig. 4

Modulation of mBDNF protein levels following prenatal stress under basal conditions or after exposure to an acute stress at adulthood. The protein levels of mBDNF were analysed in the prefrontal cortex of Ctrl or PNS adult male (**a**) and female (**b**) rats under resting condition (sham) or following exposure to an acute forced swim stress (AS). The data, expressed as a percentage of Ctrl/sham animals (set at 100 %), are the mean ± sem of 5–7 animals per group. *$p < 0.05$ and **$p < 0.01$ vs. Ctrl/sham (two-way ANOVA followed by post hoc test)



Modulation of Gadd45β Expression in Prenatally Stressed Rats

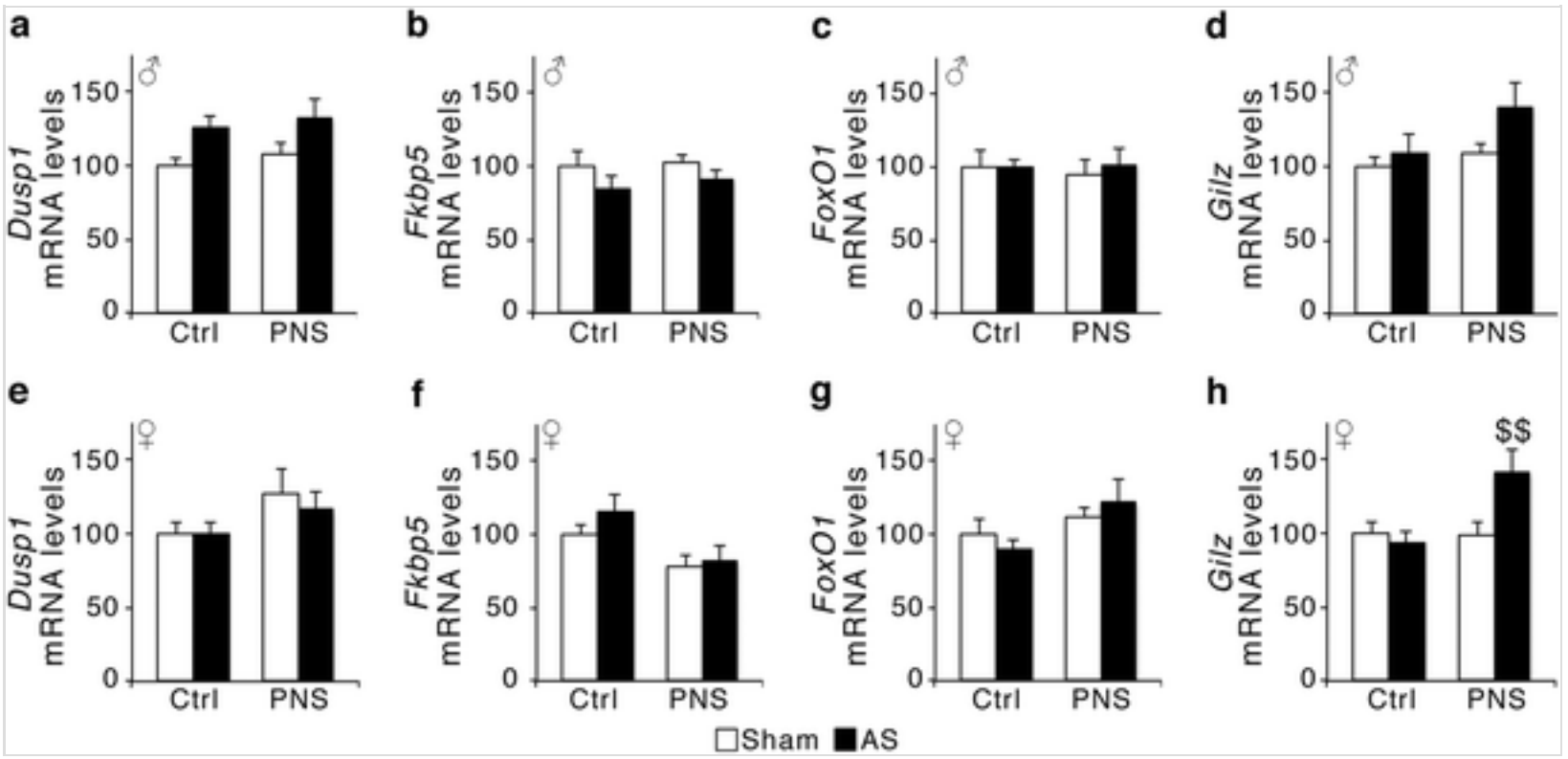
Several studies have demonstrated that *Bdnf* expression can be regulated through epigenetic mechanisms, including DNA methylation [34, 35]. In particular, it has been shown that growth arrest and DNA-damage-inducible beta (*Gadd45β*) is involved in activity-dependent neural plasticity through the epigenetic regulation of *Bdnf* [36]. On these bases, we investigated whether changes in *Gadd45β* could contribute to the differential modulation of *Bdnf* mRNA levels between Ctrl and PNS female rats exposed to an acute challenge.

As shown in Fig. ~~5~~6a, exposure to AS increased *Gadd45β* mRNA levels in the PFC of Ctrl (+42 % vs. Ctrl/sham, $p < 0.01$) as well as of PNS male rats (+60 % vs. PNS/sham, $p < 0.001$), with a significant effect of ~~the~~ AS ($F_{1,28} = 30.545$, $p < 0.001$), but no PNS × AS interaction ($F_{1,28} = 0.578$, $p > 0.05$) or PNS effect ($F_{1,28} = 0.001$, $p > 0.05$). On the contrary, female rats showed a significant effect of PNS ($F_{1,30} = 27.896$, $p < 0.001$) and AS ($F_{1,30} = 27.068$, $p < 0.001$) and a significant PNS × AS interaction ($F_{1,30} = 6.985$, $p < 0.05$). Indeed, as shown in

Fig. ~~5~~6b, while the acute stress increased the mRNA levels of *Gadd45β* in the PFC of female Ctrl rats (+50 % vs. Ctrl/sham, $p < 0.001$), no significant changes were observed in PNS rats (+22 % vs. PNS/sham, $p > 0.05$). In order to substantiate the possible relationship between the modulation of *Gadd45β* and *Bdnf* mRNA levels, data were examined for possible co-variation within the gene expression of *Gadd45β* and *Bdnf* in the PFC of male and female rats. The analyses revealed that *Gadd45β* mRNA levels correlated positively with the expression of total *Bdnf* in male ($r = 0.44$, $n = 28$, $p < 0.05$) as well as in female ($r = 0.72$, $n = 30$, $p < 0.001$) rats (Fig. ~~5~~6c, d, respectively).

Fig. 5

Modulation of glucocorticoid receptor-responsive gene expression following prenatal stress under basal conditions or after exposure to an acute stress at adulthood. The mRNA levels of *Dusp1* (**a**, **e**), *Fkbp5* (**b**, **f**), *FoxO1* (**c**, **g**) and *Gilz* (**d**, **h**) were analysed in the prefrontal cortex of Ctrl or PNS adult male (**a**–**d**) and female (**e**–**h**) rats under resting conditions (sham) or following exposure to an acute forced swim stress (AS). The data, expressed as a percentage of Ctrl/sham animals (set at 100 %), are the mean ± sem of 5–8 animals per group. $^{\$\$}p < 0.01$ vs. PNS/sham (two-way ANOVA followed by post hoc test)



Discussion

Exposure to early-life stress has been associated with an enhanced risk for ~~psychopathologies~~psychopathology, suggesting that early adversities may impair the ability to respond to or cope with environmental challenges later in life. The results of the present study, in agreement with this hypothesis,

demonstrate that exposure to prenatal stress in rats not only leads to reduced expression of brain glucocorticoid receptors, but impairs the modulation of the neurotrophin *Bdnf* following an acute challenge, with anatomical specificity and in a sex-dependent fashion. Moreover, our data suggest that epigenetic mechanisms may contribute to the inability to cope with an adverse situation, seeing the altered *Gadd45β* modulation paralleling *Bdnf* changes.

One of the main biological features shared by different psychiatric conditions and experimental paradigms is the impaired function of the hypothalamic-pituitary-adrenal (HPA) axis, possibly due to an altered function of GR in different brain regions, including the hippocampus and the prefrontal cortex [3, 37]. In line with these results, we found that the expression of the GR-encoding gene (*Nr3c1*) is significantly lower in the hippocampus and in the prefrontal cortex of adult male and female rats subjected to PNS. Since this effect becomes evident between adolescence and early adulthood, it may reflect a derangement of the developmental trajectory for GR expression in these brain regions, as we have previously shown to occur for the neurotrophin *Bdnf* [12]. Reduced expression of *Nr3c1* may alter HPA axis function, leading to increased circulating levels of corticosterone. We, and others, have previously shown that PNS rats display increased peripheral blood concentrations of corticosterone [7, 10], which may lead to a number of alterations important for neuronal plasticity and function [16, 38]. Indeed, our study reveals that the prenatal manipulation reduces the mRNA levels of total *Bdnf* selectively in the hippocampus of female rats, whereas the expression of different neurotrophin transcripts was decreased in the prefrontal cortex, with some gender specificity. Of note, Hill and colleagues have shown that maternal separation in rats, alone or in combination with chronic corticosterone treatment during late adolescence, produced gender-specific effects on *Bdnf* expression [39]. These findings suggest that perinatal manipulations could affect different brain regions depending on the sex and also on the timing of the stressful experience.

It is known that exposure to stress early in life may prime the brain and alter its ability to cope with adverse challenging situations later in life, which may lead to a precipitation of a latent pathologic condition [9, 40]. Indeed, PNS rats show brain region- and sex-specific differences in *Bdnf* modulation when exposed to an AS at early adulthood. We found that, while AS did not alter *Bdnf* expression in the hippocampus, it produced significant changes in the prefrontal cortex. Indeed, AS upregulated *Bdnf* mRNA levels in the prefrontal cortex of control animals, both males and females, although in the two genders the effect

~~is~~was sustained by different *Bdnf* transcripts. Interestingly, while male PNS rats exposed to AS showed the same responsiveness of Ctrl, female PNS rats did not, suggesting that exposure of female rats to prenatal stress may alter the ability to respond to challenging situations at adulthood. Indeed, the mRNA levels of total *Bdnf*, as well as of different neurotrophin transcripts, were significantly upregulated, after the acute challenge, in the prefrontal cortex of Ctrl but not of PNS female rats. Rapid transcriptional responses are often associated with stress and may play a role in coping with the challenging condition. For example, it has been demonstrated that contextual fear learning is associated with a significant upregulation of *Bdnf* expression that participates in the encoding of salient environmental cues [18]. The prefrontal cortex plays a key role in controlling cognition and emotion, and exposure to an acute stress may facilitate learning and memory through different mechanisms, including the action of corticosteroids and a potentiation of glutamate transmission [41–44]. We suggest that the altered upregulation of *Bdnf* seen in PNS females exposed to the acute stress may be indicative of an impairment of the coping mechanism that is usually set in motion to respond to the adverse challenging event.

Furthermore, we show that while the acute challenge leads to a decrease of mature BDNF protein levels in the crude synaptosomal fraction from PFC of control animals, such change does not occur in PNS rats. We believe that the decreased levels of mBDNF in the crude synaptosomal fraction after the acute swim stress may be indicative of a release of the neurotrophin, a mechanism that may be impaired in PNS rats.

While the expression of *Bdnf* can be regulated by glucocorticoids [32, 33], the differential responsiveness of *Bdnf* transcription found in the PFC of female rats does not appear to be strictly related to altered GR responsiveness. First, GR mRNA levels were downregulated in the PFC of both genders (see Fig. 1) and its expression was not altered by the acute swim stress (data not shown). Moreover, the analysis of different GR-responsive genes, including *Dusp1*, *Fkbp5*, *FoxO1*, and *Gilz*, does not show a profile of changes resembling the modulation of *Bdnf* mRNA levels, suggesting that other activity-dependent mechanisms may contribute to the differential regulation of *Bdnf* expression set in motion by the acute challenge.

Our results suggest the involvement of activity-dependent epigenetic mechanisms, seeing that the stress-induced modulation of growth arrest and

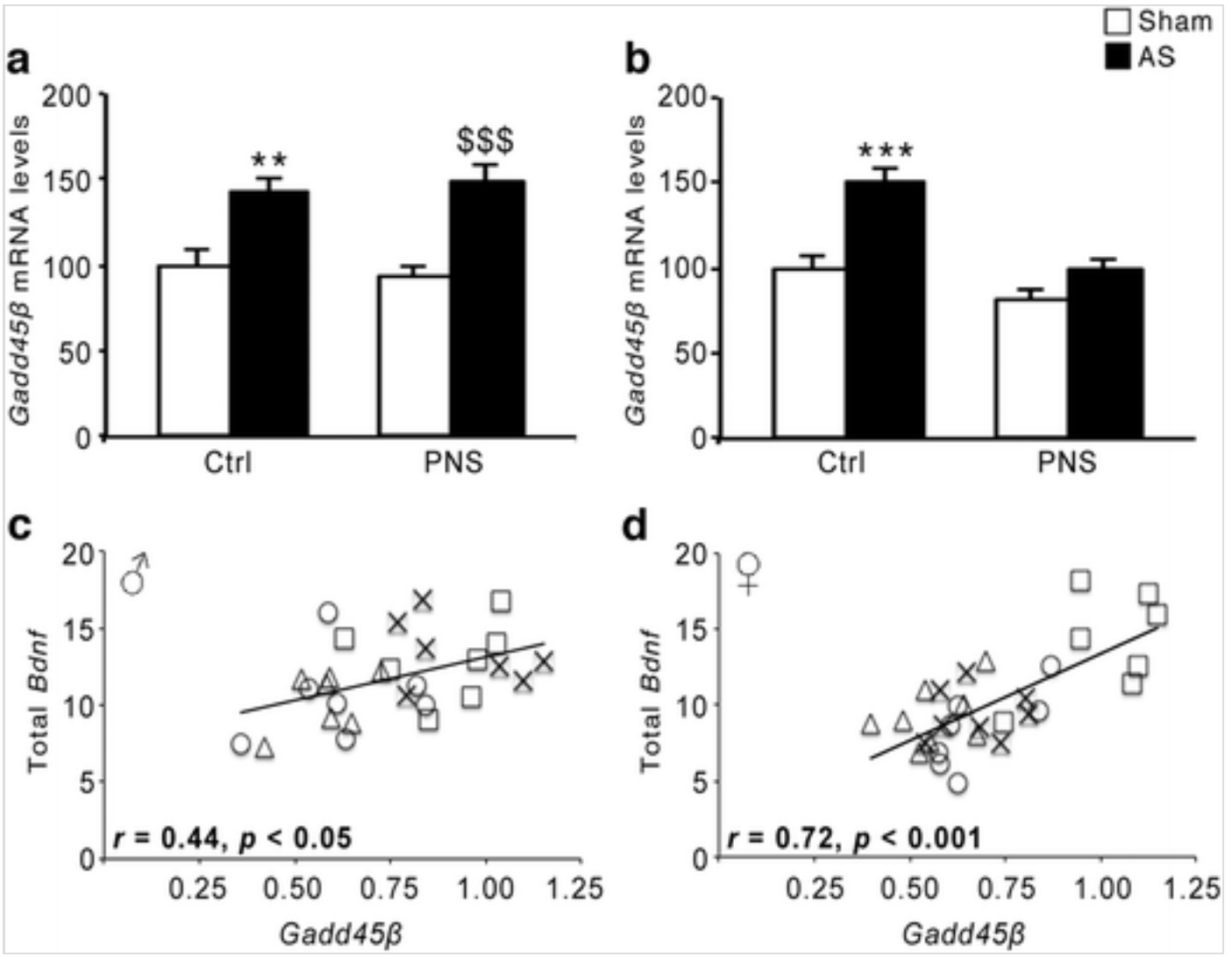
DNA-damage-inducible beta (*Gadd45β*) in PNS female rats mirrors the modulation of *Bdnf* transcription following the acute challenge. *Gadd45β* is a DNA demethylase that is able to regulate *Bdnf* expression by activity-induced demethylation of its promoter region~~s~~ [36, 45]. Indeed, we found that while *Gadd45β* mRNA levels are upregulated following exposure to AS in male rats, independently from the prenatal manipulation, such effect does not occur in PNS female animals. Moreover, we show that there is a significant correlation between the levels of *Gadd45β* in the prefrontal cortex and *Bdnf* expression, suggesting that changes in the activity-dependent modulation of the neurotrophin may be promoted by the modulation of the DNA demethylase. In agreement with this possibility, *Gadd45β*-knockout mice displayed decreased levels of *Bdnf* with important consequences for brain plasticity [45]. Moreover, reduced binding of *Gadd45β* to *Bdnf* promoter~~s~~ has been reported in psychotic patients [46]. In agreement with our data, a recent paper has shown that exposure to an acute stress at adulthood is able to decrease global DNA methylation in several brain regions and that this effect ~~seemed~~seems unlikely to be mediated by impaired DNA methylation processes, but rather by active demethylation mechanisms [47]. Of note, we and others have previously shown that psychotropic drugs, including the multi-receptor modulator lurasidone or group II glutamate metabotropic agonists, can modulate *Gadd45β* and may therefore prove useful in normalizing transcriptional defects associated with exposure to early-life stress [48, 49].

While it is difficult to establish the mechanisms that may sustain the sex specificity of the changes of *Bdnf* and *Gadd45β* expression in the PFC of PNS female rats, these results are in line with a previous study in mice showing gender differences in *Bdnf* expression in response to environmental changes [50] ~~(Fig. 6)~~. A more pronounced neuroendocrine response to stress in female rats has also been reported [51]. Furthermore, it is known that neurons in the adult rat forebrain co-express estrogen and neurotrophin receptors [52] and that estrogens regulate the expression of BDNF mRNA and protein in the developing brain [53], suggesting that hormonal variation during a critical time window may contribute to the sex-specific effects in *Bdnf* transcription.

Fig. 6

Modulation of *Gadd45β* expression following prenatal stress under basal conditions or after exposure to an acute stress at adulthood and its correlation with total *Bdnf* mRNA levels. The mRNA levels of *Gadd45β* were analysed in the prefrontal cortex of Ctrl or PNS adult male (**a**) and female (**b**) rats under resting

conditions (sham) or following exposure to an acute forced swim stress (AS). The data, expressed as a percentage of Ctrl/sham (set at 100 %), are the mean ± sem of 7–8 animals per group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. Ctrl/sham and $^{\$\$\$}p < 0.001$ vs. PNS/sham (two-way ANOVA followed by post hoc test). The correlation between mRNA levels (expressed as $2^{(-\Delta Ct)}$, where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene) of *Gadd45β* and total *Bdnf* in the prefrontal cortex of Ctrl or PNS male (**c**) and female (**d**) rats under resting conditions (sham) or following exposure to an acute forced swim stress (AS) was analysed by Pearson product-moment correlation (*r*)



AQ4

Altogether, our results indicate that exposure to early-life adversities may interfere with the proper maturation of brain circuits, thus hampering the individual ability to cope with stressful experiences later in life. In agreement with previous works [1, 2], we suggest that such impairment is associated with a programming of gene transcription mediated by epigenetic regulation. In this scenario, it will be important to establish the impact of pharmacological intervention in counteracting ~~the~~ long-term alterations in stress responsiveness, which represents an important component of different psychiatric diseases.

Acknowledgments

This work was supported by grants from the Italian Ministry of University and Research to M.A.R. (Progetti di Ricerca di Interesse Nazionale—PRIN—grant number 20107MSMA4_002), from Fondazione CARIPLO (grant number 2012-0503) to M.A.R. and F.C. and from the European Union/NEURON-ERANET to M.A.R. A.L. was supported by a fellowship from Fondazione Umberto Veronesi.

Compliance with Ethical Standards

AQ5

All animal experiments were conducted according to the authorization from the Health Ministry n. 295/2012-A (20/12/2012), in full accordance with the Italian legislation on animal experimentation (Decreto Legislativo 116/92) and adherent to EU recommendation (EEC Council Directive 86/609).

Conflict of Interest The authors Luoni A., Berry A., Raggi C., Bellisario V. and Cirulli F. have no financial interest or potential conflict of interest. Riva M.A. has received compensation as speaker/consultant from Bristol-Myers Squibb, Sumitomo Dainippon Pharma, Eli Lilly, Innova Pharma, Lundbeck, Servier, Sunovion and Takeda.

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