Sex differences in the enduring effects of social deprivation during adolescence in rats: implications for psychiatric disorders.

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Abstract

The exposure to adverse environmental situations during sensitive periods of development may induce re-organizational effects on different systems and increase the vulnerability to develop psychiatric disorders later in life. The adolescent period has been demonstrated extremely susceptible to stressful events. However, most of the studies focused on the immediate effects of stress exposure and few of them investigated sex differences. This raised the question if these modulations might also be long-lasting and how the differential maturational events taking place during adolescence between males and females might have a role in the detrimental effects of stress. Given the importance of social play for the right maturation of behaviour during adolescence, we used the preclinical model of social deprivation, based on the lack of all social contacts, for four weeks after weaning, followed by re-socialization until adulthood. We found that both male and female animals reared in isolation during adolescence developed an anhedonic phenotype at adulthood, without any impairments in the cognitive domain. At molecular level, these functional changes were associated with sex-specific impairments in the expression of neuroplastic markers as well as of hypothalamic-pituitary-adrenal axis-related genes. Lastly, we also reported anatomically-selective changes associated with the enduring effects of social isolation.

Keywords: adolescence; social deprivation; psychiatric disorders; sex

1. Introduction

Stress is considered the main environmental risk factor for psychiatric disorders. In recent years, epidemiological and preclinical studies demonstrated that the exposure to adverse experiences early in life (early life stress, ELS) can predispose to adult disease by inducing persistent changes in physiological, emotional and behavioural functions throughout life (Bale et al., 2010; Heim and Nemeroff, 2001; Maccari et al., 2014; McEwen, 2012). Most research has focused on the programming effects of stress on the developing brain taking advantage of well-established prenatal and perinatal experimental paradigms (Luoni et al., 2014; Roceri et al., 2004). These studies have shown that early-life stress exposure leads to the development of anxiety- and depressive-like phenotypes and reduces the ability to cope with stressful situations later in life (van der Doelen et al., 2014; Luoni et al., 2014; Roceri et al., 2004).

However, the adverse functional and molecular outcomes of ELS exposure depend upon the timing of the adverse experiences, which may differentially affect specific brain regions and circuits. Accordingly, adolescence represents a sensitive period of brain development when the full-blown manifestation of different psychiatric disorders often occurs (Blakemore and Mills, 2014; Fuhrmann et al., 2015). Adolescence is the transition period from childhood to adulthood and it is characterized by a series of behavioural and structural maturational events. Typical behaviours of adolescence bear similarities across different species, comprising an increase in social interaction as well as play, risk-taking and fighting behaviours (Spear, 2000). These features are linked to intensive maturational changes in the brain. The synapses go through continuous overproduction and pruning, while grey matter thins and white matter increases (Andersen and Teicher, 2008; Gogtay et al., 2004). Furthermore, whereas the hippocampus is fully organized, the amygdala and the prefrontal cortex are still developing (Lupien et al., 2009). Also, receptors of different neurotransmitter systems, including gamma-aminobutyric acid (GABA), glutamate and dopamine (DA) undergo functional changes during adolescence (Spear, 2000). Moreover, the timing of adolescence overlaps with puberty, referred to as the attainment of sexual maturation (Spear, 2000). Thereby, the pubertal activation of the hypothalamic-pituitary-gonadal axis culminating in gonadal maturation is closely related to the maturation of adult brain and behaviour (Sisk and Foster, 2004). Furthermore, taking into account that adolescence comprises a series of gradual events from childhood to adulthood, puberty could be considered one of these transitory changes.

As a result, the maturational events taking place during adolescence may determine amplified vulnerability to stressful experiences. Animal models represent a useful tool to investigate the effects of environmental and psychosocial stressors during adolescence and their long-term consequences for later neuropsychiatric disorders. Several animal models mimicking the exposure to stress during adolescence have been successfully used (Burke et al., 2017). Among others, the social deprivation paradigm consists of chronic single housing beginning on the day of weaning. Under these conditions, animals are completely deprived of social contacts but they can smell, hear and see other animals within the holding room (Leng et al., 2004; Weiss et al., 2004). The application of this paradigm affects the maturation of normal social behaviours, leading to anxiety and depressive-like behaviours, and to reduced synaptic density and myelination in the prefrontal cortex (Leussis et al., 2008; Leussis and Andersen, 2008).

Over the past years, neurotrophic factors, including Brain-Derived Neurotrophic Factor (BDNF), have emerged as essential factors in the development of psychiatric disorders (Autry and Monteggia, 2012). Interestingly an association between BDNF and glucocorticoids exists (Jeanneteau and Chao, 2013), thus linking altered plasticity with the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which is found as a consequence of stressful events (Binder and Nemeroff, 2010) and that may represent a feature of different psychiatric disorders (Cherian et al., 2019; Zorn et al., 2017). While these modulations have been well documented soon after the end of stress exposure, with animals still being during their transition phase to adulthood, the long-lasting consequences of chronic social deprivation in adolescence on adult brain and behaviour are still poorly investigated (Green and McCormick, 2013). Furthermore, the few studies on the enduring effects of social stress during a defined period of adolescence mostly focused on the hippocampus (Murínová et al., 2017) and on male animals only (McCormick et al., 2017; Murínová et al., 2017).

On these bases, in the present study, we used the preclinical model of social deprivation in male and female animals to evaluate the long-lasting behavioural effects of stress exposure during adolescence. We focused on a very specific window of vulnerability that clearly comprises age-specific behavioural discontinuities from younger and older animals (Spear, 2000). Thereby, including a period of social interaction before behavioural testing, we ensured that any enduring effects of stress exposure might arise from the lack of gaining appropriate social experiences during a particular phase of development (Lukkes, 2009). Furthermore, we aimed to characterize the pattern of changes that may sustain the behavioural impairment, including the possibility to delineate sex and anatomical specificity

as a consequence of the adverse experience during adolescence. In particular, we investigated genes related to neuronal plasticity as well as the expression of the glucocorticoid receptor (*Nr3c1*) and its co-chaperone (*Fkbp5*) in prefrontal cortex (PFC) and hippocampus (dorsal vs. ventral), which represent important brain regions for the response to stress as well as for emotion and cognition (Fanselow and Dong, 2010; McEwen et al., 2016).

2. Results

2.1. Social deprivation exposure during adolescence induces an anhedonic phenotype in adult male and female rats.

To determine whether social isolation during adolescence can determine functional outcomes at adulthood, anhedonia and cognitive performances were measured in both male and female adult rats socially isolated during adolescence, as compared to Ctrl animals.

As shown in Figure 1A, sucrose preference was significantly affected by sex and by housing condition. Although Ctrl female rats exhibited a significantly higher preference for sucrose than Ctrl males ($p<0.05$), the exposure to social isolation during adolescence indeed decreased the preference for a sucrose solution in both adult male ($p<0.001$) and female ($p<0.05$) rats. On the contrary, when investigating the cognitive performance using the novel object recognition test (Figure 1B), we did not observe any significant effect of the adverse social experience during adolescence and no sex differences.

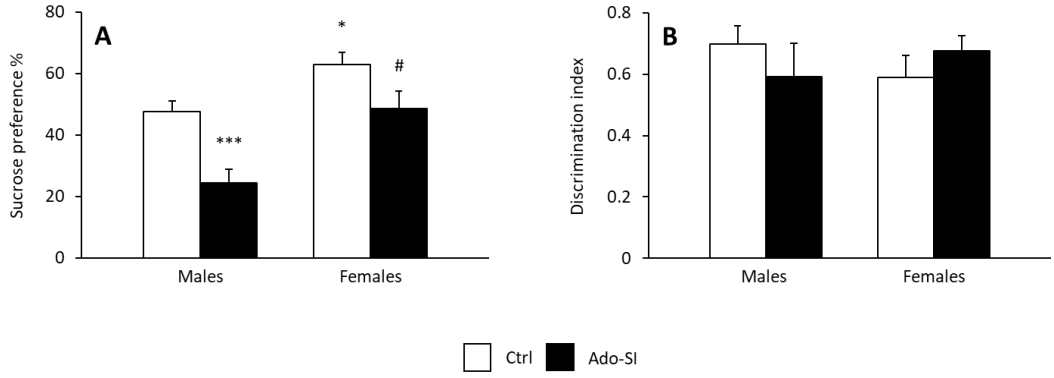

Figure 1 Effects of social isolation on anhedonia and cognition. (A) Analysis of anhedonia, as measured by the preference for sucrose, assessed in adult male and female group-housed rats (Ctrl) or in animals exposed to social isolation during adolescence (Ado-SI). Each value represents the mean ± SEM of at least 14 animals per group. (B) Analysis of cognitive impairment in Ctrl or Ado-SI rats, as measured in the novel object recognition test. The data are expressed as discrimination index, representing the difference between time spent exploring novel and familiar objects during the testing phase. Each value represents the mean ± SEM of at least 11 animals per group. *p<0.05 and ***p<0.001 vs Ctrl Males; $^{\#}$p<0.05 vs Ctrl Females (Two-way ANOVA followed by LSD post-hoc test)

2.2. *Bdnf* expression is altered in selected brain regions of adult animals exposed to social deprivation during adolescence.

Next, we wanted to investigate the biological mechanisms that may contribute to the long-lasting functional changes observed in adult male and female rats exposed to social isolation during adolescence. We assessed the expression of Brain-Derived Neurotrophic Factor (*Bdnf*) that represents an important player of neuronal plasticity (Leal et al., 2014) and whose expression is significantly affected in psychiatric disorders (Luoni et al., 2016; Molendijk et al., 2014).

In the prefrontal cortex (PFC) (Figure 2A), we found a significant main effect of the housing condition, with a strong trend toward significance of the housing X gender interaction. Indeed, the exposure to early social isolation produced a significant reduction of *Bdnf* mRNA levels only within the PFC of adult Ado-SI male animals ($p<0.05$). On the contrary, the expression of *Bdnf* in Ctrl females was significantly lower than Ctrl male rats ($p<0.05$), although such levels were not modulated by stress exposure. In the dorsal hippocampus (DH), we did not observe any statistically significant effect of gender or housing condition (Figure 2B), whereas the expression of *Bdnf* within the ventral hippocampus (VH) was significantly modulated by gender and by the housing condition (Figure 2C). Indeed, Ctrl female rats exhibited significantly lower *Bdnf* mRNA levels ($p<0.05$), as compared to Ctrl males, and the exposure to social deprivation during adolescence produced a reduction of the neurotrophin expression that was statistically significant only for adult male animals ($p<0.05$).

Seen the effects produced by early-stress exposure on total *Bdnf* mRNA levels, we assessed whether such changes could be attributable to specific *Bdnf* transcripts, including *Bdnf* exon IV and exon VI as well as the pool with long 3'-UTR.

Within the PFC, the modulation of total *Bdnf* mRNA levels was mirrored only in the expression of the *Bdnf* transcripts containing exon VI. Indeed, we did not observe any significant modulation of *Bdnf* long 3'-UTR transcripts (Figure 3A), whereas an effect of gender and housing condition only close to significance was found for *Bdnf* transcripts containing exon IV, with a slight decrease in female rats exposed to social isolation (Figure 3B). Instead, the exposure to social isolation during adolescence significantly modulated the expression of *Bdnf* transcripts containing exon VI within the PFC (Figure 3C), as confirmed by the significant downregulation of *Bdnf* exon VI mRNA levels only for adult male animals ($p<0.05$). Although not supported by a significant main effect of gender, we also observed

that Ctrl females showed significantly lower *Bdnf* exon VI mRNA levels, as compared to Ctrl males ($p<0.05$).

With regard to the dorsal hippocampus, although we did not observe any statistically significant effect of gender or housing condition on the expression of total *Bdnf* mRNA levels, our analyses revealed a differential modulation of the specific transcripts with sex specificity. Indeed, we found a significant effect of housing condition on the expression of *Bdnf* long 3'-UTR transcripts (Figure 3D). Accordingly, the exposure to stress during adolescence reduced *Bdnf* long 3'-UTR mRNA levels, with a statistical significance only for adult male animals ($p<0.05$). Opposite to this, the expression of *Bdnf* exon IV mRNA levels (Figure 3E) was statistically modulated by the housing condition with a significant housing X gender interaction. Indeed, the exposure to stress during adolescence produced a significant reduction of *Bdnf* exon IV mRNA levels only within the DH of adult female animals ($p<0.01$), an effect that was not observed for adult male Ado-SI rats. The sex specificity in the modulation of *Bdnf* transcripts within the DH is further sustained by the analysis of *Bdnf* exon VI expression (Figure 3F). Indeed, the statistical analysis showed a significant effect of the housing condition and of the housing X gender interaction. Exposure to social isolation in adolescence caused a significant reduction of *Bdnf* exon VI mRNA levels only within the DH of adult Ado-SI male animals ($p<0.01$). Instead, Ctrl females showed a significant reduction of the expression of *Bdnf* exon VI ($p<0.05$), as compared to Ctrl male rats, which was not modulated by stress exposure.

Lastly, the modulation of total *Bdnf* mRNA levels observed within the VH was confirmed only by the analysis of the expression of *Bdnf* long 3'-UTR transcripts (Figure 3G), whose expression was significantly modulated by the housing condition, with a substantial trend toward significance of the housing X gender interaction. Indeed, exposure to stress during adolescence produced a significant down-regulation of *Bdnf* long 3'-UTR mRNA levels within the VH of adult Ado-SI male animals ($p<0.01$). Instead, Ctrl females showed a significant reduction of *Bdnf* long 3'-UTR expression ($p<0.05$), as compared to Ctrl male rats, which was not affected by stress exposure. Similar to what we observed in the DH, the analysis of *Bdnf* exon IV expression showed a significant housing X gender interaction (Figure 3H). Accordingly, only adult female animals showed a significant downregulation of *Bdnf* exon IV mRNA levels as a consequence of the exposure to social stress during adolescence ($p<0.05$). On the contrary, the expression of *Bdnf* exon VI within the VH (Figure 3I) showed only a trend toward significance for the housing condition.

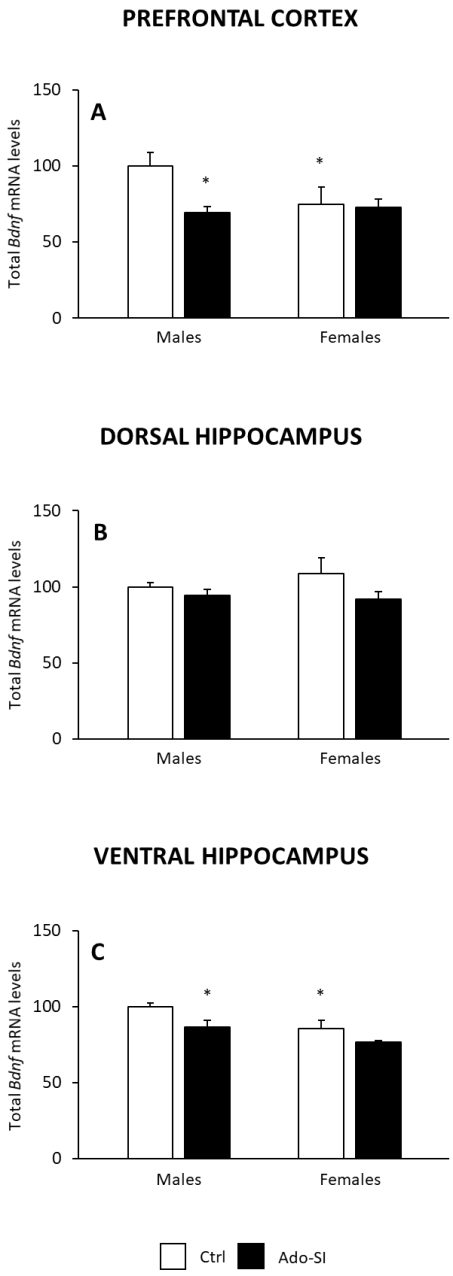

Figure 2 Long-lasting effects of social deprivation during adolescence on the expression of total *Bdnf*. The mRNA levels of total *Bdnf* were analysed in prefrontal cortex (A), dorsal and ventral hippocampus (B, C) of adult male and female rats that were lifelong group-housed (Ctrl) or exposed to a social deprivation paradigm during adolescence (Ado-SI). The data, expressed as % of Ctrl Male animals set at 100%, are the mean ± SEM of at least 4 animals per group. *p<0.05 vs Ctrl Males (Two-way ANOVA followed by LSD post-hoc test)

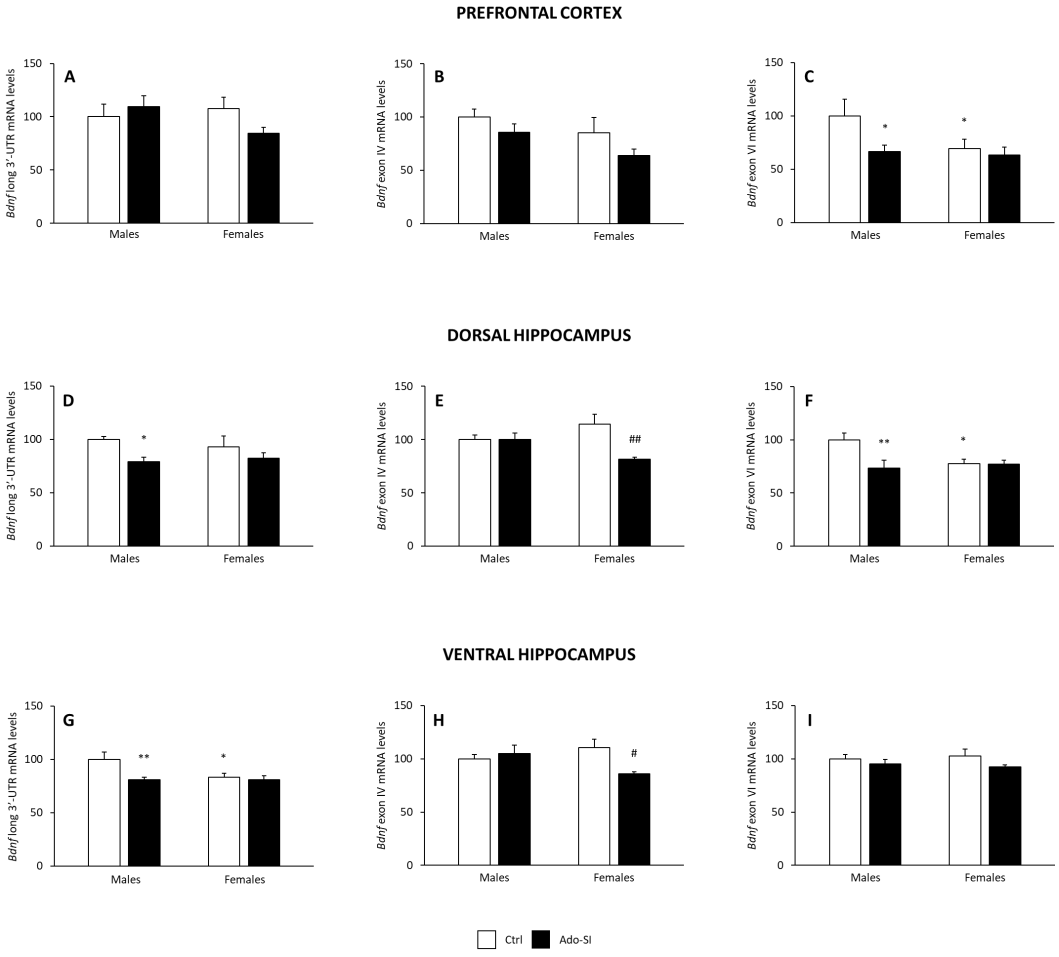

Figure 3 Long-lasting effects of social deprivation during adolescence on the expression of different *Bdnf* isoforms. The mRNA levels of *Bdnf* long 3'-UTR (A, D, G), *Bdnf* exon IV (B, E, H) and *Bdnf* exon VI (C, F, I) were analysed in prefrontal cortex (A, B, C), dorsal (D, E, F) and ventral hippocampus (G, H, I) of adult male and female rats that were lifelong group-housed (Ctrl) or exposed to a social deprivation paradigm during adolescence (Ado-SI). The data, expressed as % of Ctrl Male animals set at 100%, are the mean ± SEM of at least 4 animals per group. *p<0.05 and **p<0.01 vs Ctrl Males; $^{\#}$p<0.05 and $^{\#\#}$p<0.01 vs Ctrl Females (Two-way ANOVA followed by LSD post-hoc test).

2.3. Modulation of activity-regulated genes in the brain of adult animals exposed to social deprivation during adolescence.

We next investigated the expression of activity-dependent genes (IEGs), as markers of experience-dependent synaptic plasticity that may mediate the long-lasting adaptive changes to early life stress exposure (Jett et al., 2017; Shepherd and Bear, 2011).

As shown in Figure 4A, the expression of Activity Regulated Cytoskeleton Associated Protein (*Arc*) within the PFC was lower in adult female rats, as indicated by a significant main effect of gender. While no significant changes were observed in the DH (Figure 4C), we found a significant housing X gender interaction in the VH (Figure 4E). Indeed, exposure to social isolation during adolescence produced a significant up-regulation of *Arc* mRNA levels in adult male animals ($p<0.05$), but not in females.

With regard to the expression of the IEG early growth response 1 (*Zif268*), we did not find any significant effect of gender or housing condition within the PFC and DH (Figure 4B, D). Conversely, we observed a housing X gender interaction within the VH, although the effect did not reach statistical significance (Figure 4E). Indeed, exposure to social deprivation during adolescence down-regulated the expression of *Zif268* only in the VH of adult female rats ($p<0.05$).

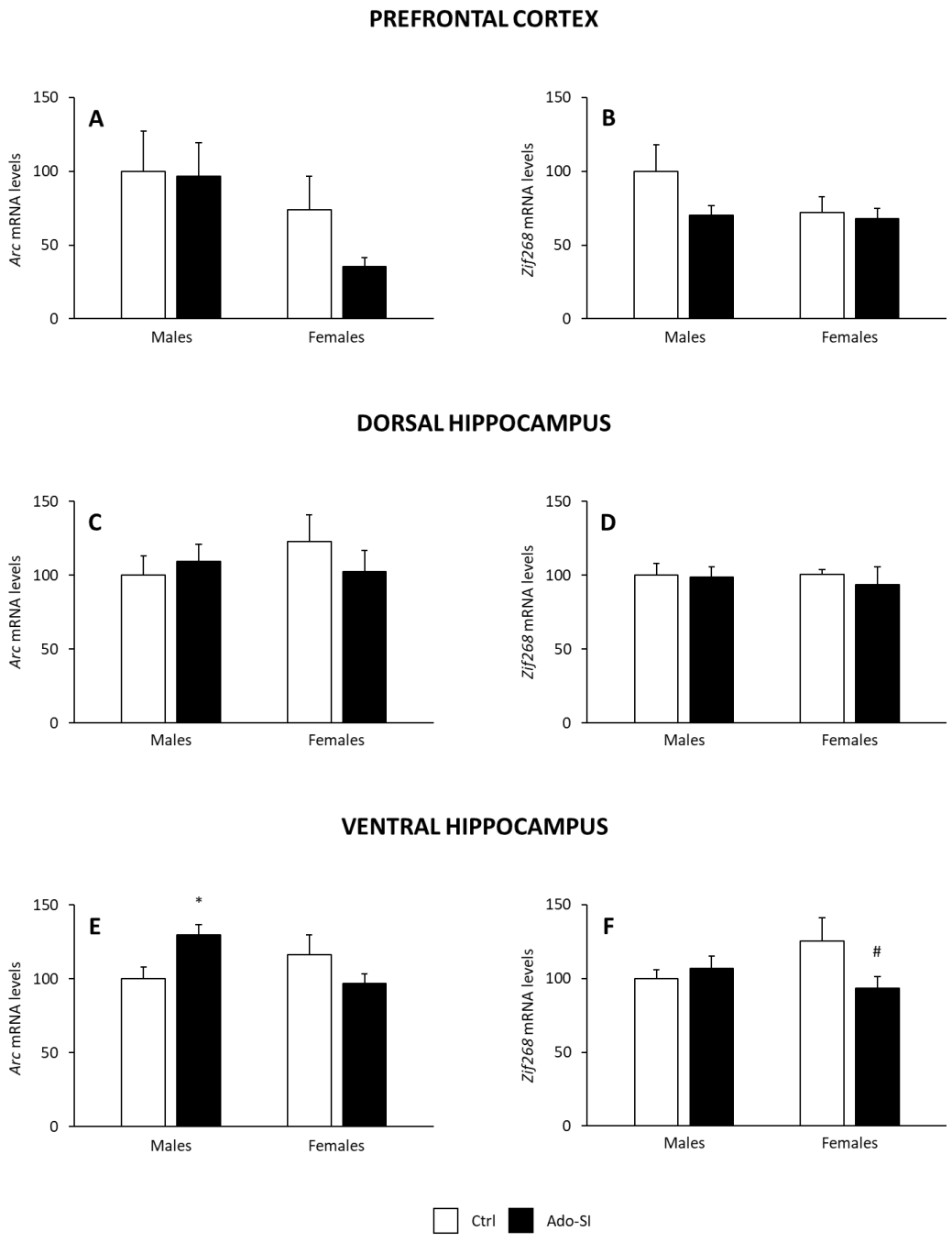

Figure 4 Long-lasting effects of social deprivation during adolescence on the expression of activity-dependent genes. The mRNA levels of *Arc* (A, C, E) and *Zif268* (B, D, F) were analysed in prefrontal cortex (A, B), dorsal (C, D) and ventral hippocampus (E, F) of adult male and female rats that were lifelong group-housed (Ctrl) or exposed to a social deprivation paradigm during adolescence (Ado-SI). The data, expressed as % of Ctrl Male animals set at 100%, are the mean ± SEM of at least 6 animals per group. *$p<0.05$ vs Ctrl Males; $^{\#}p<0.05$ vs Ctrl Females (Two-way ANOVA followed by LSD post-hoc test).

2.4. Social deprivation during adolescence produces long-term changes in the expression of the glucocorticoid receptor *Nr3c1*.

When exposed to stress, the organism activates a number of different processes aimed to cope with the challenging condition. With this respect, one key mechanism is represented by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, and glucocorticoids appear to be the first mediators of these responses (Lupien et al., 2009). Thus, in order to evaluate if adolescent stress can modulate the brain responsiveness to glucocorticoids, we investigated the expression of the glucocorticoid receptor *Nr3c1* and its co-chaperone *Fkbp5*, which regulates glucocorticoid receptor activity (Binder, 2009).

Within the PFC (Figure 5A, B), we found a significant housing X gender interaction in the modulation of *Nr3c1* expression, with no effects on *Fkbp5* mRNA levels. Indeed, exposure to stress in adolescence produced a significant up-regulation of *Nr3c1* mRNA levels in adult male ($p<0.05$), but not in female rats. Within the dorsal part of the hippocampus we did not observe any significant effect of gender or housing condition on *Nr3c1* and *Fkbp5* expression (Figure 5C, D). However, we found a significant housing X gender interaction for the expression of *Nr3c1* in the ventral hippocampus. Indeed, exposure to social deprivation during adolescence produced a significant down-regulation of *Nr3c1* mRNA levels only within the VH of adult female animals ($p<0.01$). As for the expression of *Fkbp5*, there was a significant effect of the housing condition and a significant housing X gender interaction. Accordingly, adult male animals exposed to stress during adolescence showed an increased expression of *Fkbp5*, as compared to Ctrl males ($p<0.01$), an effect that was not observed in female rats.

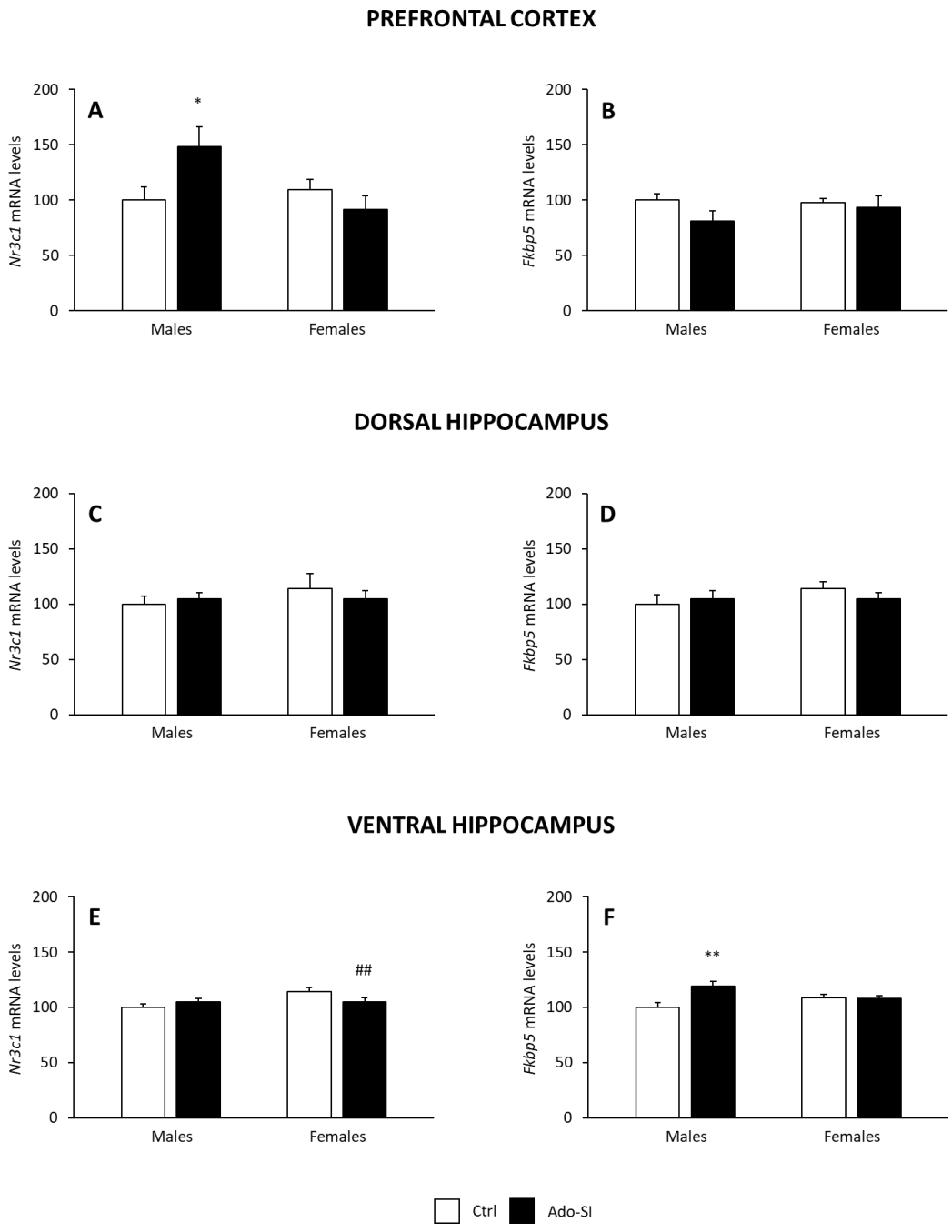

Figure 5 Long-lasting effects of social deprivation during adolescence on the expression of glucocorticoid-related genes. The mRNA levels of *Nr3c1* (A, C, E) and *Fkbp5* (B, D, F) were analysed in prefrontal cortex (A, B), dorsal (C, D) and ventral hippocampus (E, F) of adult male and female rats that were lifelong group-housed (Ctrl) or exposed to a social deprivation paradigm during adolescence (Ado-SI). The data, expressed as % of Ctrl Male animals set at 100%, are the mean ± SEM of at least 6 animals per group.

3. Discussion

The present study demonstrates that exposure to social isolation at adolescence is associated with enduring functional and molecular changes that show anatomical and gender specificity.

At the behavioural level, our results strengthen the idea that social deprivation, selectively during adolescence, produces persistent behavioural abnormalities characterized by a depressive-like phenotype. Indeed, although at basal level (non-stressed) adult female rats exhibited higher sucrose preference than males, the preference for the sucrose solution is markedly downregulated as a result of the exposure to social deprivation during adolescence, both in adult males and females. A loss of pleasure to a rewarding stimulus, such as sucrose, is representative of anhedonia, that is considered a core feature of depression (Slattery and Cryan, 2017). While such effect was observed in both sexes, the magnitude of stress-induced changes appears to be larger in male rats, as compared to socially isolated females.

However, our results failed to show a long-term impairment of the cognitive performance after exposure to social isolation during adolescence, at least with respect to the novel object recognition test. These data mirrored results obtained in other studies reporting that social deprivation induces immediate learning and memory deficits more clearly than other adolescent stressors and that these effects are attenuated when tested after a period of social housing (Green and McCormick, 2013). Indeed, the exposure to post-weaning social isolation for four or eight weeks in male rats resulted in immediate reduced spatial learning performance in the Morris water maze (Lu et al., 2003). Similarly, male animals also showed impaired reversal learning when housed in isolation from 21 to 77 or 100 days of age (Li et al., 2007; Schrijver et al., 2004). The object recognition memory was also found altered after four weeks of social deprivation in male rats, when tested immediately after the end of stress exposure (Bianchi et al., 2006). However, in these studies it might be difficult to unravel the effects of the history of social isolation from any effects of the current isolation condition on the performance compared to group housing during the test. In line with this hypothesis, the effects of social deprivation on spatial learning were no longer present when the deprivation of social contacts for four weeks was followed by a period of social housing for four more weeks (Lu et al., 2003). Similarly, male rats exposed to two weeks of social isolation at early adolescent stage showed no impairments of reversal and spatial learning when tested at early adulthood after three weeks of social housing (Han et al., 2011). All in all, these data suggest

that resocialization may be able to buffer the cognitive impairment produced after a period of social withdrawal.

Preclinical and clinical studies have provided strong evidence indicating that impairments in neuroplasticity could contribute to the behavioural phenotypes associated with depression (Nestler et al., 2002). By investigating Brain-Derived Neurotrophic Factor (BDNF) as a prototype marker of neuronal plasticity, we found gender and brain region specific changes as a consequence of social isolation during adolescence. Indeed, early stress exposure determined a strong down-regulation of *Bdnf* expression specifically within the prefrontal cortex and ventral hippocampus of adult males. No effect was observed for adult female animals exposed to adolescent social stress. Very few studies modelling chronic stress exposure during adolescence investigated the enduring effects on *Bdnf* transcription, with some discrepancies in the observed results (Han et al., 2011; Li et al., 2016; Meng et al., 2011; Weintraub et al., 2010). As an example, social isolation for two weeks, followed by resocialization for three weeks produced increased *Bdnf* mRNA levels in the prefrontal cortex of male Sprague Dawley rats associated with reduced *Bdnf* mRNA levels in the hippocampus (Han et al., 2011; Li et al., 2016). In another study, four weeks of social isolation followed by group-rearing for four more weeks determined an increase of BDNF protein levels both in the hippocampus and prefrontal cortex of male Sprague Dawley rats (Meng et al., 2011). Using the same stress paradigm, Weintraub et al. (2010) reported a reduction of *Bdnf* mRNA levels only in the hippocampus of female Sprague Dawley rats (Weintraub et al., 2010).

The *Bdnf* gene is very complex and consists of several non-coding and only one coding exon at the 3'-end, which define differently spliced transcripts. In the brain, *Bdnf* splice variants can be localized in different neuronal compartments, undergo differential transcriptional mechanisms and may subserve different functional roles within selected brain areas (An et al., 2008; Baj et al., 2011). The analysis of the main *Bdnf* transcripts confirmed some of the effects observed on total *Bdnf*, but also provided specific information that were not evident on the entire pool of *Bdnf* mRNA. Indeed, when considering the prefrontal cortex, we found that the modulation of *Bdnf* exon VI largely reflects the changes of total *Bdnf* suggesting that the impaired expression of the neurotrophin in this structure could be primarily related to an impairment in the transcriptional activity at exon VI. On the other end, the down regulation of total *Bdnf* in the VH of male rats exposed to social isolation appears to be associated with a significant decrease in the expression of the pool of transcripts with the long 3'-UTR. This pool of transcripts is preferentially targeted to dendrites, where it may contribute to the activity-dependent translation of the neurotrophin (An et al., 2008).

Interestingly, despite the fact that total *Bdnf* levels where not altered in the DH of adult male animals exposed to adolescent social deprivation, we found that *Bdnf* exon VI and *Bdnf* long 3'-UTR mRNA levels were significantly reduced in this hippocampal sub-region, suggesting that subtle changes may also be present at this level. All the changes of *Bdnf* isoforms observed in male rats exposed to stress during adolescence were not found in female animals, thus suggesting sex specificity in these mechanisms. However, the expression of *Bdnf* exon IV revealed a prominent effect of social isolation only in adult female rats. Accordingly, we found that the exposure to social deprivation during adolescence reduced *Bdnf* exon IV mRNA levels selectively within the dorsal and ventral hippocampus of adult female rats. Exon IV-containing *Bdnf* transcripts are localized to the cell body and proximal dendrites (Baj et al., 2011) and, because of the presence of three calcium responsive elements, the promoter that controls the transcription of exon IV is the most influenced by neuronal activity (Tao et al., 1998). Activity-dependent gene transcription represents an essential mechanism to adapt to the environment. Thereby, the activity-dependent modulation of BDNF may be impaired in the hippocampus of female animals that were exposed to the stressful condition during adolescence, contributing to reduced plasticity and diminished ability to cope with challenging conditions.

We have previously demonstrated that exposure to prenatal stress is also able to reduce the expression of BDNF (Luoni et al., 2014) and to alter its activity dependent transcription (Luoni et al., 2016), suggesting that impaired neurotrophin function may represent a long-lasting consequence of exposure to stress during development.

Female animals exposed to social isolation also show a significant reduction of activity-dependent genes, such as *Arc* and *Zif268*, which participate to several functions, including synaptic plasticity, regulation of AMPAR internalization and structural dendritic spine remodelling (Steward et al., 2015). It has been previously demonstrated that social isolation for 30 days induces an immediate significant down-regulation of *Arc* expression in the hippocampus (Pisu et al., 2011). Hence the persistent reduction we observed after a period of social housing may suggest an enduring deficit of synaptic plasticity as a consequence of the adverse experience during adolescence.

We have also examined the expression of the glucocorticoid receptor *Nr3c1* and of its co-chaperone *Fkbp5* in different brain regions, as a proxy for a potential dysregulation of the HPA axis, which has been found in different mental disorders, including depression (Burke et al., 2017). The picture emerging from these analyses is complex, since adult male and female animals show opposite changes for *Nr3c1* mRNA levels, with an up-regulation of

Nr3c1 within the prefrontal cortex of adult male animals, and a significant down-regulation within the ventral hippocampus of adult females. The long-lasting effects of stress during adolescence on adult HPA function appear to be quite heterogeneous (McCormick et al., 2010). Exposure to adolescent physical or social stressors can increase basal corticosterone levels with a reduction of glucocorticoid receptor expression at adulthood (Schmidt et al., 2007; Uys et al., 2006). On the contrary, a decrease of basal corticosterone levels and an increase of glucocorticoid receptor expression was observed within the hippocampus after 30-day isolation period (Boero et al., 2018; Pisu et al., 2016). The reduction of *Nr3c1* expression observed in the VH of female animals may suggest a lasting impairment of negative feedback mechanisms (Jacobson, 2014), although it remains to be tested whether this may impact HPA response to subsequent challenges at adulthood (Luoni et al., 2016). Furthermore, growing evidence indicates that glucocorticoid receptors may negatively modulate *Bdnf* expression (Chen et al., 2017; Dwivedi et al., 2006). This finding may provide a link between *Bdnf* and *Nr3c1* changes within the prefrontal cortex of adult male animals exposed to adolescent social stress, suggesting that early stress exposure may induce glucocorticoid receptor activity that in turn contributes to *Bdnf* down-regulation.

In summary, our findings demonstrate that exposure to stress during the peripubertal period, which may correspond to adolescence in humans, may lead to the development of a depressive-like phenotype in male and female rats, although the behavioural alteration appears to be sustained by sex-specific molecular mechanisms. In a translational perspective, since restoration of these molecular alterations may be critical for the functional improvement of domains that are altered in depressed patients, it may be inferred that males and females could benefit of different interventions aimed to target the specific changes found in the brain of affected individuals.

4. Materials and Methods

4.1. Animals and experimental paradigm

Pregnant adult female Sprague-Dawley rats on gestational day 16 were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, pregnant females were single housed with food and water available ad libitum (21±1°C, 60±10% relative humidity, 12/12h light/dark cycle) and monitored daily for birth (9:00 a.m.; 12:00 p.m.; 4:00 p.m.; 7:00 p.m.). When a litter was found at 9:00 a.m., we assigned the day before as the day of birth (postnatal day [PND] 0). Within 24 hours after birth, the pups were cross-fostered to reach the established number of ten animals per litter (with five animals per sex). In order to make the adoptive dam accepted also the pups from another nest, they were partially covered with the litter of the adoptive dam (Dimitsantos et al., 2007). Dams and their pups were left undisturbed in their cage until weaning with food and water available ad libitum (21±1°C, 60±10% relative humidity, 12/12h light/dark cycle).

On postnatal day (PND) 21, male and female pups were randomly assigned to control (Ctrl) or social isolation (Ado-SI) conditions. The social deprivation paradigm consisted of housing one animal per cage, allowing them to smell, hear and see other rats within the holding room but not to interact with. Ctrl animals were housed in groups of three animals per cage per sex.

On PND49, all animals underwent re-socialization. Briefly, Ado-SI rats were housed in groups of three per cage, with other two same-sex Ado-SI animals, and left undisturbed until adulthood. Partners were reassigned also within Ctrl cages. The time course of the experiments is shown in Figure 6.

In early adulthood (>PND70), rats pertaining to both Ctrl and Ado-SI groups were tested in the sucrose preference test and in the novel object recognition test. Male and female rats were killed two weeks after the end of the behavioural tests and the brain regions of interest (hippocampus, dorsal and ventral, and prefrontal cortex) were immediately dissected, frozen on dry ice and stored at -80 °C for further molecular analyses.

All animal experiments were conducted according to the authorization from the Health Ministry n. 837/2016-PR (06/09/2016), in full accordance with the Italian legislation on animal experimentation (Decreto Legislativo 26/2014) and adherent to EU recommendation (Directive 2010/63/EU). All efforts were made to minimize animal suffering and to reduce the total number of animals used, while maintaining statistically valid group numbers.

No pre-established inclusion/exclusion criteria were used for the subsequent molecular analyses. All samples were processed and analysed by investigators blind to the housing conditions.

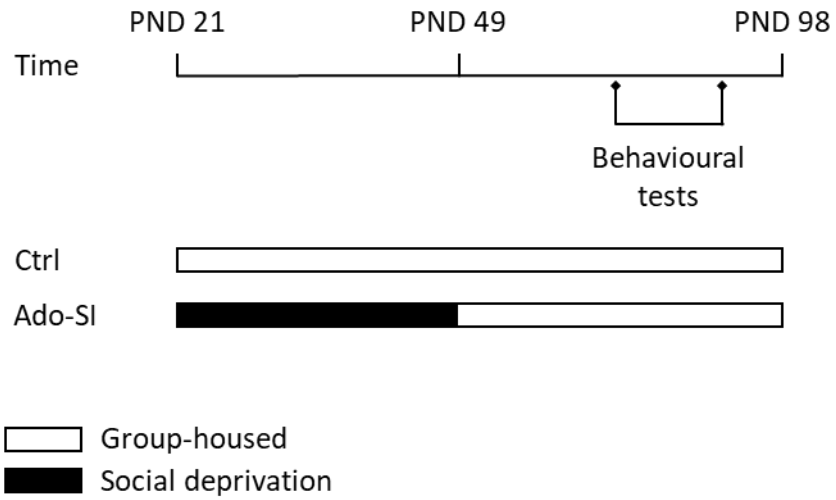

Figure 6 Summary of the experimental design. Timeline of stress exposure and behavioural testing. Ctrl = lifelong group-housed animals; Ado-SI = animals exposed to social isolation during adolescence

4.2. Behavioural testing

Sucrose preference test procedure

On the day of testing, rats were single housed and given, for 1 h, a free choice between two bottles, one with 1% sucrose solution and another with tap water. The water and sucrose intakes were measured by weighing pre-weighted bottles containing the solutions, at the end of the test. Sucrose preference was calculated as the percentage of consumed sucrose solution on the total amount of liquid drunk.

Novel object recognition test procedure

On the day of testing, animals were habituated in a quiet room dimly illuminated for 15 min before the experimental procedure began. The test consisted of a first phase of habituation during which the rat was allowed to explore an open-field box made of Plexiglas for 10 min followed by two other sessions, the familiarization and the test phase. During the familiarization phase, two identical objects were presented to the animal for 5 minutes. During an inter-trial delay of 3 min, that the rat spent in its home cage, one of the two familiar objects was replaced by a novel, previously unseen object (with different shape, colour and

texture). The rat was then allowed to explore the two objects, the familiar and the new one, for 5 min. For both sessions, object exploration time was manually measured by a trained observer blind to the experimental conditions and a discrimination index was calculated for each animal and expressed as follows: [(time spent with the novel object – time spent with the familiar object)/(time spent with the novel object + time spent with the familiar object)]

Total RNA purification and quantitative Real-Time PCR analyses

Total RNA was isolated from the prefrontal cortex and the dorsal and ventral hippocampus using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Italy), according to the manufacturer's instructions.

RNA was analysed by 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 triplicate as multiplexed reactions with a normalizing internal control (ß-Actin). Relative target gene expression was calculated according to the 2^(−ΔΔC(T)) method. Probe and primer sequences of *Bdnf* long 3'-UTR (Assay ID: Rn02531967_s1), *Bdnf* Transcript IV (Assay ID: Rn01484927_m1), *Bdnf* Transcript VI (Assay ID: Rn01484928_m1) were purchased from Thermo Fisher Scientific, while the other TaqMan gene expression assays were purchased from Eurofins Genomics and are summarized in Table 1.

Statistical analyses

Changes produced by housing condition and gender were analysed using a two-way ANalysis of VAriance (ANOVA), followed by Fisher's LSD post-hoc comparisons. A probability level of $p<0.05$ was taken as significant in every test. The outcomes of the statistical analyses are summarized in Supplementary Table 1.

Table 1 Sequences of forward and reverse primers and probes used in qRT-PCR analysis and purchased from Eurofins Genomics

Gene	Forward Primer	Reverse Primer	Probe
Total Bdnf	AAGTCTGCATTAC ATTCCTCGA	GTTTTCTGAAAGAGG GACAGTTTAT	TGTGGTTTGTTGCCG TTGCCAAG
Arc	GGTGGGTGGCTCT GAAGAAT	ACTCCACCCAGTTCT TCACC	GATCCAGAACCACA TGAATGGG
Zif-268	GAGCGAACAACC CTACGAG	GTATAGGTGATGGG AGGCAAC	TCTGAATAACGAGA AGGCGCTGGTG
Nr3c1	GAAAAGCCATCG TCAAAAGGG	TGGAAGCAGTAGGT AAGGAGA	AGCTTTGTCAGTTGG TAAAACCGTTGC
Fkbp5	GAACCCAATGCT GAGCTTATG	ATGTACTTGCCTCCC TTGAAG	TGTCCATCTCCCAGG ATTCTTTGGC

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Abbreviations

ELS	Early Life Stress
BDNF	Brain-Derived Neurotrophic Factor
HPA	Hypothalamic-Pituitary-Adrenal
PFC	Prefrontal Cortex
DH	Dorsal Hippocampus
VH	Ventral Hippocampus
PND	Postnatal Day

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