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LONG-LASTING BEHAVIOURAL AND MOLECULAR ALTERATIONS FOLLOWING STRESS EXPOSURE DURING ADOLESCENCE IN RATS: IMPLICATIONS FOR PSYCHIATRIC DISORDERS

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ABSTRACT

The exposure to adverse events early in life is associated with long-lasting neurochemical, structural and behavioural changes that may enhance the vulnerability to mental disorders, such as major depression. Our laboratory, among others, has been investigating the reprogramming effects of stress on the developing brain, showing that exposure to stress during gestation leads to anxiety- and depressive-like disturbances and reduces the ability to cope with a later stressful situation (Fumagalli et al., 2004; Luoni et al., 2014; Roceri et al., 2004; Roceri et al., 2002; van der Doelen et al., 2014). In the present study, we investigated the effects of stress exposure during adolescence, a sensitive period of structural and behavioural reorganization (Spear, 2000). Given the importance of social play for the right maturation of behaviour during adolescence, we used the paradigm of social isolation based on deprivation of all social contacts (Burke et al., 2017). The purpose of this study was to evaluate the long-lasting effects of stress exposure during adolescence on neuronal plasticity as well as on the functional activity and responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis, which are known to be defective in several psychiatric disorders (Burke et al., 2005; Calabrese et al., 2009). In order to do this, we compared control group-housed animals with rats that were exposed to isolation rearing for four weeks after weaning, followed by re-socialization until adulthood. We found that the exposure to social isolation during a specific time window of adolescence induced an anhedonic phenotype in males and females, without producing any significant effect in cognitive function, as measured by the novel object recognition test. At molecular level, these functional changes were associated with an impairment in the expression of brain-derived neurotrophic factor (BDNF) and in the BDNF/TrkB mediated phospholipase C-γ (PLCγ) signalling, primarily within the prefrontal cortex.

Since exposure to adverse events early in life may change the susceptibility toward subsequent stressors at adulthood (Fumagalli et al., 2004; Grissom and Bhatnagar, 2009; McEwen, 2003; Roceri et al., 2004; Roceri et al., 2002), we also tested whether exposure to stress during adolescence may alter the ability to cope with an acute immobilization stress. We found that social deprivation did not impair the responsiveness of activity-induced genes as well as the expression and phosphorylation of selected glutamatergic receptor subunits in the prefrontal cortex and hippocampus. Moreover, while social deprivation during

adolescence did not alter the responsiveness of the HPA axis, as measured by circulating corticosterone levels following the acute challenge, the analyses on glucocorticoid receptor-related genes revealed that the exposure to social isolation during adolescence produced a different modulation of key genes involved in the negative feedback of HPA, particularly within the prefrontal cortex and dorsal hippocampus.

In summary, our results demonstrated that exposure to social isolation during adolescence produced a depressive like phenotype, associated with impairments in the neuroplastic marker BNDF and PLCγ related signalling as well as in stress response related genes. Moreover, our data suggested that there were anatomically-selective changes associated with the enduring effects of social isolation as well as some degree of gender specificity. We believe that a better understanding of the molecular mechanisms associated with long-term alterations following stress exposure during adolescence may guide future research efforts toward the identification of novel strategies for therapeutic intervention.

RIASSUNTO

L'esposizione ad eventi avversi nelle prime fasi della vita è associata all'instaurarsi di alterazioni a lungo termine a livello neurochimico, strutturale e comportamentale che possono aumentare la vulnerabilità dell'individuo a sviluppare malattie mentali come la depressione maggiore. La maggior parte della ricerca si è focalizzata sugli effetti dello stress a livello prenatale e perinatale grazie a consolidati paradigmi sperimentali preclinici. Questi studi hanno mostrato che l'esposizione a eventi traumatici durante la gestazione porta allo sviluppo di fenotipi simil-ansiosi e simil-depressivi ed è in grado di ridurre l'abilità di affrontare successive situazioni avverse nel corso della vita (Fumagalli et al., 2004; Luoni et al., 2014; Roceri et al., 2004; Roceri et al., 2002; van der Doelen et al., 2014). In questo studio abbiamo investigato gli effetti dello stress durante l'adolescenza, un periodo di riorganizzazione strutturale e comportamentale particolarmente delicato (Spear, 2000). Considerata l'importanza delle interazioni sociali per un corretto sviluppo emotivo-comportamentale durante l'adolescenza, abbiamo utilizzato un consolidato paradigma sperimentale di stress durante l'adolescenza che si basa sulla deprivazione di qualsiasi contatto sociale (Burke et al., 2017). Lo scopo di questo studio è stato quello di investigare gli effetti a lungo termine di una esposizione a stress durante l'adolescenza sui meccanismi di plasticità sinaptica e di risposta allo stress dell'asse ipotalamo-ipofisi-surrene (HPA), alterati in molte patologie psichiatriche (Burke et al., 2005; Calabrese et al., 2009). Quindi, gli effetti dello stress sono stati valutati confrontando animali controllo alloggiati in gruppo con animali esposti al paradigma di isolamento sociale per quattro settimane, seguito da un periodo di risocializzazione fino all'età adulta. Dal punto di vista comportamentale, abbiamo osservato che l'esposizione ad una condizione di deprivazione sociale durante l'adolescenza ha indotto lo sviluppo di un fenotipo anedonico sia in maschi che in femmine, senza però alterare la funzione cognitiva nell'età adulta, come evidenziato dal test di riconoscimento dell'oggetto. A livello molecolare invece, soprattutto per quanto riguarda la corteccia prefrontale, abbiamo osservato disfunzioni nell'espressione del fattore neurotrofico BDNF e nel signaling della fosfolipasi C-γ (PLCγ).

Considerato che l'esposizione a eventi avversi nelle prime fasi della vita può determinare una alterata risposta a successivi eventi stressanti nel corso della vita (Fumagalli et al., 2004; Grissom and Bhatnagar, 2009; McEwen, 2003; Roceri et al., 2004; Roceri et al., 2002), abbiamo inoltre valutato se l'esposizione all'isolamento sociale durante l'adolescenza possa alterare

l'abilità di affrontare uno stress acuto da immobilizzazione in età adulta. Abbiamo osservato che l'isolamento sociale non ha influenzato la responsività di geni precoci così come l'espressione e la fosforilazione di alcune subunità dei recettori glutamatergici sia a livello della corteccia prefrontale che dell'ippocampo. Inoltre, mentre l'isolamento durante l'adolescenza non ha alterato la responsività dell'asse HPA, come evidenziato dalle concentrazioni di corticosterone circolante in seguito allo stress acuto, l'analisi di geni legati al recettore dei glucocorticoidi ha evidenziato che l'isolamento sociale durante l'adolescenza ha prodotto una differente modulazione di geni chiave coinvolti nel feedback negativo dell'asse HPA, soprattutto a livello della corteccia prefrontale e dell'ippocampo dorsale.

In conclusione i nostri risultati hanno dimostrato che l'esposizione all'isolamento sociale durante l'adolescenza determina lo sviluppo di un fenotipo simil-depressivo, associato a disfunzioni del marcatore neurotrofico BDNF e del signaling della PLCγ così come di geni coinvolti nella risposta allo stress. Inoltre i nostri dati hanno mostrato che le alterazioni molecolari indotte dallo stress a lungo termine presentano un certo grado di specificità anatomica e di genere. Ulteriori studi volti a identificare i meccanismi molecolari associati ad alterazioni a lungo termine indotte dall'esposizione a stress durante l'adolescenza potranno guidare la ricerca di nuove strategie terapeutiche.

1. INTRODUCTION

In any given year, mental disorders affect about 25% of people (Kessler et al., 2005). The most prevalent psychiatric disorders are anxiety and mood disorders, with a lifetime prevalence of about 30% and a typical onset in adolescence (Kessler et al., 2005). Thus, in last years, great attention was focused in examining and characterizing the etiopathological mechanisms that may contribute to the development of these disorders. With this respect, animal models have been a very useful tool to investigate such mechanisms in order to identify relevant players for psychiatric disorders. Despite the well documented genetic predisposition for anxiety and depression (Sullivan et al., 2000), exposure to stressful experiences account for about half of the risk for depression (Kendler et al., 2001). Most importantly, there is an interaction between genes and environmental adversities. Moreover, exposure to stressful events may represent a predisposing element, which will eventually lead to full-blown pathologic conditions, when an individual is re-exposed to adverse challenging conditions. The consequences of stress can depend on several factors besides the genetic background, including severity, timing, duration and nature of the stressful experience. With this respect, the growing interest in the effects of stress on the development of psychiatric disorders has revealed a significant role of the timing of the stress on resilience and risk factors. It is well known that stressful life events during sensitive periods of neural development can have long term functional outcomes by altering brain trajectories. The brain undergoes maturation during gestation and continues its development into adolescence; new-born neurons undergo extensive pruning and organize functional networks. Thus, due to extensive and dynamic cell proliferation, the embryonic and early postnatal brain is highly plastic and more vulnerable to adverse events. Indeed, disturbances by environmental factors during these critical periods of development may induce re-organizational effects on different biological systems that increase the susceptibility to psychiatric diseases. However, there is not a specific vulnerable period and the effects of early stress may depend on the developmental stage of specific brain areas, neuronal circuits and stress system.

1.1 Adolescence: maturational events and sensitive periods

The term adolescence (lat. *adolescere*; to grow up) refers to a highly important period of postnatal maturation, established by behavioural and cognitive transitions from childhood to adulthood. These maturational events contribute to the typical age-specific behavioural characteristics of adolescence, including an increase in risk-taking and propensity to use drugs and alcohol. Adolescence, being a period of transitions, comprises a series of gradual events and so it is difficult to determine the precise start and stop of this critical maturational period. In this way, the term puberty (lat. *pubertas*; maturity) cannot be used to refer to adolescence while it can be considered one of these adolescent changes. In fact, puberty, even though overlaps temporally adolescence, is defined exactly by the acquisition of sexual maturity. Although the terms are not synonymous, puberty and adolescence are closely related through many interactions between the nervous system and gonadal steroid hormones (Sisk and Foster, 2004).

No single event signals the onset or termination of adolescence and the timing of physiological and socio-behavioural transitions may vary depending on several conditions (Savin-Williams and Weisfeld, 1989). In this way, the exact timing of adolescence is difficult to define in humans and even more difficult in laboratory animals, considering the actual nature of the term adolescence. One approach to characterize margins of adolescence could be referring to physical and behavioural discontinuities from childhood and adulthood. In humans, this age range is considered to be from 12 to 18 years. The beginning of adolescence thought to be around the onset of puberty, characterized by profound psychological changes, while the end is said to occur when adult behavioural and maturational abilities are acquired (Blakemore et al., 2010). In laboratory animals, certain time points can be used to define the age range as weaning occurs at 21 days of life and puberty is at about 35 days of age in females and about 42 days of age in males (McCormick and Mathews, 2010). When assessing the time frame of adolescence in laboratory animals, such as rats, these boundaries may vary with gender and the growth rate of animals and also the transition under examination must be considered. In general, the age ranges between postnatal days (PND) 28-42 but under some specific experimental conditions such time window should be prolonged (Spear, 2000).

1.1.1 Maturational events

For the successful transition from childhood to adulthood, adolescents must undergo a series of behavioural and structural maturational events.

Behavioural changes

Typical behaviours of adolescents bear similarities across different species, comprising an increase in social interactions and in risk taking. Both in human adolescents as well as in other species, social behaviours are highly addressed (Spear, 2000). Adolescents spend more time with peers, shifting their social orientation from adults to peers. Similarly, adolescent rodents spend more time in social interactions and play behaviours. These increased interactions provide an important source of experiences that may help to develop social skills and promote independence.

The transitional adolescent period is also characterized by important increase in risk taking behaviours, including misconduct, both at school and at home, alcohol and drug abuse, theft and fighting behaviours. With respect to laboratory animals, adolescent rodents actualize these risk-taking behaviours seeking out novelty of their environment. Indeed, adolescent mice exhibit increased hyperactivity and exploration in a novel situation and are hyper-reactive to novel stimuli (Spear, 2000).

Neural development

These behavioural features common in adolescence are linked to intensive maturational changes in the brain. The adolescent brain is exposed to continuous regressive and progressive structural changes, characterized by overproduction and pruning of synapses and signalling mechanisms (Andersen and Teicher, 2008). Indeed, adolescence is characterized by a massive loss of synapses in neocortical brain regions, associated with an increase in specific and less widespread activation of the brain during task performances (Spear, 2000). Adolescence-related changes in brain development are also evident in magnetic resonance imaging (MRI) of cortex where it can be seen a shift from grey to white matter; grey matter thins while white matter increases, probably due to the elimination of unnecessary connections and the addiction of myelin to the axon connections (Fig. 1.1). This indicates that networks between subcortical and cortical regions in the brain are in a highly transitional state during adolescence (Andersen and Teicher, 2008). Also, receptors of different neurotransmitter systems, including gamma-aminobutyric acid (GABA), glutamate and

dopamine (DA) undergo these transitional structural changes in the adolescent brain. Both the prefrontal cortex (PFC) and other limbic regions, such as the hippocampus, experience a substantial elimination of glutamatergic excitatory inputs during adolescence (Spear, 2000). In contrast, DA concentration and fiber density has been shown to increase in the PFC (Spear, 2000).

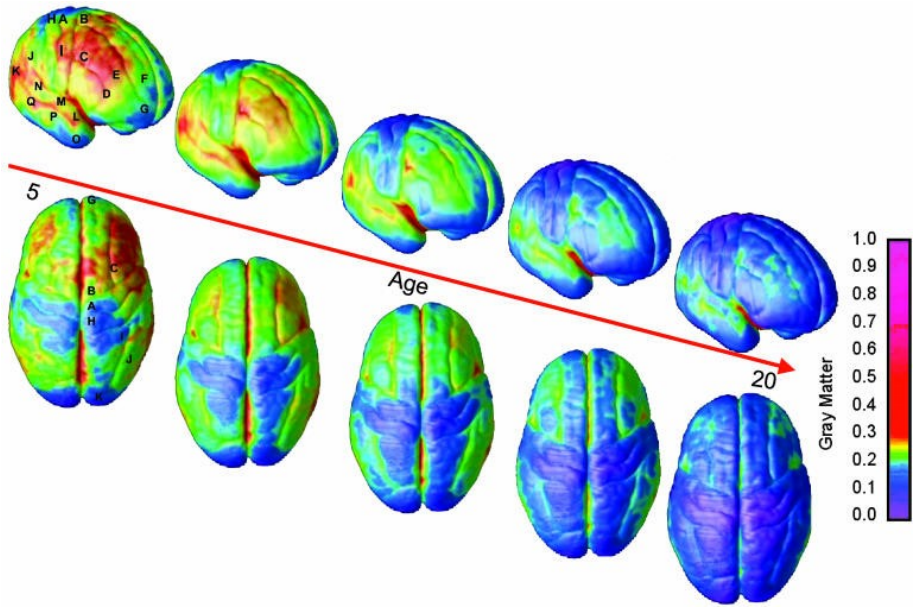

Fig. 1.1 *Right lateral and top views of the dynamic sequence of grey matter maturation over the cortical surface (from (Gogtay et al., 2004)).* The side bar shows a colour representation in units of grey matter volume.

1.1.2 Sensitive periods

These maturational events and increased plasticity may result in amplified vulnerabilities. Neural plasticity refers to the ability of the brain to adapt in response to environmental stimuli, experiences and physiological changes. Several researches hypothesized that adolescence, being characterized by pronounced neurocognitive changes, may represent a sensitive and thus critical period in brain development (Leussis and Andersen, 2008; Paus et al., 2008). They suggested that severe stress exposure during adolescence can affect the proper maturational processes taking place in this period. Chronic administration of corticotropin-releasing factor (CRF), one key player in stress response, during adolescence sets back the onset of puberty (Kinsey-Jones et al., 2010) and this might alter the correct trajectories of brain development. Exposure to severe stress, as abuse, during adolescence has significant negative effects on dendritic spine morphology and on the overall volume of the prefrontal cortex (Garrett and Wellman, 2009; Leussis and Andersen, 2008). Moreover, the transition from childhood to adult independence can per se represent a stressful period. Adolescents indeed are usually more exposed to several social stressors as bullying behaviours including both physical aggression, group exclusion and malicious gossip (Gladstone et al., 2006). These aberrations of normal neurodevelopmental changes in the adolescent brain may thereby increase the risk for the onset of neuropsychiatric disorders.

1.2 Molecular and functional alterations following exposure to stress during adolescence

Exposure to stress during sensitive periods is associated with the activation of several systems, involving the hypothalamic-pituitary-adrenal (HPA) axis as well as neurotransmitters, neurotrophic factors, hormones and cytokines. This complex response results in long-term effects on behaviour, metabolism, reproduction, immune response and cardiovascular function. Under physiological conditions, exposure to stress leads to adaptive changes aimed to maintain homeostasis whilst the exposure to chronic and intense stressors can lead to prolonged activation of the stress system with a 'pathologic' elevation of glucocorticoids and other neuromodulators. This hypersecretion of stress hormones can induce insulin hypersecretion, growth and sex steroid hormone hyposecretion, resulting in long-term accumulation of fat, loss of muscle, arterial hypertension, metabolic syndrome and type 2 diabetes mellitus (Pervanidou and Chrousos, 2012). On the other hand, elevated glucocorticoid concentrations act also on the central nervous system, impairing synaptic connectivity, memory retrieval and working memory (Green and McCormick, 2013). Accordingly, exposure to severe chronic stress during adolescence can alter multiples mechanisms and all these changes can, in turn, enhance the vulnerability to develop psychopathologies later in life.

1.2.1 Neuronal plasticity and brain-derived neurotrophic factor

The brain-derived neurotrophic factor (BDNF) is a member of the family of neurotrophic factors, which includes nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (Begni et al., 2017). 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. Moreover, the coding exon has two polyadenylation sites that lead to the production of a short or a long 3' untranslated region (UTR). The expression of *Bdnf* transcripts is tissue-driven whilst the pull of transcripts with the short 3'-UTR are concentrated in the soma and the ones with the long 3'-UTR are preferentially targeted to dendrites (Vicario et al., 2015). All *Bdnf* transcripts, within the endoplasmic reticulum, are translated into a precursor protein, proBDNF, from which proteolytic cleavage defines the mature form of BDNF, referred to as mBDNF. BDNF is thus sorted into a regulated secretory pathway and transported to releasing sites; BDNF

expressed in the soma is important for dendritic spine formation while in dendrites BDNF is expressed for spine head growth and spine pruning (Orefice et al., 2013). BDNF is expressed mainly in neuronal tissues, as in the hippocampus by excitatory neurons, though it can be produced also by astrocytes (Hofer et al., 1990). The secretion process of BDNF can be induced by several stimuli including high levels of glutamate, intracellular Ca^{2+} and BDNF itself (Goodman et al., 1996). Once secreted, BDNF can bind two receptors: the high-affinity TrkB receptor or the low-affinity p75 neurotrophin receptor (p75 NTR) (Chao, 2003). BDNF binding to TrkB is followed by receptor dimerization and auto-phosphorylation leading to the activation of different signalling pathways (Fig. 1.2): the phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase C-γ (PLCγ) pathways. All these pathways lead to the phosphorylation and activation of the transcription factor cAMP-response element binding protein (CREB) which induces the transcription of genes essential for cell survival, growth and differentiation and synaptic plasticity (Begni et al., 2017).

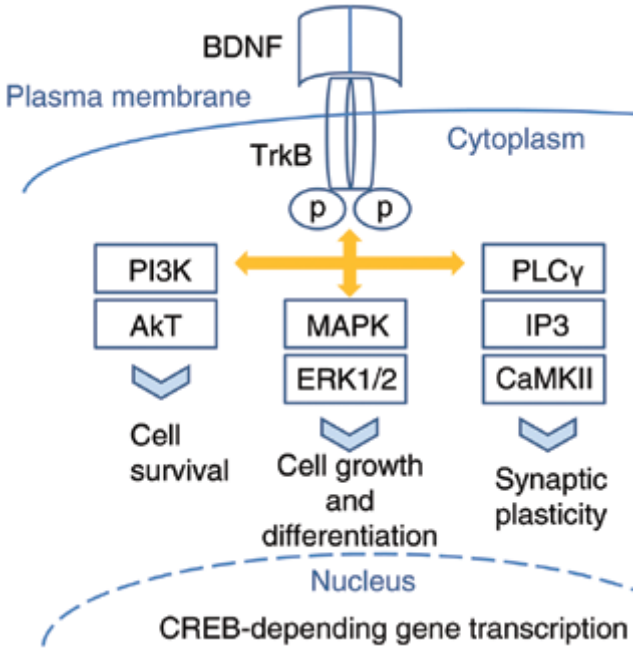

Fig. 1.2 *BDNF signalling pathways (adapted from (Zaletel et al., 2017))*

BDNF plays an important role in a multitude of biological functions, including neuronal plasticity processes, differentiation of neuronal precursor cells, synaptogenesis and neuronal survival. As already mentioned, neuronal plasticity refers to a series of processes aimed to respond to different challenges that lead to neuronal remodelling. As a matter of fact, the influence of BDNF on contextual and spatial learning, emotion and cognition was well documented in literature. BDNF has been shown implicated in the induction of synaptic plasticity through long-term potentiation (LTP) processes, thus inducing memory formation (Xu et al., 2000). The contribution of BDNF to LTP occurs through the modulation of glutamate and the activation of glutamate receptors that in turn elevate intracellular Ca^{2+} and activate different downstream mechanisms, including the secretion of BDNF (Bramham and Messaoudi, 2005). In this way, BDNF can regulate the activity-dependent synaptic plasticity, promoting the formation and maturation of synapses, both in the developing and mature nervous system (Lu, 2003). Functional changes at synapses can lead to structural changes at dendritic spines; thus, learning and memory processes are associated with activity-dependent changes not only in the number of dendritic spines but also in their size and shape (Yuste and Bonhoeffer, 2001). In fact, increasing evidence suggests that BDNF may induce long-lasting structural remodelling of dendritic spines (Poo, 2001; Tanaka et al., 2008). Moreover, BDNF contributes in the process of adult neurogenesis (Waterhouse et al., 2012). During this process, new neurons in the subgranular zone of the dentate gyrus are generated and increasing evidence supports a role for the neurotransmitter GABA in this mechanism. Given the role of BDNF on the maturation of GABAergic networks, the neurotrophin may contribute to adult neurogenesis. Furthermore, several studies suggested the involvement of BDNF in adult neurogenesis (Lee et al., 2002; Li et al., 2008; Scharfman et al., 2005).

Impairments in these mechanisms have been shown to alter the ability to adapt to environmental tasks, enhancing the vulnerability to psychopathologies (Calabrese et al., 2009; Pittenger and Duman, 2008). Given its role in crucial brain physiological conditions that are impaired in mental diseases, BDNF has been extensively investigated in the pathogenesis of psychiatric disorders. Indeed, BDNF expression is reduced in major depression, both in central regions as cerebral cortex and hippocampus and peripherally in serum. With this regard, several studies suggested that BDNF, neurogenesis and synaptic plasticity have an important role in the development of depression (Monteggia et al., 2007; Shirayama et al., 2002).

Moreover, antidepressant treatments ameliorate depressive symptoms and increase BDNF expression (Duman and Monteggia, 2006).

As mentioned before exposure to stressful experiences accounts for about half of the risk for depression (Kendler et al., 2001) and may represent a predisposing element, which will eventually lead to full-blown pathologic conditions, when an individual is re-exposed to adverse challenging conditions. Early life sensitive periods of maturation can be particularly vulnerable to adverse events and thus several studies investigated the expression of BDNF in animal models of early life stress. The most widely used and powerful experimental paradigms to study early life stress consist in prenatal or perinatal stress procedures. The prenatal model of early life stress is based on the exposure of pregnant dams to repetitive stress, usually restraint stress, from embryonic day 14 until delivery. We have demonstrated that adult prenatally stressed rats showed a significant reduction of *Bdnf* mRNA levels in prefrontal cortex and striatum, with no changes in the hippocampus, suggesting a long-term and anatomically specific effect of exposure to stress in utero on BDNF expression (Fumagalli et al., 2004). Moreover, prenatal stress induced a down-regulation of the pool of *Bdnf* transcripts with long 3'UTR in the prefrontal cortex, suggesting a selective impairment in transcripts targeting to dendrites that contribute to local, activity-dependent, neurotrophin synthesis (Luoni et al., 2014). When exposed to a chronic stress procedure during adult life, prenatally stressed rats revealed a significant increase of *Bdnf* gene expression in prefrontal cortex whilst the chronic manipulation produced a reduction of *Bdnf* mRNA levels in control animals (Fumagalli et al., 2004). As said before, the other experimental paradigm of perinatal stress in rodents is based on the disturbance of mother-pup interaction, namely maternal separation. The central characteristic of this model consists in separating the pups from the mother for a short period of time. We have shown that a strong single 24h maternal separation on PND 9 reduced BDNF mRNA and protein levels in the hippocampus of adult animals with no changes in other brain regions. The exposure to an acute challenging situation later in life determined a decrease of *Bdnf* mRNA levels only in control animals while stressed animals did not show any alterations following the acute manipulation at adulthood (Roceri et al., 2002). On the contrary, the exposure to repeated maternal deprivation from PND2 until PND14 caused a significant reduction of BDNF levels in prefrontal cortex and not in the hippocampus. In line with previous results, when exposed to a chronic stress in adult life, we observed a significant reduction of BDNF only in not-maternally stressed animals (Roceri et al., 2004). Altogether

these data suggest that the timing and duration of stressful manipulation are important for the anatomical specificity of the long-term alterations in BDNF expression.

The effects of stressful experiences during adolescence on BDNF levels has been investigated in few studies. Majority of studies report a rather consistent decrease of BDNF in the hippocampus of male rats following social deprivation (Han et al., 2011; Li et al., 2016; Pisu et al., 2016), in line with the findings from other stress paradigms (Duman and Monteggia, 2006). However, due to differences in the protocols used, there are contradictory and unexpected results. Moreover, none of these works investigated the pathways downstream of BDNF/TrkB that arbitrate the effects of BDNF on plasticity and neurogenesis (Murinova et al., 2017). Very few working groups tried to find the possible mechanisms mediating the effects of stress during adolescence on BDNF. Li et al., (2016) suggested that histone modifications could play a role as they showed an increase of histone H3 acetylation of *Bdnf* gene in PFC matched with an increase in BDNF mRNA and protein levels in the PFC, and a reduction of histone H3 acetylation of *Bdnf* gene in the hippocampus matched with a reduction in BDNF mRNA and protein levels in the hippocampus (Li et al., 2016).

1.2.2 The hypothalamic-pituitary-adrenal axis

When exposed to stress, the organism activates a series of processes aimed to cope with stressors, during which the HPA axis is one of the main players. First the HPA response to stress is adaptive and is able to influence learning and memory systems (McEwen, 2012). When chronic stressors occur, the HPA response may be maladaptive and, if the nervous system is more vulnerable, it could impair the proper trajectories of brain development (McEwen, 2012). In the presence of stressors, the paraventricular nucleus of the hypothalamus causes the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), that in turn determine the release of adrenocorticotropic hormone (ACTH) in the anterior pituitary. Then, ACTH induces the release of glucocorticoids from the adrenal cortex into the general circulation. Circulating glucocorticoids (corticosterone in rats) act in the brain through two receptors: the mineralocorticoid and glucocorticoid receptors (MR and GR). The MR is more relevant for basal tone as the affinity for glucocorticoids is about 10-fold higher than that of GR. In response to stress, corticosterone (CORT) binds GR leading to receptor dimerization. In turn, GR enters in the nucleus where, binding to specific DNA sequences (GR-responsive elements), it can enhance or repress the expression of different responsive genes, comprising BDNF. When the stress ends, different brain structures, including hypothalamus, hippocampus and medial PFC efficiently activate a negative feedback, aimed to limit the exposure to glucocorticoids (McEwen, 2012).

Impairments in HPA axis have been found in several mental disorders including depression (Burke et al., 2005). The major hypothesis is based on the hyperactivity of HPA axis associated with GR resistance and reduced negative feedback, leading to elevation of CRF (Juruena, 2014). These dysfunctions of HPA axis are highly relevant in the pathophysiology of depression and alterations in the expression levels of GR and MR have been associated with mental disorders. Indeed, reduced GR and MR mRNA levels have been found in major depression as well as in subjects with schizophrenia (Medina et al., 2013; Webster et al., 2002). As already mentioned before, adverse events play a crucial role in depression and an association between early life stress and HPA axis dysregulation has been reported. Although there is a discrepancy in basal plasma corticosterone levels in adult rats exposed to prenatal stress, several studies demonstrated HPA axis reprogramming after prenatal stress exposure (Maccari et al., 2003; Weinstock, 2005). Indeed, the exposure to adverse events early in life

impairs the ability of HPA axis to respond to stress in adulthood. Several evidences showed an effect of maternal care, through the frequency of pup licking/grooming, on the regulation of HPA axis function. Offspring of high licking/grooming mothers showed, as adults, reduced plasma ACTH and corticosterone responses to acute stress, increased hippocampal GR expression, enhanced negative feedback sensitivity and lower CRF expression in the hypothalamus (Meaney, 2001). Under basal condition, offspring of high kicking/grooming mothers showed enhanced hippocampal-dependent learning and memory. Whilst, after stressful manipulation, learning processes were increased only in offspring of low licking/grooming mothers as exposure to high levels of CORT inhibited LTP in adult offspring of high licking/grooming offspring (Bagot et al., 2012; Champagne et al., 2008).

Rodents are characterized by a period, from PND 4 to PND 14, namely stress hyporesponsive period (SHRP), during which corticosterone levels are maintained stable and low, for the normal development of the nervous system. The presence of the mother preserves this period against stress induced stimulation of GR and thus, maternal separation procedure may be a useful tool to investigate HPA response to stress. Repetitive maternal separation daily during SHRP increases CORT levels in adulthood (Nishi et al., 2014). Animals exposed to maternal separation showed increased HPA responsivity to acute stress at adult life, mediated by altered glucocorticoid receptor expression in brain regions crucial for the negative feedback (Meaney, 2001). As adults, rats that were separated from their mothers showed increased CRF expression in the hypothalamus, enhanced secretion of ACTH and CORT in response to an acute stimulus and a delayed return to baseline (Huot et al., 2004). Furthermore, exposure to high CORT during gestation results in dysfunctions in the regulation of HPA axis and of brain areas involved in HPA regulation, as stress hormones may be transported through the placenta (Beijers et al., 2014). These effects of prenatal stress on HPA responsiveness has been shown in a variety of stress paradigms throughout gestation, ranging from a single session of immobilization to thrice daily, starting during the second or the last week of gestation (Cannizzaro et al., 2006; Chung et al., 2005; Fujioka et al., 2006; Koenig et al., 2005; Maccari et al., 2003), indicating that the stress must be of sufficient intensity and administered at least once daily during the last week of gestation. As already mentioned, mental disorders are very complex and characterized by a strong interaction between genes and environment. With this regard, we have investigated the effects of the interaction between early life stress and serotonin transporter gene allelic variant on HPA axis function (van der Doelen et al., 2014).

The short allelic variant of serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR), characterized by a reduction in the promoter activity of the gene, has been already associated with an increased vulnerability to depression when coupled with early life adverse events (Caspi et al., 2003). We thus exposed 5-HTT knock-out rats, considered a valid tool to study the consequences of the short allelic variant of 5-HTT, to daily 3h sessions of maternal separation from PND2 to PND14. Our data showed that 5-HTT genotype interacts with early life stress affecting the expression of both GR and MR and their balance as well as of FK506 binding protein 5 (FKBP5) in specific brain areas. 5-HTT $^{-/-}$ rats showed reduced GR mRNA levels in the dorsal hippocampus following exposure to early life stress while 5-HTT $^{+/+}$ showed reduced GR mRNA levels in the dorsal medial prefrontal cortex (mPFC) following exposure to early life stress. 5-HTT $^{-/-}$ rats showed also reduced *Fkbp5* mRNA levels in the dorsal mPFC following exposure to early life stress. In the ventral mPFC 5-HTT $^{+/+}$ showed reduced *Fkbp5* mRNA levels while 5-HTT $^{-/-}$ showed increased expression levels following exposure to early life stress (van der Doelen et al., 2014).

Few studies investigated the effects of stress during adolescence on HPA response. Social isolation for 4 weeks from weaning reduced basal levels of corticosterone in both male and female rats (Pisu et al., 2016). Moreover, social deprivation exposure caused an increased expression of GR in the hippocampus of both male and female rats (Pisu et al., 2016). The reduction of corticosterone levels found by Pisu and colleagues was not in line with a previous study of Serra et al., (2000) reporting a 30% increase in the levels of circulating corticosterone in male rats (Serra et al., 2000). Weintraub et al., (2010) found that, after a 1h acute restrain stress at PND 50, immediately after the isolation procedure, isolated males exhibited lower corticosterone than group-housed counterpart while females exhibited higher corticosterone than control females. The same results were obtained when animals were tested on PND 70, after resocialization (Weintraub et al., 2010).

1.3 Rat models of adolescent stress

Animal models represent a useful tool to investigate the effects of environmental and psychosocial stressors during adolescence and the long-term consequences for later neuropsychiatric disorders. As said before, rodents are highly social animals and adolescent rats tend to spend more time in social behaviours than adult animals do. These features are linked with the correct development of social competence and proper cognitive maturation (Douglas et al., 2004). Several animal models mimicking the exposure to stress during adolescence have been successfully used (Fig. 1.3), with the aim to manipulate the rat social experience in order to investigate the behavioural and molecular alterations relevant to anxiety, depression as well as to other neuropsychiatric conditions.

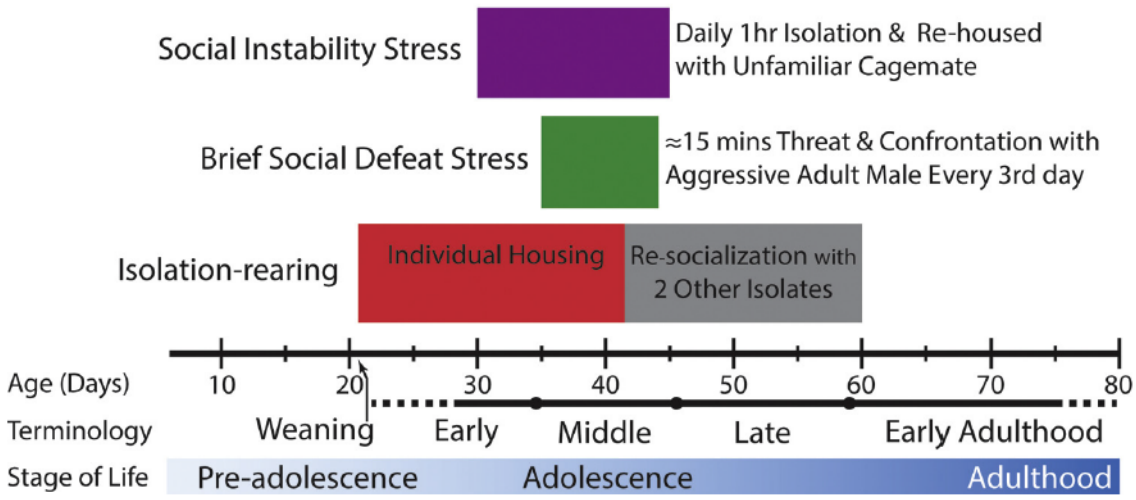

Fig. 1.3 *Schematic timeline of the experimental procedures and terminology (adapted from (Burke et al., 2017))*

Social Instability

The social instability paradigm usually consists of daily social isolation for 1h followed by pair housing with an unfamiliar partner (McCormick et al., 2008). Sometimes it also involves periods of isolation and periods of overcrowding (Herzog et al., 2009). This condition increases adult anxiety in the elevated plus-maze, suppresses hippocampal cell proliferation and impairs adult object recognition in a spatial memory test (McCormick et al., 2010; McCormick et al., 2008).

Social Defeat

The social defeat paradigm is used to model exposure to a physical abuse and subordination in bullying and commonly involves a resident-intruder procedure. The animal (intruder) is introduced into the cage of an aggressive conspecific (resident) that rapidly, defending its territory, investigates and attacks the intruder. This paradigm has been shown to induce several long-lasting behavioural and neurochemical alterations related to depression (Buwalda et al., 2005). However, finding a proper resident animal to act as an aggressor can be challenging, considering also that females are not so territorials as males and residents are less aggressive toward an adolescent animal as compared to an adult intruder (McCormick et al., 2017).

Social Deprivation

The social deprivation paradigm consists of chronic single housing condition beginning on the day of weaning. Under these conditions, animals are completely deprived of social contacts but they can smell, hear and see other rats within the holding room (Leng et al., 2004; Weiss et al., 2004). Deprivation of social contacts is able to affect the maturation of normal social behaviours and reduce synaptic density and myelination in prefrontal cortex (Leussis and Andersen, 2008; Leussis et al., 2008). In the majority of studies (Table 1), animals are housed individually for 2 to 4 weeks during adolescence and then immediately tested, while, more recently, the attention was focused on very specific and defined time windows of vulnerability (Table 2). This allows to investigate the effects of abnormal social experience during a particular phase of brain maturation (Lukkes et al., 2009a). Animals are single housed and then return to group rearing condition before testing in order to evaluate long lasting consequences of the exposure to social stress during specific developmental time frames. Exposure to isolation rearing during adolescence is able to increase the vulnerability to develop anxiety disorders and major depression during adulthood (Burke et al., 2017). Isolation condition in adolescent rats induces the development of anxiety behaviours, with decreased exploration of novel objects, reduced time spent in the open arms of the elevated plus maze and increased freezing during a social interaction test (Chappell et al., 2013; Lukkes et al., 2009a). Increased anxiety behaviours are often present in comorbidity with depression. Indeed, isolation rearing during adolescence causes increased depressive-like behaviours in the forced swim test (Amiri et al., 2015; Jahng et al., 2012). Social deprivation also results in learning and memory deficits, as indicated by reduced performance in the Morris water maze

and impaired object recognition memory (Bianchi et al., 2006; Lu et al., 2003b). When tested after a period of group rearing, some of these deficits are attenuated (Han et al., 2011; Lu et al., 2003b). Although the diversity of protocols used, isolation during adolescence seems to impair serotoninergic activity in various forebrain regions in adulthood (Lukkes et al., 2012). 5-HT_{1A} and 5-HT_{2A} serotonin receptor binding, serotonergic turnover as well as serotonin concentrations have been found increased in several isolation protocols (Brenes and Fornaguera, 2009; Gunther et al., 2008). Serotonergic activity in the prefrontal cortex seems to be linked to stress-coping response; serotonergic neurons located in the dorsal raphe nucleus are selectively activated by anxiety-related stimuli and project to the PFC, modulating anxiety states and anxiety-related responses. In turn, serotonergic activity in the PFC is required for anxiety-like behaviours (Amat et al., 2004). Moreover, isolation during adolescence up-regulates corticotropin-releasing factor CRF_2 receptors expression in the dorsal raphe nucleus, resulting in increased serotonergic activity (Lukkes et al., 2009b).

Table 1.1 *Immediate effects of social deprivation in adolescence on learning and memory (adapted from* (Green and McCormick, 2013))

Sex/strain	Age at social dep.	Adult comparison	Experimental measures	Compared to controls	Reference
Male SD – CD	25–55	No	Morris water maze Acquisition learning Probe Hippocampus BDNF and Arc protein expression	 ↑ ↑ ↓	(Pisu et al., 2011a)
Male L	21–63	No	Morris water maze Acquisition learning Reversal learning	 ↑ ↑	(Wongwitdecha and Marsden, 1996)
Male L	21–84	No	Object spatial location test Object recognition test Morris water maze Acquisition learning Probe	= = = =	(Schrijver et al., 2002)
Male L	21–100 No	No	Morris water maze Acquisition learning Probe Two-way discrimination Simple discrimination Compound discrimination Reversal	 = = = = ↓	(Schrijver et al., 2004)
Male SD	22–49 or 22–77	No	Morris water maze Acquisition learning DG of hippocampus Cell proliferation Number of granule cells Volume of granule cells CA1 of hippocampus LTP	 ↓ ↓ = = ↓	(Lu et al., 2003a)
Male W	21–77	No	Morris water maze Acquisition learning Probe trial Reversal Memory retention (8 days later) Prelimbic subregion of mPFC LTP Hippocampus Outward potassium currents	 = ↓ ↓ ↓ ↓ ↑	(Quan et al., 2011)
Male mice	24–52	No	Morris water maze Acquisition learning Probe Hippocampus Cell proliferation Cell survival Cell differentiation	 ↓ ↓ = ↓ ↓	(Ibi et al., 2008)
Female and male SD	21–150	No	Fear conditioning Training Cue recall Context recall (females)	 = = ↓	(Weiss et al., 2004)
Male WT, CRF2 KO, & C57BL/67 mice	28–90	No	Fear conditioning Cue recall Context recall	 = ↓	(Gresack et al., 2010)
Female L	28–70	No	Object recognition test Attentional set-shifting Simple discrimination Complex discrimination	↓ = =	(McLean et al., 2010)

			Intra-dimensional shift	=	
			Extra-dimensional shift	↓	
			Reversal	=	
Male L	21–70	No	Attention set-shifting		(Schrijver and Wurbel, 2001)
			Simple discrimination	=	
			Reversal	=	
			Extra-dimensional	↓	
Female L	21–105	No	Operant conditioning		(Abdul-Monim et al., 2003)
			Training	=	
			Reversal	=	
Male SD	21–77	No	Rotating T maze		(Li et al., 2007)
			Acquisition learning	=	
			Reversal learning	↓	
			Prepulse inhibition	↓	
Female L	21–90	No	Conditional visual discrimination	↓	(Jones et al., 1991)
			Distractability	↓	
			Extinction	↓	
Male LE	21–120	No	Maze learning	↓	(Holson, 1986)
Male L	28–58	No	Object recognition test	↓	(Bianchi et al., 2006)
			Hippocampus		
			MAP-2	↓	
			*Several changes in markers of microtubule dynamics		
Male L	28–85	No	Object recognition test		(Bianchi et al., 2009)
			P57	↓	
			P86	↓	
Male L	23–63	No	Object recognition test	↓	(Jones et al., 2011)
			Prepulse inhibition	=	
Male L	21–50	No	Object recognition test	=	(Lapiz et al., 2000)
			Noradrenaline		
			Cortical	=	
			Hippocampus	=	

Table 1.2 *Delayed and long-lasting effects of social deprivation in adolescence on learning and memory (adapted from (Green and McCormick, 2013))*

Sex/strain	Age at social dep.	Age at test	Adult comparison	Experimental measures	Compared to controls	Reference
Male SD	22–49	77	No	Morris water maze		(Lu et al., 2003a)
				Acquisition	=	
				Dentate gyrus		
				Cell proliferation (BrdU)	=	
				Number of granule cells	=	
				Volume of granule layer	=	
				LTP in CA1 (in vivo and in vitro)	=	
Male SD	21–34	56 to 64	No	Morris water maze		(Han et al., 2011)
				Acquisition	=	
				Probe	=	
				Reversal	↓	
				BDNF protein expression		
				NAcc	↓	
				CA1 and DG of hippocampus	↓	
				mPFC	↑	
Male SD	21–34	56	No	Latent inhibition	↓	(Han et al., 2012)
				D2 receptors		
				mPFC	↑	
				NAcc	↑	
Male & female W albino & female LH	23–45 or 23–60	54 or 150 90	Yes Stressed: 45–150 Tested: 150	Radial arm maze	Adol ↓ Adult =	(Einon, 1980)
Male SD	21–42	56	No	Fear conditioning		(Lukkes et al., 2009a)
				Training	=	
				Cue recall	=	

2. AIM OF THE PROJECT AND EXPERIMENTAL STRATEGY

In last years, great attention was focused in examining and characterizing the etiopathological mechanisms that may contribute to the development of psychiatric disorders. Despite the well documented genetic predisposition (Sullivan et al., 2000), exposure to stressful experiences account for about half of the risk for depression (Kendler et al., 2001) and most importantly, exposure to stressful events early in life may represent a predisposing element, which will eventually lead to full-blown pathologic conditions, when an individual is re-exposed to adverse challenging conditions. There is now consistent evidence that stressful life events during sensitive periods of neural development can interact with functional outcomes, altering the course of brain development. With this respect, animal models represent a very useful tool to investigate relevant players for psychiatric disorders, with the advantage of controlling the influence of various factors such as the timing and intensity of the adverse condition, as well as other environmental conditions. Accordingly, preclinical studies showed that stressful experiences during gestation or early in life can lead to increased vulnerability for mental disorders (Fumagalli et al., 2007).

In the present study, we investigated the effects of stress exposure during adolescence, a critical developmental period characterized by intensive neural and behavioural changes and thus highly vulnerable to adverse events. Rodents are highly social animals and adolescent rats tend to spend more time in social behaviours then adult animals do (Spear, 2000). Due to their predilection for social behaviours, we used social deprivation, a well-established paradigm of stress for adolescent rats based on deprivation of all social contacts (Burke et al., 2017). The paradigm consists in manipulating the rat social experience (social versus isolation rearing) in order to investigate behavioural and molecular alterations relevant to anxiety, depression as well as to other neuropsychiatric conditions.

In order to ensure that any alteration detected can be ascribed to long-lasting isolation rearing-induced disturbance of specific time window of development, male and female pups were weaned at postnatal day (PND) 21 and housed either in group or individually until PND 49, followed by re-socialization in groups of 3 rats per cage until adulthood. Since anhedonia and cognitive disabilities are common symptoms of different psychiatric conditions (Green and McCormick, 2013), adult animals underwent the sucrose preference test in order to establish the presence of a depressive-like phenotype, whereas the cognitive performance

was evaluated with the novel object recognition test. Beyond the long-term effects on several systems, exposure to chronic severe stressful events early in life has revealed altered responses to subsequent stressors later in life (Fumagalli et al., 2004; Grissom and Bhatnagar, 2009; McEwen, 2003; Roceri et al., 2004; Roceri et al., 2002). In order to investigate if the stressful experience during adolescence may alter the ability to cope under a challenging condition, a group of animals (grouped or single-housed as adolescents) was exposed to a 1h-session of acute immobilization stress immediately before killing.

Next, we investigated three candidate systems, whose deterioration may contribute to the development of the diseased phenotype, namely the neurotrophin brain-derived neurotrophic factor (BDNF), the hypothalamic-pituitary-adrenal (HPA) axis and the glutamatergic signalling pathway. We performed analyses at gene and protein levels under resting conditions as well as following the acute immobilization challenge. BDNF has emerged as an important player in facilitating brain connectivity, neuronal plasticity and promoting adult neurogenesis and its expression is reduced by early life stress manipulation and in depressed patients (Duman and Monteggia, 2006; Luoni et al., 2014). A clear interplay between neurotrophic factors and neurotransmitters mediates adaptive responses to stressful situations, with BDNF and the excitatory neurotransmitter glutamate being key regulators of synaptic plasticity throughout the central nervous system. 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 HPA axis. The aim of these analyses was 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.

3. MATHERIALS AND METHODS

3.1 Animals

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).

All animal experiments were conducted 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.

Within 24 hours of 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).

3.1.1 Social isolation

At PND 21, pups were housed in groups of 3 rats per cage per sex or individually until PND 49. Rats group-housed in the same cages belonged to different litters such that each litter provided 2/3 males group-housed and 2/3 females group-housed in order to avoid any litter effect. Animals housed individually were located, one rat per cage, in the same room of group-housed ones, allowing them to see other animals without any interaction.

All rats were habituate to the experimenters in order to reduce stress induced by manipulation, with food and water ad libitum (21±1°C, 60±10% relative humidity, 12/12h light/dark cycle). Noise and disturbance were kept to a minimum. Males and females were housed in two different rooms and all rats received weekly husbandry.

3.1.2 Re-socialization

At PND 49, all animals underwent re-socialization. Group-housed rats remained in groups of 3 rats per cage but the partners were reassigned within cages. As before, group-housed rats in the same cage belonged to different litters in order to avoid any litter effect. In this way, each rat was re-grouped with 2 new group-housed rats reared in two different cages. Isolated animals were housed in groups of 3 rats per cage, allowing them to interact with other 2 isolated animals. All the cages after re-socialization were composed by rats coming from different litters.

All rats were habituate to the experimenters in order to reduce stress induced by manipulation, with food and water ad libitum (21±1°C, 60±10% relative humidity, 12/12h light/dark cycle). Noise and disturbance were kept to a minimum. Males and female were housed in two different boxes and all rats received weekly husbandry.

From PND21 until the end of all procedures, water consumption and weight gain were measured weekly.

3.2 Behavioural testing

At PND 70-77, half animals, both group- and single-housed, underwent behavioural assessment. We tested animals in the sucrose preference test and, after a week of wash-out, in the novel object recognition test.

The sucrose preference test allows to investigate the development of anhedonia, namely the decreased ability to experience pleasure, one of the core features of depressive-like phenotype. It is known that rodents are interested in sweet foods or solutions, thus reduced preference for sweet solution represents anhedonia.

The aim of the novel object recognition test is to assess the cognitive performance of animals. The test evaluates differences in the exploration time between novel and familiar objects, which may indicate learning and memory impairments. Based on the timing of testing, it is also possible to assess the influence of specific brain regions in the process of recognition.

3.2.1 Sucrose preference test

Sucrose preference test was carried out individually, in a new clean cage, at PND 70 at the same time for all tested animals. Prior to the beginning of the test, animals were habituated to the presence of two drinking bottles of tap water in their home cage for 24 h, in order to prevent possible effects of side preference in drinking behaviour during the test session. No previous food or water deprivation was applied. Following this habituation, on the day of the test, 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. During the test, noise and disturbance were kept to a minimum. At the end, water and sucrose solution intake was measured. Sucrose preference was calculated as the percentage of consumed sucrose solution (in mg) on the total amount of liquid drunk (in mg).

3.2.2 Novel object recognition test

Animals belonging to each experimental group, both males and females, were subjected to the novel object recognition test (ORT) at PND77. This test exploits the natural tendency of rodents to explore novel items more than familiar ones. The experimental apparatus used for

the test was an open-field box made of Plexiglas, placed in a quiet room dimly illuminated. On the day of testing, animals were habituated in the room for 15 min before the experimental procedure began. The floor of the apparatus was covered with some clean litter in order to reduce stress induced by a novel environment. Equally, the walls were covered with an opaque black film to reduce any disturbance from outside. After every test session, the apparatus was cleaned at first with a 0.1% acetic acid solution, followed by a 10% ethanol solution, water and it was finally air-dried. In this way, the behaviour of animals was not influenced by smell cues. The test consisted in a first phase of habituation during which the rat was allowed to explore the new field 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, and it is allowed to explore them for 7 minutes. The objects were placed in the corners of the apparatus equidistantly from the walls and the animal placed in the center to start the experiment. After an inter-trial delay of 3 min during which the animal was returned to its cage, one of the two familiar objects was replaced by a novel, previously unseen object (with different shape, colour and texture). 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 measured 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)] X 100

We defined exploration of an object as orienting the nose at a distance $\leq$1 cm to the object or touching it with the nose and sniffing, while climbing on it was not considered exploration. A positive score indicates more time spent with the novel object, while a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference.

3.3 Acute stress procedure

In order to evaluate the ability to cope a challenging condition during adulthood, a group of animals underwent, immediately before killing, an acute immobilization stress. Animals were restrained in individual semi cylindrical plastic restrainer. Each restraint (8 cm inner diameter, 20 cm long) had a hole on the top and a slotted tail piece and front clots provided ample ventilation. Animals not exposed to the acute stress condition were left undisturbed in their home cages. The restraint stress session lasted for 1 h and the animals were sacrificed immediately after.

3.4 Molecular analyses

3.4.1 Collection of brain samples

At the end of all experiments animals were killed by beheading and brain samples (hippocampus and prefrontal cortex) were rapidly dissected, frozen on dry ice and stored at -80 °C for later analyses. Dissections were performed according to the atlas of Paxinos and Watson (Paxinos and Watson, 1996). In detail, the hippocampus (including both the ventral and dorsal parts) was dissected from the whole brain, while the prefrontal cortex was dissected from 2-mm thick slices (PFC defined as Cg1, Cg3 and IL subregions corresponding to the plates 6–9).

3.4.2 Collection of peripheral samples

Samples of blood from each rat were collected in heparinised tubes. Plasma was separated by centrifugation (3000 g for 20 min at 4°C) and stored at -80 °C for later analyses.

3.4.3 RNA preparation and quantitative Real-Time PCR analyses

Total RNA was isolated from brain tissues of prefrontal cortex (PFC) and dorsal and ventral hippocampi using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Italy), according with the manufacturer's instructions. Subsequently, RNA was quantified by spectrophotometric analysis, measuring its absorbance at 260 nm, which corresponds to the region of the electromagnetic spectrum within nucleic acids show a strong absorbance.

After total RNA extraction, the samples were processed for quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) to determine the levels of mRNA.

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 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (β-actin). We chose β-actin as internal standard for gene expression analyses since its expression was not altered by housing conditions and neither by the acute stress procedure.

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), and *Gadd45β* (Assay ID: Rn01452530_g1) were purchased from Thermo Fisher Scientific and are available on request, while the other TaqMan gene expression assays were purchased from Eurofins Genomics (Vimodrone, Italy) and are summarized in Table 4.1. Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retro-transcription) 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.

We used the qRT-PCR technique to quantify the mRNA levels of our genes of interest, as this technique is highly sensitive, specific and reproducible. It allows the correlation of the fluorescent signals, accumulating during the cycles of PCR, directly with the amount of starting material (mRNA). Indeed, it is possible to monitor the amplification of the target gene in real time by quantifying the amount of fluorescent product during the exponential phase of the reaction. To avoid possible biases due to pipetting errors, a housekeeping gene (β-actin), with a different fluorophore, is also detected and the fluorescence values of the target gene are normalized to the ones of the housekeeping gene. In the real time PCR assay it is possible to identify the Ct (cycle threshold), that is the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). The threshold cycle is inversely proportional to the starting amount of target gene in the sample (the lower the Ct level the greater the amount of starting target nucleic acid in the sample). Our analyses, in particular, were conducted exploiting TaqMan probes complementarity to the target sequence. This kind of probes, indeed, are capable of emitting fluorescence when, after hybridization with the cDNA, they are hydrolyzed by the polymerase during the elongation step. The duplication in the intensity of fluorescence is therefore observed and measured after each cycle, allowing its quantification. Data analysis was performed by comparing the values of the Ct of the target gene and β-actin (ΔCt = Ct target gene – Ct β-actin) and the data were expressed as $2^{(-\Delta\Delta Ct)}$ (which represents the fold change enrichment vs. Ctrl group), or as a percentage over Ctrl (setting Ctrl at 100%).

Gene	Forward Primer	Reverse Primer	Probe
Total Bdnf	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Arc	GGTGGGTGGCTCTGAAGAAT	ACTCCACCCAGTTCTTCACC	GATCCAGAACCACATGAATGGG
Zif-268	GAGCGAACAACCCTACGAG	GTATAGGTGATGGGAGGCAAC	TCTGAATAACGAGAAGGCGCTGGTG
Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGAGA	AGCTTTGTCAGTTGGTAAAACCGTTGC
Sgk-1	GACTACATTAATGGCGGAGAGC	AGGGAGTGCAGATAACCCAAG	TGCTCGCTTCTACGCAGC
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG	TGTCCATCTCCCAGGATTCTTTGGC

Table 3.1 *Sequences of forward and reverse primers and probes used in qRT-PCR analysis and purchased from Eurofins Genomics (Vimodrone, Italy)*

3.4.4 Protein extraction and western blot analyses

Prefrontal cortex samples were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer, containing 0.32 M sucrose, 0.1 mM EGTA, 1mM HEPES solution and 0.1mM phenylmethylsulfonyl fluoride, in presence of a complete set of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors and then sonicated for 10s at a maximum power of 10-15% (Bandelin Sonoplus). The homogenate was clarified (1000 *g*; 10min), obtaining a pellet (P1) enriched in nuclear components, which was re-suspended in a buffer (1mM HEPES, 0.1mM dithiothreitol, 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. The supernatant (S1) was then centrifuged (13000 *g*; 15min) to obtain a clarified fraction of cytosolic proteins (S2). The pellet (P2), corresponding to the crude membrane fraction, was re-suspended in the same buffer used for the nuclear fraction. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Protein analyses were performed on homogenate and crude membrane fraction (pTrkB Y816, TrkB, proBDNF, mBDNF, pPLC Y783, PLC, pCaMKII T286, CaMKII, pAKT S473, AKT, pGSK3ß S9, GSK3ß, pERK1/2 T202/Y204 T185/Y187, ERK1/2, pGluA1 S845, pGluA1 S831, GluA1, GluA2, pGluN2B Y1472, pGluN2B S1303, GluN2B, GluN2A). Equal amounts of protein (10µg) were run under reducing conditions on Any Kd Criterion TGX precast gels (Bio-rad Laboratories) and on 8% polyacrylamide gels. The gels were then transferred by electrophoresis onto polyvinylidene fluoride (PVDF) and nitrocellulose membranes. Unspecific binding sites were blocked for 1h in 10% non-fat dry milk in Tris-buffered saline and the membranes were then incubated with the primary antibodies listed in table 4.2. Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:750 for PLC; 1:800 for GluA2; 1:1000 for pPLC Y783, GluN2A, pGluA1 S845; 1:2000 for pTrkB Y816, TrkB, pAKT S473, ERK1/2, AKT, pGluN2B Y1472, pGluN2B S1303; 1:3000 for proBDNF, pGluA1 S845; 1:4000 for pGSK3ß S9 Cell Signaling), anti-mouse IgG (1:1000 for mBDNF; 1:2000 for GluA1; 1:4000 for GSK3ß; 1:5000 for pCaMKII T286; 1:7000 for CaMKII, Sigma-Aldrich) or anti-goat IgG (1:2000 for GluN2B, Santa Cruz). Immunocomplexes were then visualized by chemiluminescence using 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 probing the membranes with a polyclonal antibody (1:10000,

Sigma–Aldrich) followed by a 1:20000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma–Aldrich). In order to visualize the immunocomplexes, the membranes were incubated for 2 minutes with one of the following enhanced chemiluminescent substrate: LiteAblot PLUS (Euroclone), ECL Star (Euroclone) and Clarity western ECL substrate (Bio-Rad Laboratories). Protein levels were calculated using the Chemidoc MP imaging system together with Image Lab software (Bio-Rad Laboratories).

Primary antibody	Primary antibody condition
pTrkB Y816 (Immunological Sciences)	1:1000 in 5% non-fat dry milk, 4°C o/n
TrkB (Cell Signaling)	1:750 in 5% non-fat dry milk, 4°C o/n
proBDNF (Genetex)	1:2000 in 5% non-fat dry milk, 4°C o/n
mBDNF (Icosagen)	1:500 in 3% non-fat dry milk, 4°C o/n
pPLC Y783 (Cell Signaling)	1:500 in 5% Bovine Serum Albumine, 4°C o/n
PLC (Cell Signaling)	1:750 in 5% non-fat dry milk, 4°C o/n
pCaMKII T286 (Thermo Scientific)	1:2000 in 3% non-fat dry milk, 4°C o/n
CaMKII (Millipore)	1:5000 in 3% non-fat dry milk, 4°C o/n
pAKT S473 (Cell Signaling)	1:1000 in 5% non-fat dry milk, 4°C o/n
AKT (Cell Signaling)	1:500 in 5% non-fat dry milk, 4°C o/n
pGSK3ß S9 (Cell Signaling)	1:1000 in 5% Bovine Serum Albumine, 4°C o/n
GSK3ß (BD Biosciences)	1:1000 in 5% non-fat dry milk, 4°C o/n
pERK1/2 T202/Y204 T185/Y187 (Cell Signaling)	1:1000 in 3% non-fat dry milk, 4°C o/n
ERK1/2 (Cell Signaling)	1:1000 in 3% non-fat dry milk, 4°C o/n
pGluA1 S845 (Millipore)	1:1000 in 5% Bovine Serum Albumine, 4°C o/n
pGluA1 S831 (Thermo Scientific)	1:500 in 5% non-fat dry milk, 4°C o/n
GluA1 (Neuromab)	1:1000 in 5% non-fat dry milk, 4°C o/n
GluA2 (Cell Signaling)	1:1000 in 3% non-fat dry milk, 4°C o/n
pGluN2B Y1472 (Millipore)	1:1000 in 3% non-fat dry milk, 4°C o/n
pGluN2B S1303 (Millipore)	1:1000 in 5% Bovine Serum Albumine, 4°C o/n
GluN2B (Santa Cruz)	1:1000 in 3% non-fat dry milk, 4°C o/n
GluN2A (Thermo Scientific)	1:1000 in 3% non-fat dry milk, 4°C o/n

Table 3.2 *Primary antibodies used in western blot analyses. (o/n: overnight).*

3.4.5 Analysis of plasma corticosterone levels

Samples of blood were collected in heparinized tubes and plasma was separated by centrifugation. Corticosterone was determined using a commercially available kit (IBL-International Corticosterone ELISA Kit), based on the principle of competitive binding. The microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the corticosterone molecule. Endogenous corticosterone of a sample competes with a corticosterone-horseradish peroxidase conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of corticosterone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of corticosterone in the sample.

The samples were diluted as follows: dilution 1:5 for Male/Group/Ctrl/Sham, Male/Group/Test/Sham, Male/Iso/Ctrl/Sham and Male/Iso/Test/Sham; dilution 1:50 for Male/Group/Ctrl/AS, Male/Group/Test/AS, Male/Iso/Ctrl/AS and Male/Iso/Test/AS; dilution 1:20 for Female/Group/Ctrl/Sham, Female/Group/Test/Sham, Female/Iso/Ctrl/Sham and Female/Iso/Test/Sham; dilution 1:100 for Female/Group/Ctrl/AS and Female/Group/Test/AS; dilution 1:80 for Female/Iso/Ctrl/AS and Female/Iso/Test/AS.

3.4.6 Statistical analysis

Changes produced by housing condition were analysed with Student's *t* test. Changes produced by housing condition and acute stress were analysed using a two-way ANalysis of VAriance (ANOVA), followed by Fisher's LSD post-hoc comparisons. Changes produced by housing condition, acute stress and gender were analysed using a three-way ANalysis of VAriance (ANOVA), followed by Fisher's LSD post-hoc comparisons. SPSS for Mac OS X (Release 24.0.0) was used to perform the statistical analyses.

A probability level of $p<0.05$ was taken as significant in every test.

4. RESULTS

4.1 Effects of social isolation on behaviours

4.1.1 Adolescent isolation rearing does not influence body weight gain

We first evaluated weight gain to investigate whether body weight was influenced by the housing condition. Body weights were determined weekly from PND 21 till the end of the experiments. Repeated measurements two-way ANOVA was applied for the whole period and revealed significant effects of time, gender and interaction (Time: $F_{9,1044}$= 18453, p<0.001; Gender: $F_{1,116}$= 1512, p<0.001; Interaction: $F_{9,1044}$= 2158, p<0.001). Statistical analyses showed no significant effects of housing condition and time*housing interaction (Housing: $F_{1,116}$= 0.295, p>0.05; Interaction: $F_{9,1044}$= 0.354, p>0.05).

Indeed, as shown in fig. 4.1, all animals increased weight over time significantly (p<0.001), demonstrating the expected body weight gain consistent with age-appropriate growth over the duration of the study. Post hoc analyses revealed that starting from PND 35 males gained more weight than females, independently from housing condition (p<0.001). Only at PND 56 and 63 single-housed male rats weighted less than group-housed ones (p<0.05), although this effect was not found in all other time points.

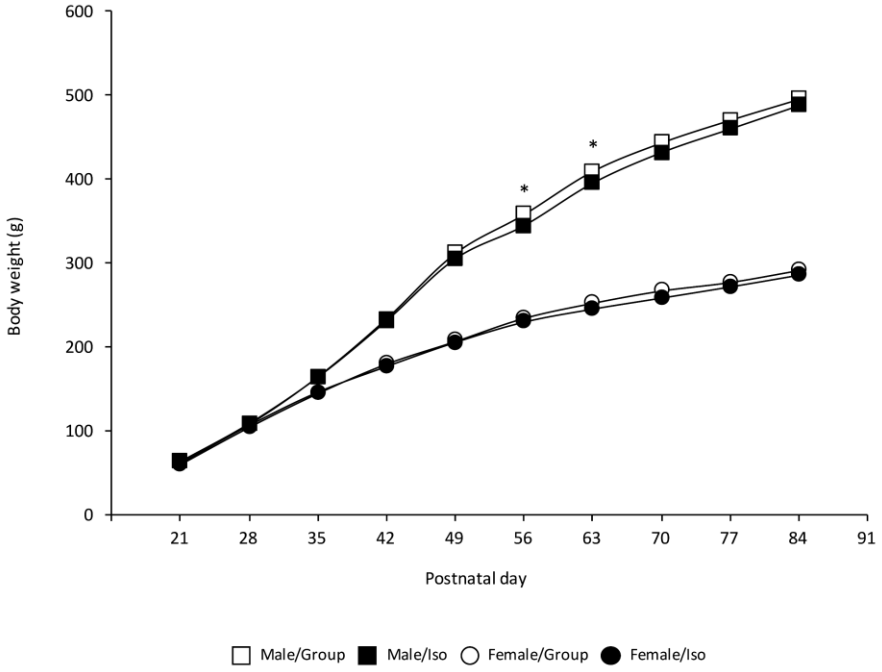

Fig. 4.1 *Effects of social isolation on body weight*
Weekly body weight (g) was measured from PND 21. Data are expressed as mean ± SEM of at least 29 animals per group.
* $p<0.05$ vs Males/Group (Repeated measurements two-way ANOVA followed by post-hoc test)

4.1.2 Animals housed individually during adolescence display an anhedonic phenotype later in life

Depression is a very complex psychiatric disorder comprising several symptoms as low mood, anhedonia, feeling sad, hopeless and helpless. In rodents, it is not possible to directly identify and quantify these feelings. Thus, researches have used some behavioural tests as the forced swim test and the sucrose preference test as a measure of anhedonia. Moreover, the power of these tests is confirmed when conducted in response to antidepressants (Moreau, 2002).
We have evaluated the development of depressive-like behaviour at adulthood in animals socially isolated during adolescence by measuring sucrose preference. After a 24h period of habituation, 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. Sucrose preference was calculated as the percentage of consumed sucrose solution (in mg) on the total amount of liquid drunk (in mg). Two-way ANOVA revealed significant effects of gender and housing condition (Gender: $F_{1,58}$= 23.235, p<0.001; Housing: $F_{1,58}$= 13.452, p<0.01). As shown in fig. 4.2, post-hoc analyses revealed that social isolation during adolescence decreased the preference for a sucrose solution later in life in males (** p<0.01) with a tendency toward a reduction also in females (p=0.05). Female rats, both group-housed and isolated, showed an increased preference for sucrose compared to male counterparts (§§ p<0.01 vs group-housed male animals; $^{###}$ p<0.001 vs isolated male animals).

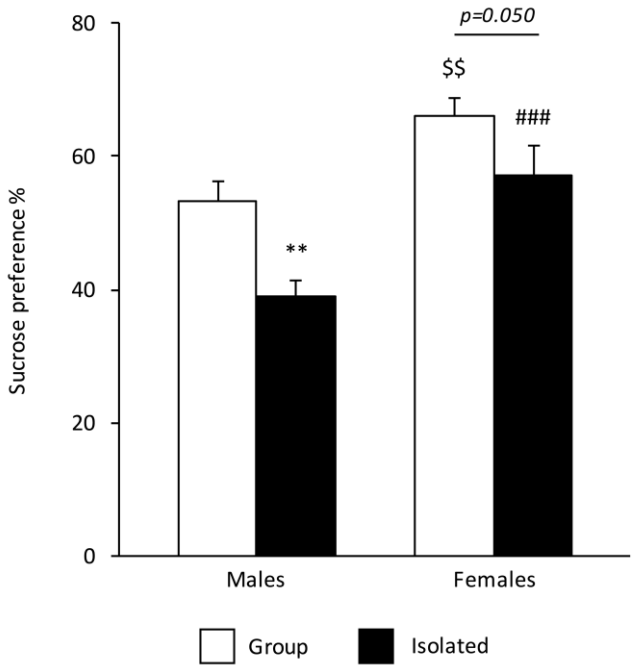

Fig. 4.2 *Effects of social isolation on sucrose preference measured at adulthood as degree of depressive-like phenotype*

Percentage of sucrose preference, calculated as indicated in Methods. Data are expressed as mean ± SEM of at least 14 animals per group.

** $p<0.01$ vs. Males/Group; ### $p<0.001$ vs Males/Isolated; $^{\$\$}$ $p<0.01$ vs Males/Group (2-way ANOVA followed by post-hoc test)

4.1.3 Adult rats single-housed during adolescence report cognitive performance similar to group-housed animals

After evaluating the anhedonic phenotype, we assessed the effects of isolation rearing during adolescence on cognitive functionality at adulthood by subjecting animals to the novel object recognition test (ORT). Indeed, cognitive disabilities are one of the common symptoms of psychiatric disorders (Disner et al., 2011; Lewis et al., 2012) and ORT is widely used to assess cognitive performance in animal models of mental disorders (Lyon et al., 2012). The test is based on the spontaneous behaviour of rodents to explore novelty and the choice to explore the new object reflects the use of working and recognition memories. The percentage of exploration time during the test phase was calculated as a discrimination index *[(new-familiar)/(new+familiar)]x100* and as a recognition index *[(new)/(new+familiar)]x100*.

As shown in fig. 4.3 (a, b) group-housed and isolated animals, of both genders, spent the same time exploring the two identical objects during the familiarization phase, showing no preference for either of the two positions (left or right). During the test phase (Fig. 4.3 c, d, e, f) group-housed and isolated animals, of both genders, differentiated the familiar and the novel object as they spent more time exploring the novel one compared to the familiar. In this way, animals exposed to isolation rearing during adolescence showed a similar cognitive performance, at adulthood, to group-housed animals, as we didn't observe a significant reduction both in the discrimination and recognition indexes ($p>0.05$).

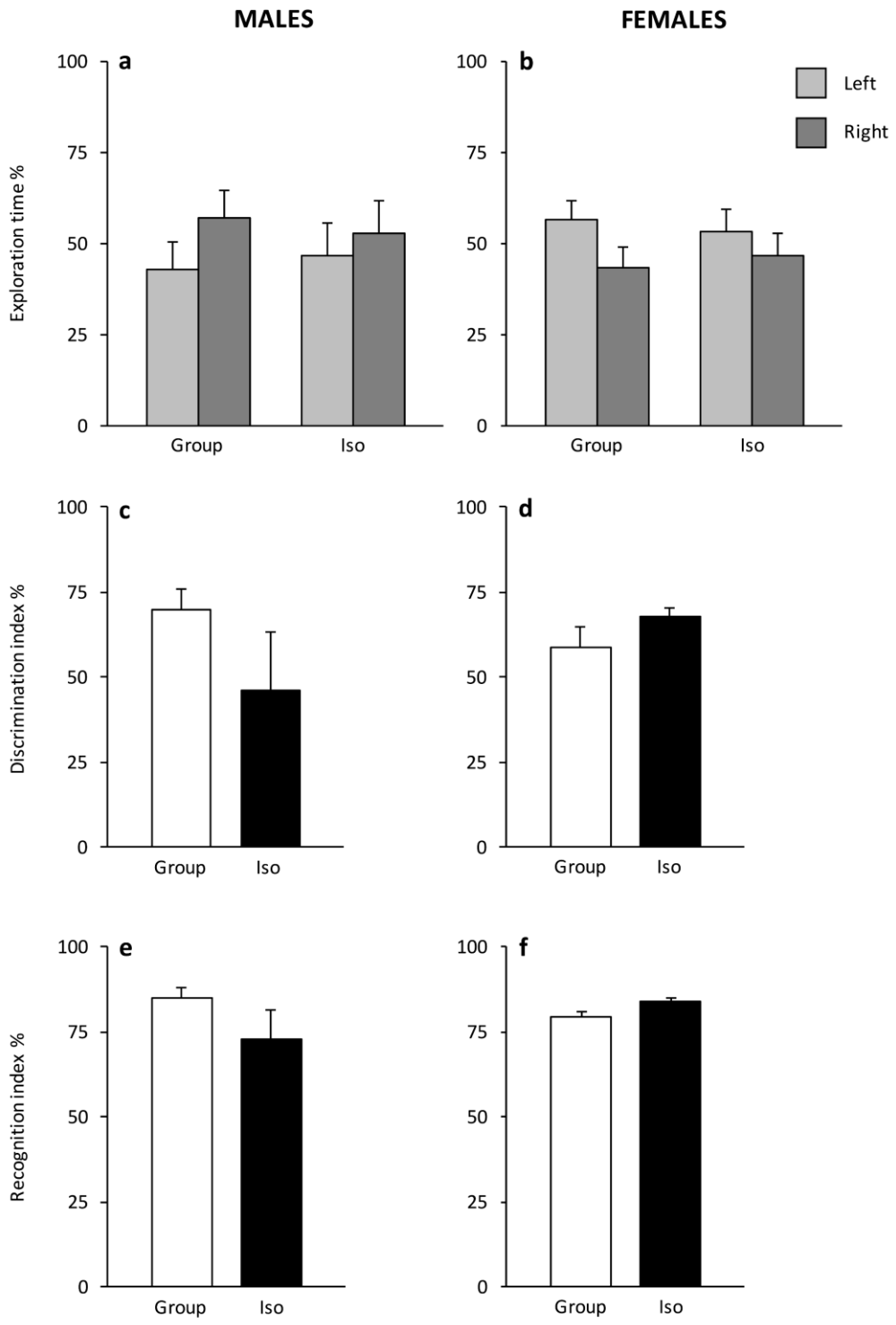

Fig. 4.3 *Effects of social isolation on recognition memory measured at adulthood with the novel object recognition test*

Percentage of exploration time of identical objects during the habituation phase (a, b); Percentage of discrimination index, calculated as indicated in Methods (c, d); Percentage of recognition index, calculated as indicated in par 4.1.3 (e, f)

Data are expressed as mean ± SEM of at least 12 animals per group.

4.2 Isolation rearing, neuronal plasticity and BDNF

Brain-Derived Neurothrophic Factor (BDNF) and other members of the neurotrophin family have emerged as important players in facilitating brain connectivity, neuronal plasticity and promoting adult neurogenesis. Due to its clear role in central processes, several studies have evaluated the long-lasting effects of early life stress on BDNF expression, showing a reduction of mRNA and protein levels as a consequence of the exposure to stressful situations early in life (Berry et al., 2015; Calabrese et al., 2015; Duman and Monteggia, 2006; Luoni et al., 2014; Roceri et al., 2004).

On these bases, we decided to investigate the expression of the neurothrophin BDNF in prefrontal cortex and dorsal and ventral hippocampus of rats exposed to social isolation during adolescence.

4.2.1 Analysis of *Bdnf* mRNA levels in the prefrontal cortex of single-housed rats

We first analysed the mRNA levels of total *Bdnf* and its transcripts containing the long 3'-UTR that are usually targeted to dendrites and may contribute to synaptic functions (An et al., 2008). Moreover, in order to further investigate the effects of isolation on *Bdnf* expression, we analysed the mRNA levels of two major brain splice transcripts of the neurothrophin, namely exon IV and VI. The presence of multiple variants defines a local spatial, temporal and stimulus-specific *Bdnf* transcripts production; exon IV-containing *Bdnf* transcripts are localized to the cell body and proximal dendrites, while exon VI-containing transcripts are found in distal dendrites (Baj et al., 2011). We performed the analyses in adult male and female rats exposed to isolation rearing during adolescence focusing on the prefrontal cortex, a brain region crucial in psychopathology and memory.

As shown in fig. 4.4, total *Bdnf* mRNA levels were not altered by social isolation within the prefrontal cortex of adult male rats, while female isolated animals showed a significant reduction in total *Bdnf* mRNA levels compared to group-housed counterpart ($p<0.05$) (Fig. 4.4a). In line with these results, social isolation did not cause any alterations in the pool of *Bdnf* transcripts with the long 3'-UTR in adult male animals within the prefrontal cortex while in females there is a tendency to reduction as a consequence of the isolation ($p=0.057$) (Fig. 4.4b). When investigating exon IV and VI-containing transcripts, we found that isolation

rearing produced a significant reduction only of exon IV-containing variants in the prefrontal cortex of adult female isolated rats (p<0.05), but not on exon VI (Fig. 4.4 c, d). Conversely, socially-isolated male animals did not show any changes in the expression of *Bdnf* exon IV and VI-containing transcripts (Fig. 4.4 c, d).

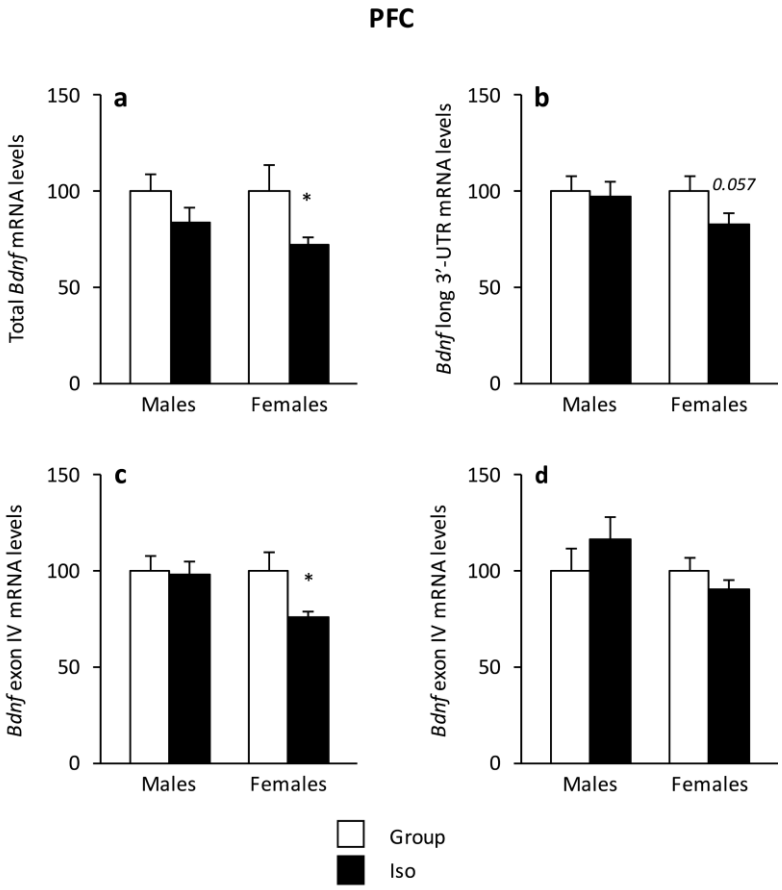

Fig. 4.4 *Effects of social isolation on Bdnf mRNA expression in the prefrontal cortex of adult male and female rats*

The mRNA levels of total (a), long 3'UTR (b), exon IV (c) and exon VI (d) *Bdnf* were analysed in adult male and female rats exposed to social isolation during adolescence (Iso), as compared to group-housed animals (Group). The data, expressed as a percentage of Group (set al 100%), are the mean ± SEM of at least 11 animals per group.

* $p<0.05$ vs Group of the same sex (Student's t test)

4.2.2 Analysis of *Bdnf* mRNA levels in the hippocampus of single-housed rats

Prolonged and traumatic stressors have been shown to influence the hippocampus at various levels, impairing neuronal morphology, suppressing neuronal proliferation and reducing hippocampal volume (Kim et al., 2015). Stress-associated long-term potentiation (LTP) deficits have been demonstrated in several regions of the hippocampus (Pavlides et al., 2002).
We thus investigated mRNA levels of total *Bdnf* and its transcripts containing the long 3'-UTR in adult male and female rats exposed to isolation rearing during adolescence focusing on the dorsal and ventral hippocampus. As shown in fig. 4.5, total *Bdnf* mRNA levels were not altered by social isolation in the dorsal hippocampus of male stressed animals, with a tendency to reduction in isolated female rats (p=0.061). Within the ventral hippocampus, isolation rearing did not induce any changes in total *Bdnf* mRNA expression in adult isolated animals, of both genders. Focusing on the *Bdnf* transcripts with the long 3'-UTR, the stressful experience during adolescence produced a significant reduction in the dorsal hippocampus of female isolated animals, compared to group-housed females (p<0.05). Isolated male animals did not show any changes in *Bdnf* transcripts with the long 3'-UTR within the dorsal hippocampus. Lastly, *Bdnf* long 3'-UTR mRNA levels were not altered by social isolation in the ventral hippocampus of adult animals, for both genders.

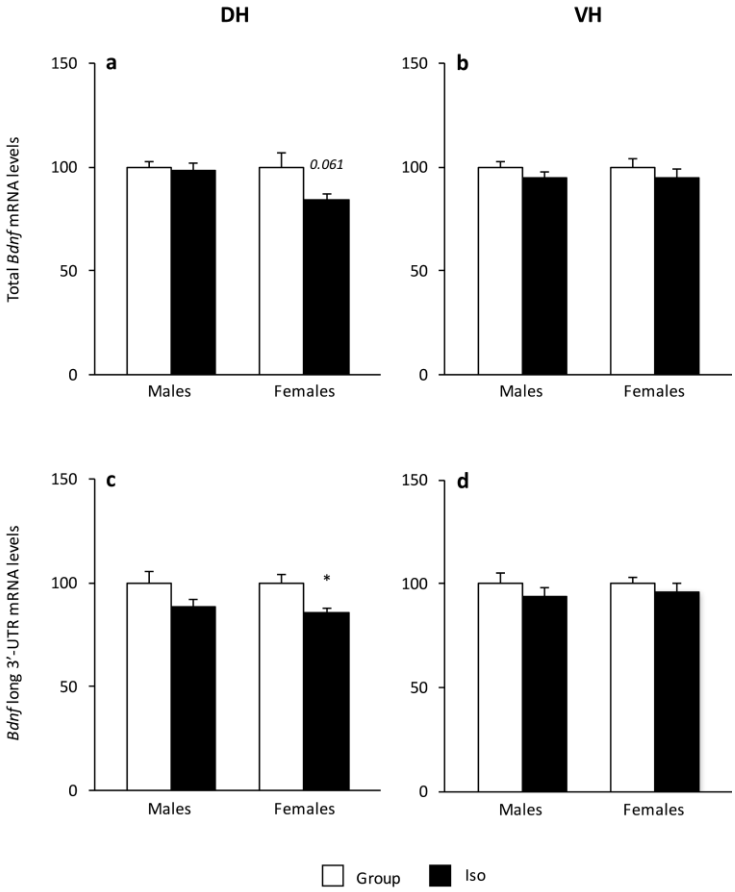

Fig. 4.5 *Effects of social isolation on Bdnf mRNA expression in the dorsal and ventral hippocampus of adult male and female rats*

The mRNA levels of total (a, b) and long 3'UTR (c, d) *Bdnf* were analysed in the dorsal (a, c) and ventral (b, d) hippocampus of adult male and female rats exposed to social isolation during adolescence (Iso), as compared to group-housed animals (Group). The data, expressed as a percentage of Group (set al 100%), are the mean ± SEM of at least 11 animals per group. * $p<0.05$ vs Group of the same sex (Student's t test)

4.3 Social isolation and stress-response at adulthood

Stress represents a major element of vulnerability to mental illness, possibly because of an inability to cope with the adverse experience. Along this line of reasoning, exposure to an acute stress may be used to investigate the functional responsiveness and the ability to activate immediate and adaptive response, which engages multiple neuronal and non-neuronal systems to cope with the challenging condition. A clear interplay between neurotransmitters and neurotrophic factors mediates adaptive responses to stressful situations, with the excitatory neurotransmitter glutamate and the neurotrophic factor BDNF being key regulators of synaptic plasticity throughout the central nervous system. Challenging situations in neurons result in the release of glutamate from the presynaptic compartment that, activating differential receptors on the membrane of dendrites, induces the influx of Na^+ and Ca^{2+}. Ca^{2+} in turn influences the production of numerous proteins involved in synaptic plasticity, including BDNF. BDNF exerts its effects, through binding with the high affinity receptor TrkB, by activating several downstream signalling cascades including the phosphatidylinositol-3 (PI3K) and mitogen-activated protein (MAPK) kinases (Reichardt, 2006). Moreover, exposure to an initial stressor may cause several changes that modify responses to subsequent stressors.

4.3.1 Analysis of *Bdnf* mRNA levels in response to acute stress in the prefrontal cortex of animals exposed to isolation rearing during adolescence

First, we investigated the mRNA levels of stress-responsive gene *Bdnf*, in adult male and female rats exposed to isolation rearing during adolescence and required to cope a challenging condition at adulthood, focusing on the prefrontal cortex and hippocampus. Indeed, *Bdnf* expression can undergo rapid changes following severe situations, as a marker of adaptive plasticity under challenging conditions (Fumagalli et al., 2012).

We thus measured the mRNA levels of total *Bdnf* and its transcripts containing the long 3'-UTR, as well as exon IV and exon VI-containing *Bdnf* transcripts, in the prefrontal cortex of isolated animals exposed to an acute restrain stress at adulthood. Two-way ANOVA revealed a significant effect of acute stress in the expression of total *Bdnf* in the prefrontal cortex of male rats (AS: $F_{1,55}$= 5.622, p<0.05). Indeed, total *Bdnf* mRNA levels were not altered by the

acute stress in group-housed male rats while isolated male rats showed a significant increase of total *Bdnf* in response to the acute manipulation (p<0.05). As for total *Bdnf* mRNA levels in the prefrontal cortex of females, we found significant effects of both housing condition and AS but not of their interaction (Housing: $F_{1,57}$= 4.471, p<0.05; AS: $F_{1,57}$= 10.284, p<0.01). Similar to male animals, total *Bdnf* mRNA levels within the prefrontal cortex of female group-housed rats didn't change after the challenging condition while isolated female rats showed a significant increase of total *Bdnf* in response to the acute manipulation (p<0.01).

Two-way ANOVA revealed no significant effects of housing condition and acute stress or their interaction in the expression of *Bdnf* long 3'-UTR in the prefrontal cortex of male rats. Indeed, *Bdnf* long 3'-UTR mRNA levels were not altered in response to the restrain stress, in both group-housed and isolated male rats. Conversely, we observed a significant effect of acute stress in the expression of *Bdnf* long 3'-UTR in the prefrontal cortex of female rats (AS: $F_{1,48}$= 8.074, p<0.01). In particular, we found that the exposure to an acute manipulation at adulthood did not alter *Bdnf* long 3'-UTR mRNA levels within the prefrontal cortex of group-housed female rats. Whilst, we detected a significant up-regulation of *Bdnf* long 3'-UTR mRNA levels within the prefrontal cortex of isolated female rats in response to the challenging condition (p<0.01).

As for exon IV-containing *Bdnf* transcripts, the statistical analysis revealed a significant effect of acute stress in the prefrontal cortex of male rats (AS: $F_{1,52}$= 26.298, p<0.001). Notably, we found an up-regulation of exon IV-containing *Bdnf* mRNA levels in response to stress in the prefrontal cortex of both group-housed (p<0.01) and isolated male animals (p<0.001). Two-way ANOVA detected a significant effect of acute stress and a tendency for housing condition in the expression of exon IV-containing *Bdnf* transcripts in the prefrontal cortex of female animals (Housing: $F_{1,52}$= 3.951, p=0.053; AS: $F_{1,52}$= 47.531, p<0.001). Indeed, exon IV-containing *Bdnf* mRNA levels increased after the restrain stress manipulation in the prefrontal cortex of both group-housed (p<0.001) and isolated (p<0.001) female rats.

Regarding exon VI-containing *Bdnf* transcripts we did not observe any effects of acute stress and housing condition or their interaction in the prefrontal cortex of male rats. Exon VI-containing *Bdnf* mRNA levels did not change in neither group-housed nor isolated male animals after acute stress. The statistical analysis showed only a significant effect of housing condition in the expression of exon VI-containing *Bdnf* transcripts within the prefrontal cortex of females (Housing: $F_{1,52}$= 6.739, p<0.05). Indeed, isolated females showed significantly lower

levels of exon VI-containing *Bdnf*, when exposed to acute manipulation, compared to group-housed female rats following the acute stress ($p<0.05$).

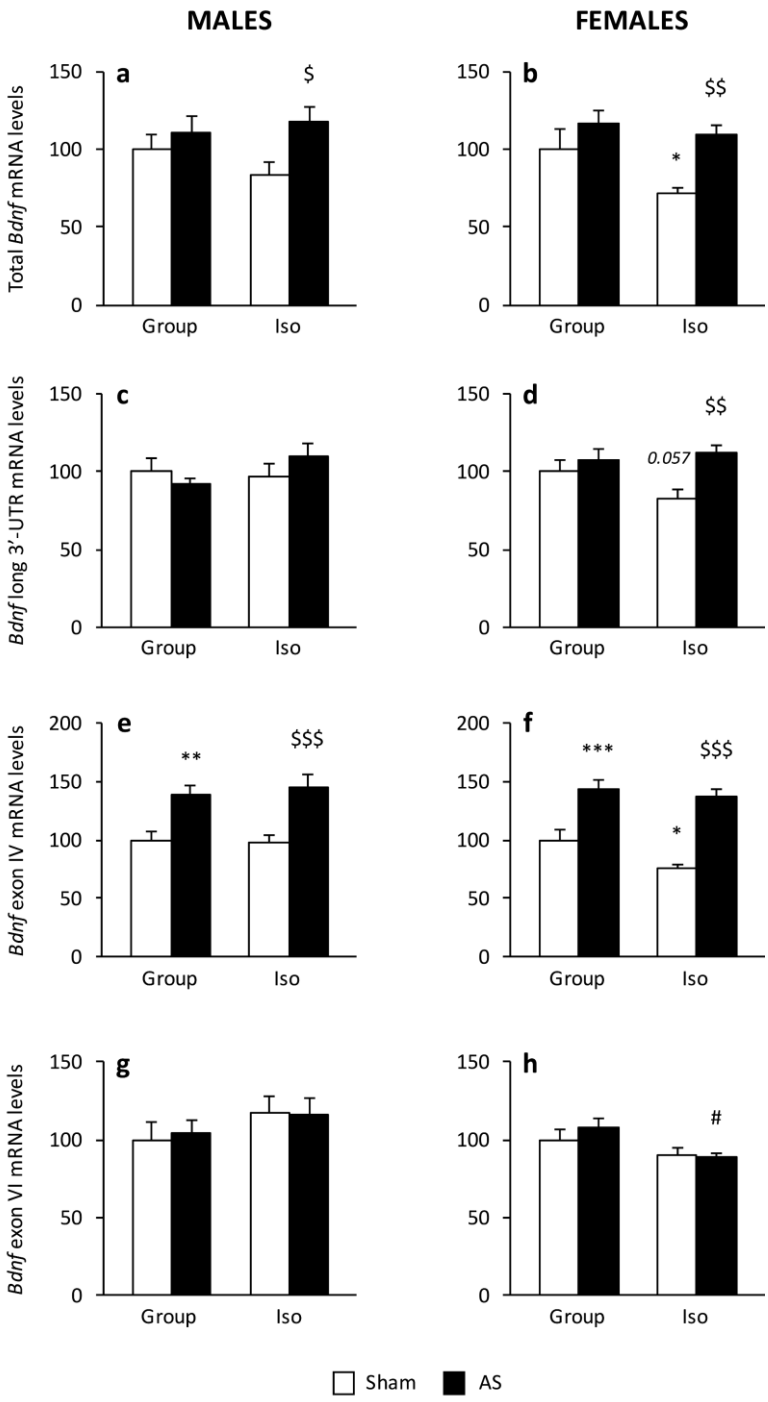

Fig. 4.6 *Analysis of Bdnf responsiveness to an acute stress during adult life in prefrontal cortex of animals exposed to isolation rearing during adolescence*

The mRNA levels of total (a, b), long 3'UTR (c, d), exon IV (e, f) and exon VI (g, h) *Bdnf* were analysed in the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male (a, c, e, g) and female (b, d, f, h) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 9 animals per group.

* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs Group/Sham; $^{\$}$ $p<0.05$, $^{\$\$}$ $p<0.01$ and $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham; $^{\#}$ $p<0.05$ vs. Group/AS (2-way ANOVA followed by post-hoc test)

4.3.2 Analysis of *Bdnf* mRNA levels in response to acute stress in the hippocampus of animals exposed to isolation rearing during adolescence

We thus measured the mRNA levels of total *Bdnf* and its transcripts containing the long 3'-UTR in the dorsal and ventral hippocampus of isolated animals exposed to an acute restrain stress at adulthood. Two-way ANOVA showed no effects of housing condition, acute stress and their interaction in the expression of total *Bdnf* in the dorsal hippocampus of male rats. Indeed, the acute manipulation did not cause any alterations in total *Bdnf* mRNA levels of both group-housed and isolated male animals. Statistical analysis revealed a significant effect of housing*acute stress interaction in the expression of total *Bdnf* in the dorsal hippocampus of female animals (Interaction: $F_{1,55}$= 6.857, p<0.05). In particular, we observed a down-regulation of total *Bdnf* mRNA levels only in the dorsal hippocampus of group-housed female rats in response to acute stress (p<0.05), an effect not found in isolated animals.

As for *Bdnf* long 3'-UTR mRNA levels, we observed only an effect of housing condition in the dorsal hippocampus of males (Housing: $F_{1,56}$= 6.860, p<0.05). Indeed, in response to the acute manipulation we did not observe any alterations in the *Bdnf* long 3'-UTR mRNA levels of both group-housed and isolated animals within the dorsal hippocampus. Two-way ANOVA revealed significant effects of housing condition and acute stress in the expression of *Bdnf* long 3'-UTR transcripts in the dorsal hippocampus of female animals (Housing: $F_{1,54}$= 4.676, p<0.05; AS: $F_{1,54}$= 6.538, p<0.05). Notably, we observed a significant down-regulation of *Bdnf* long 3'-UTR mRNA levels only in the dorsal hippocampus of group-housed female rats in response to acute stress (p<0.01), an effect not found in isolated animals.

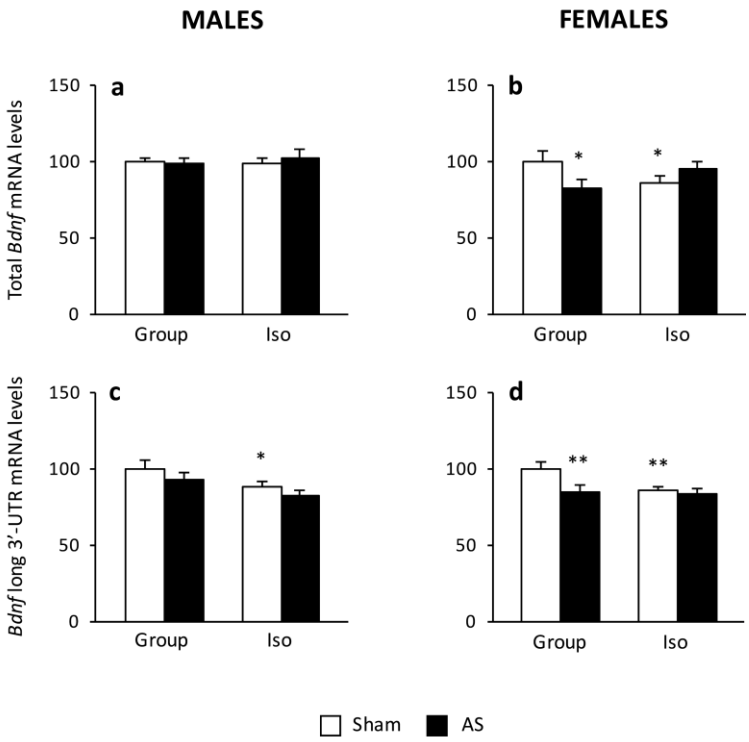

Fig. 4.7 *Analysis of Bdnf responsiveness to an acute stress during adult life in dorsal hippocampus of animals exposed to isolation rearing during adolescence*

The mRNA levels of total (a, b) and long 3'UTR (c, d) *Bdnf* were analysed in the dorsal hippocampus of group-housed (Group) or isolated (Iso) adult male (a, c) and female (b, d) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 10 animals per group.

* $p<0.05$ and ** $p<0.01$ vs Group/Sham (2-way ANOVA followed by post-hoc test)

Statistical analysis as for total *Bdnf* mRNA levels within the ventral hippocampus did not reveal any effects of housing condition or acute stress or their interaction in both group-housed and isolated animals, of both genders. Indeed, we did not found any significant change in the mRNA levels of total *Bdnf* in response to acute stress, in both the experimental groups. Moreover, we did not observe any effects of housing condition, acute stress and interaction in the expression of *Bdnf* long 3'-UTR transcripts in the ventral hippocampus of male rats. The exposure to an acute restrain stress did not modify *Bdnf* long 3'-UTR mRNA levels in the ventral hippocampus of isolated males as well as of their counterpart. Two-way ANOVA revealed an effect of housing condition and housing*AS interaction in the expression of *Bdnf* long 3'-UTR transcripts in the ventral hippocampus of female rats (Housing: $F_{1,52}$= 11.366, $p<0.01$; Interaction: $F_{1,52}$= 5.109, $p<0.05$). In particular, we detected a significant down-regulation of *Bdnf* long 3'-UTR mRNA levels in the ventral hippocampus of isolated female rats after the challenging condition as compared to group-housed counterpart subjected to the acute manipulation ($p<0.001$).

In summary, similarly to mRNA levels, we suggested that prefrontal cortex appears to be the brain region primarily affected by exposure to social isolation during adolescence. For this reason, we focused our attention on this region for the next analyses.

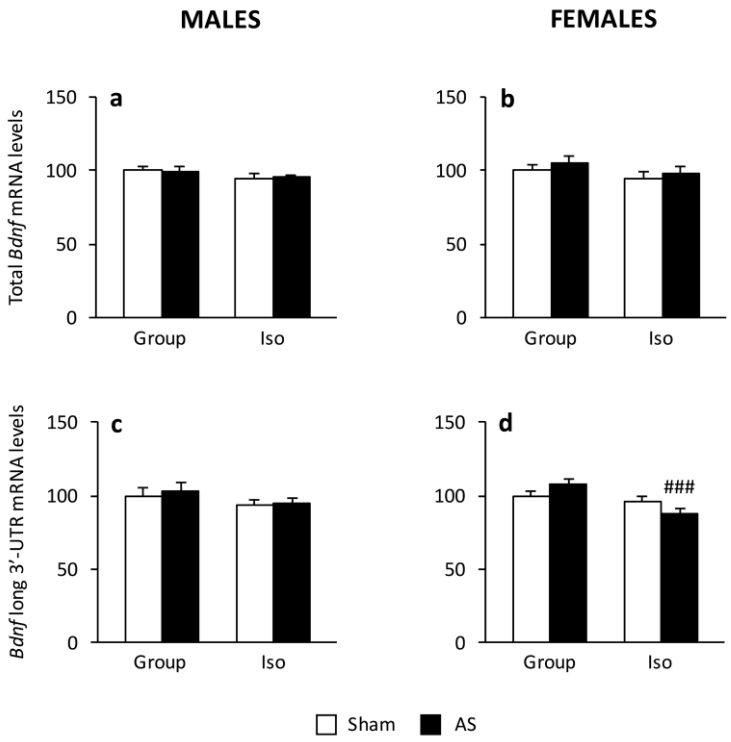

Fig. 4.8 *Analysis of Bdnf responsiveness to an acute stress during adult life in ventral hippocampus of animals exposed to isolation rearing during adolescence*

The mRNA levels of total (a, b) and long 3'UTR (c, d) *Bdnf* were analysed in the ventral hippocampus of group-housed (Group) or isolated (Iso) adult male (a, c) and female (b, d) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 10 animals per group.

$^{###}$ $p<0.001$ vs Group/AS (2-way ANOVA followed by post-hoc test)

4.4 Analysis of BDNF protein, TrkB levels and downstream signalling pathways

We next investigated whether the changes observed at transcriptional level were paralleled by modifications of BDNF protein levels in the prefrontal cortex of male rats. We focused on the levels of the precursor protein, proBDNF, and of the mature form of the neurotrophin, mBDNF, in the crude synaptosomal fraction, as changes in this cellular compartment may reflect changes in the pool of 'synaptic' BDNF.

As shown in fig. 4.9, we found a significant effect of housing condition in the expression levels of proBDNF within the crude membrane fraction of prefrontal cortex (Housing: $F_{1,19}$= 8.399, p<0.05). Indeed, post-hoc analysis revealed a significant up-regulation of proBDNF levels in single-housed animals compared to group-housed ones, under resting condition (p<0.05). On the contrary, exposure to acute stress (AS) did not alter protein levels in both group-housed and isolated animals (p>0.05). As for the mature form of BDNF, we observed a significant effect of housing*AS interaction in the mBDNF protein levels within the prefrontal cortex (Interaction: $F_{1,21}$= 4.879, p<0.05). In detail, isolated animals during adolescence showed significant increased mBDNF protein levels at adulthood, under resting condition, as compared to group-housed animals (p<0.05). When exposed to a challenging condition at adult life, single-housed animals displayed a tendency for down-regulation of mBDNF levels (p=0.056), while group-housed animals did not.

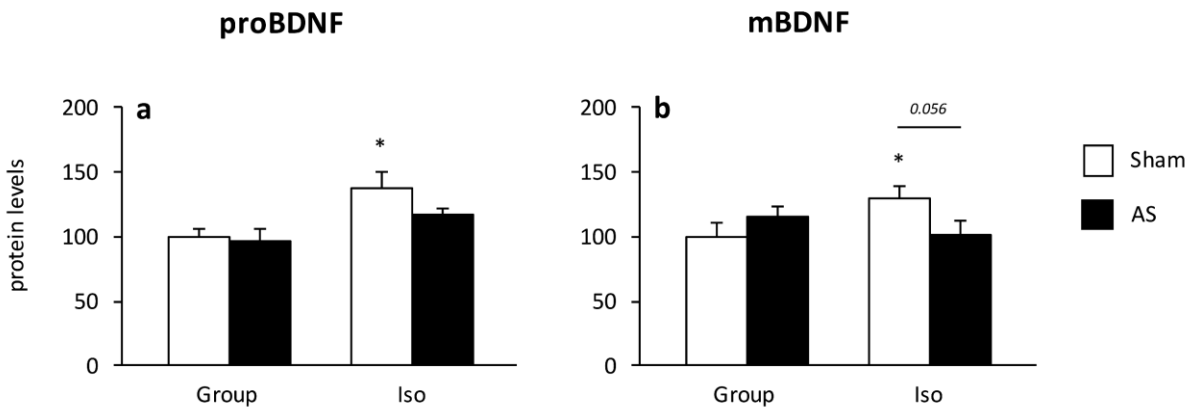

Fig. 4.9 *Modulation of BDNF protein levels following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood*

The protein levels of proBDNF (a) and mBDNF (b) were analysed in crude membrane fraction of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

* $p<0.05$ vs. Group/Sham (2-way ANOVA followed by post-hoc test)

Once secreted, BDNF can bind two receptors: the high-affinity TrkB receptor or the low-affinity p75 neurotrophin receptor (p75 NTR) (Chao, 2003). BDNF binding to TrkB is followed by receptor dimerization and auto-phosphorylation leading to the activation of three signalling pathways: the phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase C-γ (PLCγ) pathways. All these pathways lead to the phosphorylation and activation of the transcription factor cAMP-response element binding protein (CREB) which induces the transcription of genes essential for cell survival, growth and differentiation and synaptic plasticity (Begni et al., 2017).

We then measured the protein levels for TrkB as well as its phosphorylation in the crude membrane fraction of prefrontal cortex of isolated male animals, under resting condition and following an acute stress at adulthood. As shown in fig. 4.10, as for the auto-phosphorylation site Tyr816 (pTrkB Y816) and full-length TrkB (TrkB) protein levels, neither housing condition nor acute stress or their interaction produced significant effects. Indeed, under resting condition or after the acute stress, both group-housed and isolated animals did not show any significant changes in protein levels. However, when focusing on the ratio of pTrkB/TrkB protein levels, we observed a significant effect of housing*AS interaction (Interaction: $F_{1,21}$= 4.941, p<0.05). Indeed, although social isolation produced only a marginal, non significant reduction in the ratio of pTrkB/TrkB under resting condition, when exposed to acute stress at adult life, isolated animals showed increased ratio of pTrkB/TrkB protein levels as compared to group-housed animals, even though this effect did not reach the statistically significance (p=0.062).

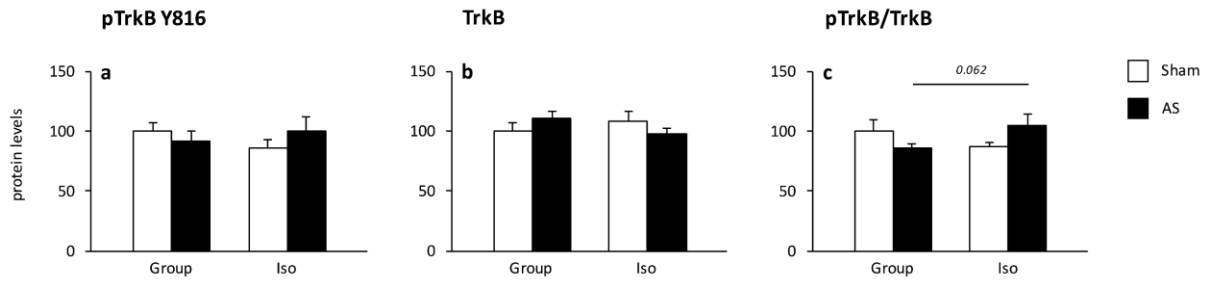

Fig. 4.10 *Modulation of TrkB phosphorylation and protein levels following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood*
The protein levels of phospho-TrkB in Tyr816 (a), full length Trkb (b) and pTrkB/TrkB ratio (c) were analysed in crude membrane fraction of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

We next investigated specific key players of BDNF-TrkB downstream signalling pathways, implicated in several forms of synaptic plasticity. MAPK- and PI3K-related cascades play crucial roles in translation and trafficking of proteins induced by synaptic activity while PLCγ-related signalling regulates intracellular Ca^{2+} that can drive transcription via cyclic adenosine monophosphate (cAMP) and a Protein Kinase C (PKC). Phosphorylated TrkB recruits and activates PLCγ which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate, to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates PKC and IP3 releases Ca^{2+} from intracellular stores, leading to activation and phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII). On the other hand, TrkB recruits Shc that interacts with an adaptor protein Grb2, leading to the activation of Ras. In turn, Ras activates downstream MEK and MAPK/Erk kinases. Recruitment of Shc also influences the stimulation of PI3K pathway, leading to Akt translocation and activation. In turn, activated Akt promotes cell survival, growth and proliferation by phosphorylating a range of intracellular proteins including glycogen synthase kinase 3 (GSK-3).

Fig. 4.11 shows phosphorylation and protein levels of PLCγ-induced signalling players, as PLCγ itself and CaMKII, in the whole homogenate of prefrontal cortex of single-housed animals during adolescence, under resting condition and after an acute stress at adulthood. Two-way ANOVA showed a significant effect of housing*AS interaction in the phosphorylation levels of PLCγ at Tyr783 (Interaction: $F_{1,23}$= 10.118, p<0.01). Notably, under resting condition, social isolation caused a significant reduction of PLCγ phosphorylation levels in males compared to group-housed animals (p<0.05). Moreover, when exposed to acute stress, isolated rats showed a significant increase of phosphorylation levels at Tyr783 as compared to animals under resting condition (p<0.05), an effect that was not present in group-housed animals (p>0.05). As for PLCγ protein levels, no effect of housing condition, acute stress or their interaction was detected by statistical analysis. Indeed, both under resting condition and after acute stress, all animals did not show any changes induced by social isolation or stress exposure. The statistical analysis on the ratio of pPLCγ/PLCγ protein levels revealed a significant effect of housing*AS interaction, as already seen for PLCγ phosphorylation levels (Interaction: $F_{1,21}$= 9.806, p<0.01). Under resting condition, social isolation caused a significant down-regulation of protein levels in isolated animals, as compared to group-housed ones (p<0.01). Following a challenging condition in adult life, group-housed and isolated animals

showed opposite responses. In particular, group-housed animals showed a trend toward a reduction in ratio protein levels after the acute manipulation, as compared to group-housed animals under resting condition (p=0.066). On the contrary, exposure to acute stress determined a significant increase of pPLCγ/PLCγ ratio protein levels of isolated animals, compared to single-housed rats under resting condition (p<0.05).

Regarding CaMKII phosphorylation and protein levels, statistical analyses showed no effects of housing, acute stress and their interaction in the expression levels of phosphorylated CaMKII in Thr286 and total protein in the prefrontal cortex of isolated male rats after an acute manipulation later in life. As shown in fig. 4.11 (d, e, f), both phosphorylated, total protein and ratio levels were not altered in both group-housed and isolated animals, under resting condition and after the acute stress.

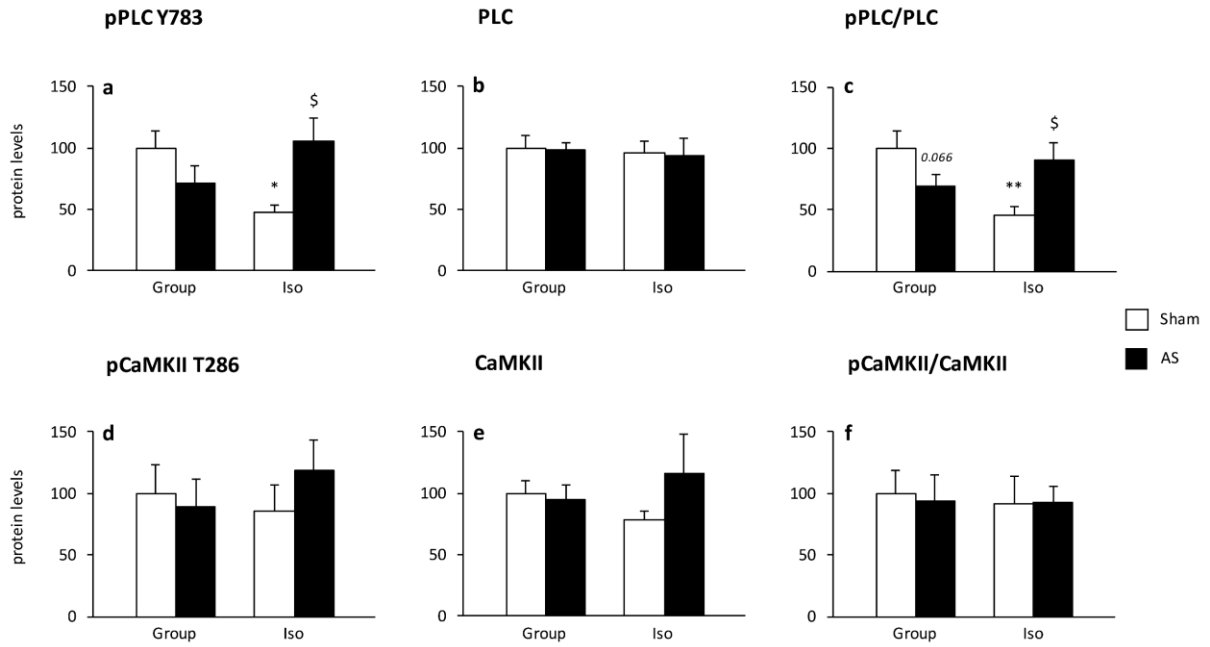

Fig. 4.11 *Modulation of PLCγ and CaMKII phosphorylation and protein levels following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood*

The protein levels of phospho-PLC in Tyr783 (a), PLC (b), pPLC/PLC ratio (c), phospho-CaMKII in Thr286 (d), CaMKII (e) and pCaMKII/CaMKII ratio (f) were analysed in whole homogenate of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

* $p<0.05$ and ** $p<0.01$ vs. Group/Sham; $^{\$}$ $p<0.05$ vs. Iso/Sham (2-way ANOVA followed by post-hoc test)

Next, as shown in Fig. 4.12, we investigated the phosphorylation and protein levels of Erk1 and Erk2, the key players of MAPK signalling cascade. The analysis was carried out in the whole homogenate from prefrontal cortex of single-housed male animals during adolescence, under resting condition and after an acute stress at adulthood.

Statistical analyses revealed no effects of housing condition, acute stress or their interaction in the expression of both phosphorylated and total Erk1 and Erk2 protein levels. Thus, both under resting condition and after acute stress, social isolated animals showed similar protein levels of group-housed animals.

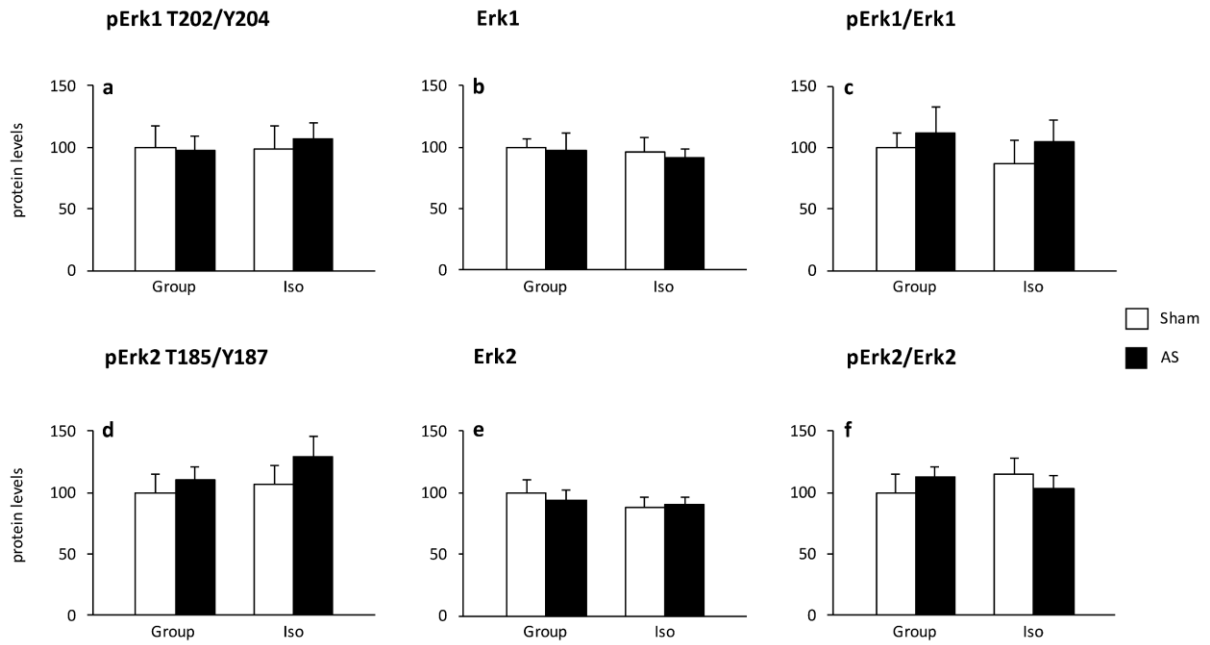

Fig. 4.12 *Modulation of Erk1 and Erk2 phosphorylation and protein levels following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood*

The protein levels of phospho-Erk1 in Thr202/Tyr204 (a), Erk1 (b), pErk1/Erk1 ratio (c), phospho-Erk2 in Thr185/Tyr187 (d), Erk2 (e) and pErk2/Erk2 ratio (f) were analysed in whole homogenate of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

Last, we investigated the phosphorylation and protein levels of Akt and GSK3B, involved in PI3K signalling cascade, in the whole homogenate of prefrontal cortex of single-housed male animals during adolescence, under resting condition and after an acute stress at adulthood. As summarized in Fig. 4.13, we did not find any significant effects of housing condition, acute stress or their interaction in the expression of both phosphorylated and total Akt and GSK3B protein levels. Thus, both under resting condition and after acute stress, social isolated animals showed similar protein levels of group-housed animals.

In summary, the data on BDNF-related signalling pathways suggested an impaired action of PLCγ and not of other downstream key players, as a consequence of exposure to isolation during adolescence.

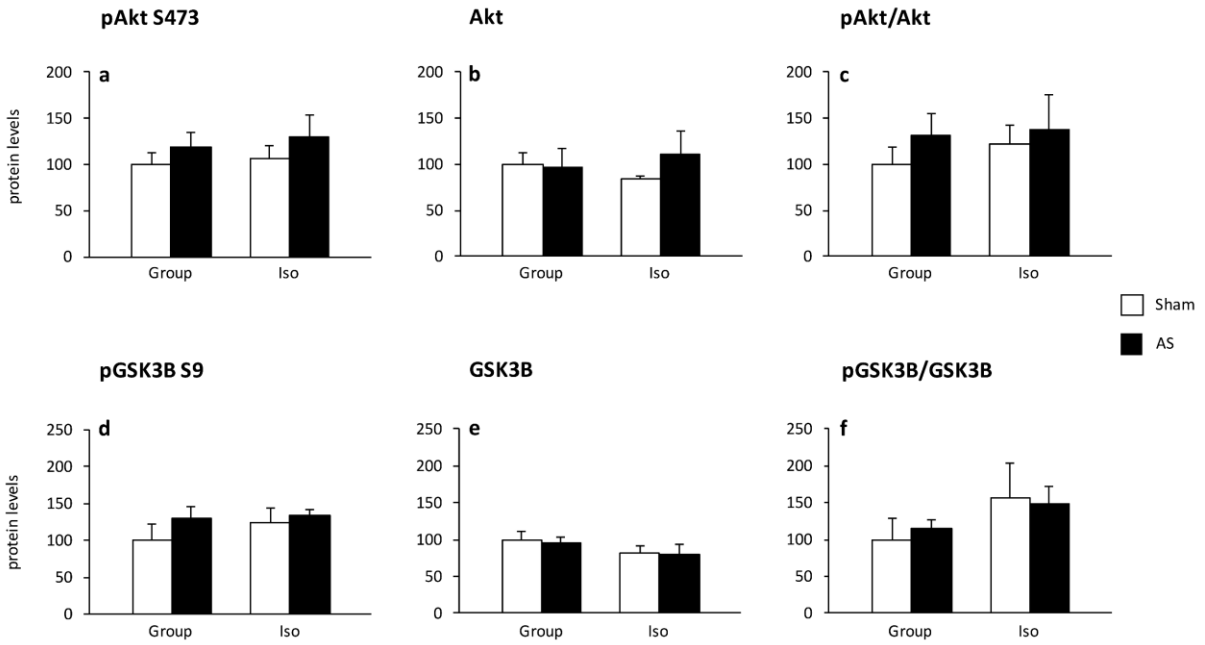

Fig. 4.13 *Modulation of Akt and GSK3B phosphorylation and protein levels following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood.*

The protein levels of phospho-Akt in Ser473 (a), Akt (b), pAkt/Akt ratio (c), phospho-GSK3B in Ser9 (d), GSK3B (e) and pGSK3B/GSK3B ratio (f) were analysed in whole homogenate of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

4.5 Analysis of selected glutamatergic receptor subunits in the prefrontal cortex of animals exposed to isolation rearing during adolescence

As mentioned above, exposure to acute stress is associated with an activation of glutamatergic system and its signalling pathways. We thus analysed selected subunits of the glutamatergic receptors AMPA and NMDA in the crude membrane fraction of prefrontal cortex of single-housed male animals during adolescence, under resting condition and after an acute stress at adulthood.

Fig. 4.14 shows the levels of total and phosphorylated GluN2B NMDA receptor subunit, total GluN2A NMDA receptor subunit, total and phosphorylated GluA1 AMPA receptor subunit and total GluA2 AMPA receptor subunit. As for total protein levels, statistical analyses revealed no effects of housing condition, acute stress and their interaction. Social isolation did not cause any changes in non-phosphorylated forms of selected glutamatergic receptor subunits, neither under resting condition nor following an acute manipulation at adulthood. The same results were obtained with respect to phosphorylated GluN2B and GluA1 subunits, suggesting that social isolation had no long-term effect on glutamatergic receptors expression and that the acute manipulation later in life did not activate glutamatergic receptors, regardless of housing condition during adolescence.

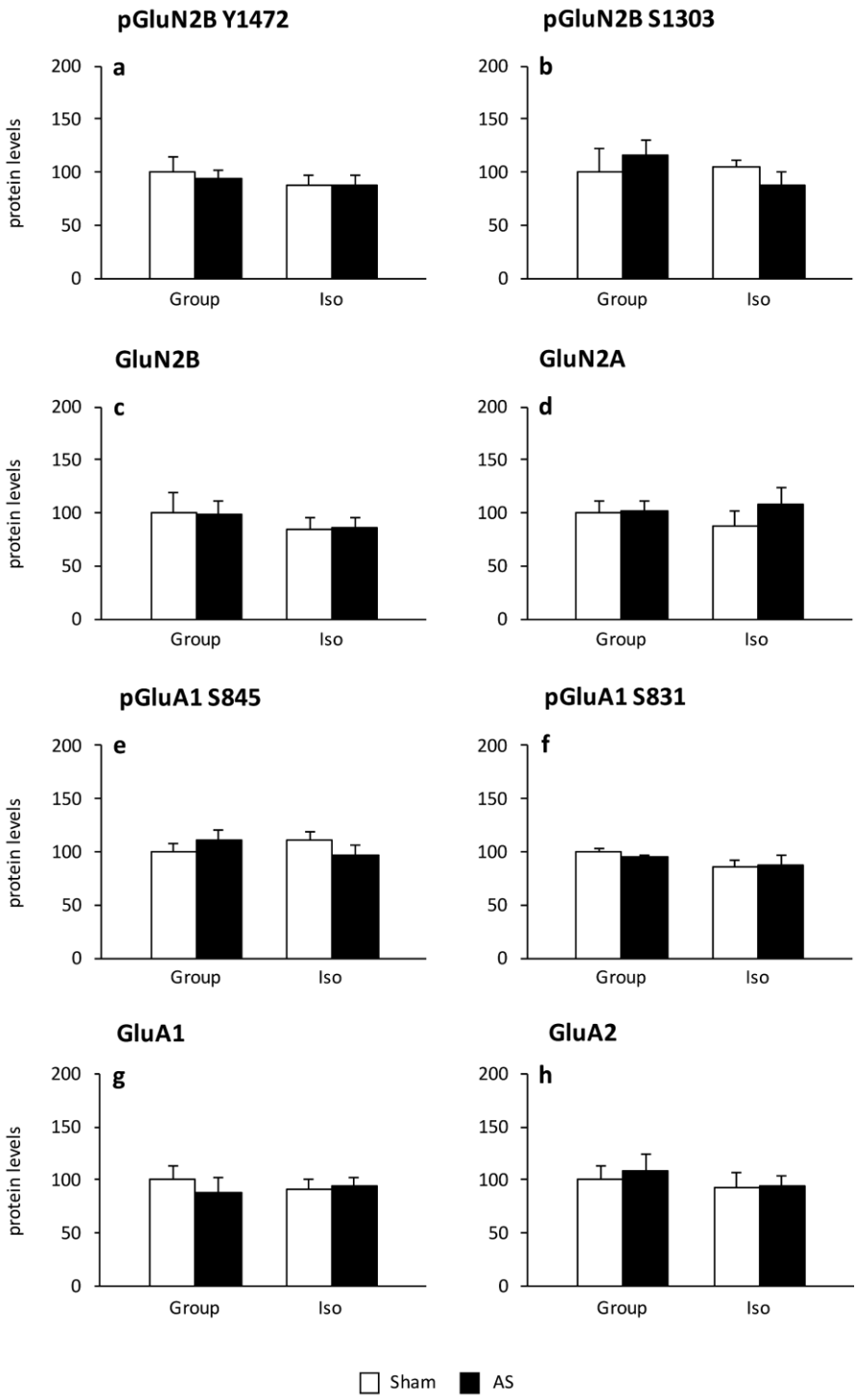

Fig. 4.14 *Modulation of phosphorylation and protein levels of selected glutamatergic receptor subunits following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood*

The protein levels of phospho-GluN2B in Tyr1472 (a) and Ser1303 (b), GluN2B (c), GluN2A (d), phospho-GluA1 in Ser845 (e) and Ser831 (f), GluA1 (g) and GluA2 (h) were analysed in the crude membrane fraction of the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham animals (set at 100%), are the mean ± SEM of at least 5 animals per group.

4.6 Effects of social isolation on the activation of immediate early genes in response to a challenging condition later in life

It is well known that stress-induced synaptic plasticity influences transcription of selective genes in neurons, including the immediate early genes *Gadd45ß*, *c-fos*, *Zif-268* and *Arc*, identified for rapid response to differential stimuli. These genes are necessary for long-term potentiation and establishment of certain forms of memory, affecting multiple cellular functions at different organizational levels (Loebrich and Nedivi, 2009).

4.6.1 Quantification of *Arc* and *Zif-268* mRNA levels in response to acute stress in the prefrontal cortex of animals exposed to isolation rearing during adolescence

In response to a challenging situation, neurons are able to undergo activity-dependent synaptic changes. This process of synaptic plasticity, as already said, consists of activation of selective genes that, inducing protein synthesis, can translate synaptic activity into persistent changes of synaptic strength (Flavell and Greenberg, 2008; Mastwal et al., 2016). Several studies found that *Arc* and *Zif-268* expression is induced in an activity-specific manner and examining *Arc* and *Zif-268* activation dynamics in brain regions may provide some evidences of activation of specific neural structures in response to different stimuli.

We measured the mRNA levels of *Arc* and *Zif-268* focusing on the prefrontal cortex of isolated animals exposed to an acute restrain stress at adulthood.

Two-way ANOVA revealed significant effects of both housing condition and acute stress in the expression of *Arc* in the prefrontal cortex of male rats (Housing: $F_{1,51}$= 6.126, p<0.05; AS: $F_{1,51}$= 49.449, p<0.001). Notably, isolated male rats under resting condition showed a significant up-regulation of *Arc* mRNA levels compared to group-housed counterpart (p<0.05). When subjected to acute manipulation both group-housed (p<0.001) and isolated (p<0.001) male animals presented a significant increase of *Arc* expression within the prefrontal cortex. As for female animals, we observed a significant effect of acute stress (AS: $F_{1,57}$= 65.062, p<0.001) in the expression of *Arc* within the prefrontal cortex. In particular, we found a significant up-regulation of *Arc* mRNA levels in the prefrontal cortex of both experimental groups in response to acute restrain stress, compared to their counterparts (p<0.001).

Similarly, statistical analysis showed a significant effect of acute stress in the expression of *Zif-268* in the prefrontal cortex of both genders (AS (Males): $F_{1,55}$= 19.617, p<0.001; AS (Females): $F_{1,55}$= 69.585, p<0.001). In detail, we observed no changes under resting condition for both males and females and a significant up-regulation of *Zif-268* mRNA levels following the acute stress in both group-housed and isolated animals, of both genders, as compared to their counterparts (p<0.001 vs group-housed males and females; p<0.05 vs isolated males; p<0.001 vs isolated females).

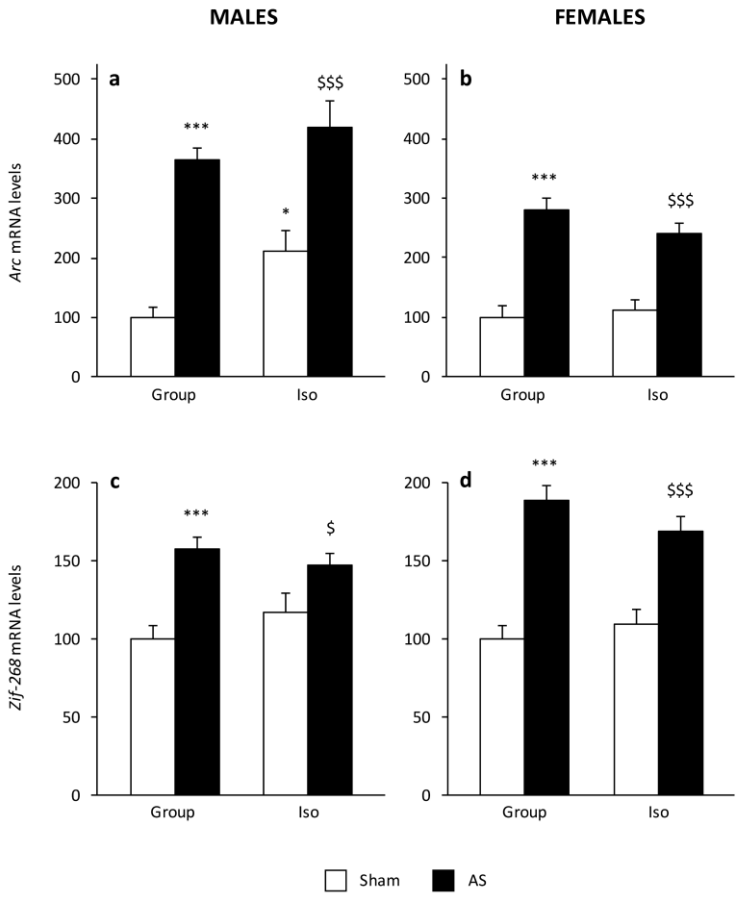

Fig. 4.15 *Analysis of immediate early genes activation following an acute stress during adult life in prefrontal cortex of animals exposed to isolation rearing during adolescence*
The mRNA levels of *Arc* (a, b) and *Zif-268* (c, d) were analysed in the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male (a, c) and female (b, d) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 11 animals per group.
* $p<0.05$ and *** $p<0.001$ vs Group/Sham, $^{\$}$ $p<0.05$ and $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham (2-way ANOVA followed by post-hoc test)

4.6.2 Quantification of *Gadd45ß* mRNA levels in response to acute stress in the prefrontal cortex and hippocampus of animals exposed to isolation rearing during adolescence

Gadd45ß (growth arrest and DNA-damage-inducible protein 45 beta) is an immediate early gene in mature hippocampal neurons whose expression has been found induced by neuronal activity, such as electroconvulsive treatment (Ma et al., 2009). Furthermore *Gadd45ß* promotes rapid DNA demethylation at specific regions of BDNF (Wu and Sun, 2009). We measured the mRNA levels of *Gadd45ß* in the prefrontal cortex and dorsal and ventral hippocampus of single-housed animals exposed to an acute restrain stress at adulthood.

Two-way ANOVA revealed a tendency for an effect of housing condition in the mRNA levels of *Gadd45ß* within the prefrontal cortex of males (Housing: $F_{1,50}$= 3.964, p=0.052) and a significant effect of acute stress in the expression of *Gadd45ß* in the prefrontal cortex of both genders following a challenging condition (AS (Males): $F_{1,50}$= 55.894, p<0.001; AS (Females): $F_{1,54}$= 68.846, p<0.001). Indeed, post-hoc analyses showed no changes under resting condition and a significant increase of mRNA levels after the acute stress in both group-housed and isolated male rats (p<0.001). As for females, no alteration was found under resting condition as a function of social isolation while we observed a significant up-regulation of *Gadd45ß* expression in response to the acute manipulation in both experimental groups (p<0.001).

Similarly, statistical analysis showed a significant effect of acute stress in the dorsal hippocampus of both isolated male and female animals exposed to an acute restrain stress at adulthood (AS (Males): $F_{1,50}$= 59.882, p<0.001; AS (Females): $F_{1,57}$= 30.167, p<0.001). Notably, under resting condition we did not observe any modifications induced by social isolation in both males and females, while both group-housed as well as isolated animals of both genders showed increased *Gadd45ß* mRNA levels after the acute stress, as compared to their counterparts (p<0.001 vs Males/Group/Sham; p<0.01 vs Females/Group/Sham; p<0.001 vs Iso/Sham).

Lastly, as for ventral hippocampus, we noted significant effects of acute stress and housing*AS interaction in male animals (AS: $F_{1,56}$= 40.131, p<0.001; Interaction: $F_{1,56}$= 5.009, p<0.05). In detail, we observed a significant up-regulation of *Gadd45ß* mRNA levels in the ventral hippocampus of isolated male rats compared to group-housed animals, under resting condition (p<0.05). Moreover, animals of both experimental groups showed increased *Gadd45ß* expression in response to acute stress compared to resting animals (p<0.001 vs

Group/Sham; $p<0.01$ vs Iso/Sham). Regarding female rats, statistical analysis showed a significant effect of acute stress (AS: $F_{1,51}= 69.618$, $p<0.001$). Social isolation did not cause any changes in the expression of *Gadd45ß* in the ventral hippocampus and the exposure to a restrain stress at adulthood determined a significant up-regulation of *Gadd45ß* mRNA levels in both experimental groups ($p<0.001$).

We can summarize that exposure to social isolation during adolescence did not produce major changes in the expression of the activity-dependent genes, such as *Gadd45ß*, *Zif-268* and *Arc*, and that their acute modulation in response to a challenging condition at adulthood later in life was not affected by the rearing condition during adolescence.

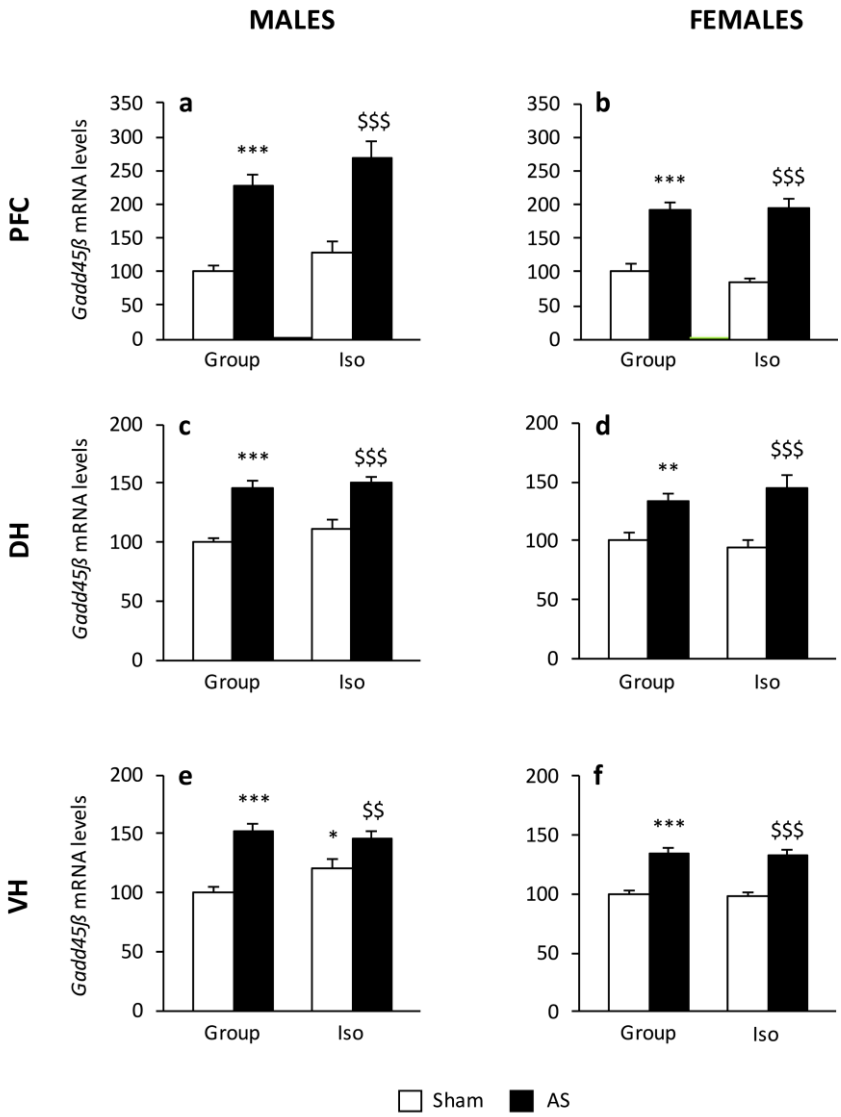

Fig. 4.16 *Analysis of Gadd45ß expression following social isolation during adolescence, under basal condition and after exposure to an acute stress at adulthood.*
The mRNA levels of *Gadd45ß* were analysed in the prefrontal cortex (a, b), dorsal (c, d) and ventral (e, f) hippocampus of group-housed (Group) or isolated (Iso) adult male (a, c, e) and female (b, d, f) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 10 animals per group.
* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs Group/Sham, $^{\$\$}$ $p<0.01$ and $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham (2-way ANOVA followed by post-hoc test)

4.7 The hypothalamic-pituitary-adrenal axis and stress-responsiveness

Stress is implicated in the emergence of several pathologies, including depression and schizophrenia. However, it is clear that exposure to a stressful situation often determines altered responses to subsequent stressors. As mentioned above, 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 HPA axis. Following exposure to stressors, the paraventricular nucleus of the hypothalamus releases the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which in turn determine the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH induces the release of glucocorticoids (corticosterone in rats, cortisol in humans) from the adrenal cortex into the general circulation. Classically, termination of the stress response involves a negative feedback, including fast non-genomic feedback at the level of the hypothalamus as well as negative control of limbic system structures as the hippocampus and the prefrontal cortex (de Kloet et al., 2005; De Kloet and Reul, 1987). Impaired control of the HPA activation or negative feedback may result in a hypo- or hypercortisolism, thus leading to pathological conditions.

4.7.1 Analysis of corticosterone following social isolation during adolescence under basal condition and after exposure to an acute stress at adulthood

We thus measured the plasma corticosterone levels of adult rats housed in isolation during adolescence and then exposed to a challenging condition in adult life. As shown in fig. 4.17, three-way ANOVA revealed significant effects of gender, acute stress and their interaction but not of housing condition (Gender: $F_{2,103}$= 135.294, p<0.001; AS: $F_{2,103}$= 370.036, p<0.001; Gender*AS: $F_{2,103}$= 14.479, p<0.001; Housing: $F_{2,103}$= 1.253, p>0.05). Notably, female animals, both in group and isolated, showed significant higher corticosterone levels that male animals, both under resting condition (p<0.001) and following acute stress (p<0.001). After the acute stress, we observed that male animals, both group housed and isolated as adolescents, had increased corticosterone concentration compared to males under resting condition (p<0.001). Similarly, also female rats showed up-regulated corticosterone levels following acute stress, regardless of housing condition (p<0.001). The analysis revealed that animals subjected to

isolation rearing during adolescence, of both genders, released the same amount of corticosterone into the general circulation in response to the stressful condition to which they have been exposed at adulthood, compared to control animals housed in group.

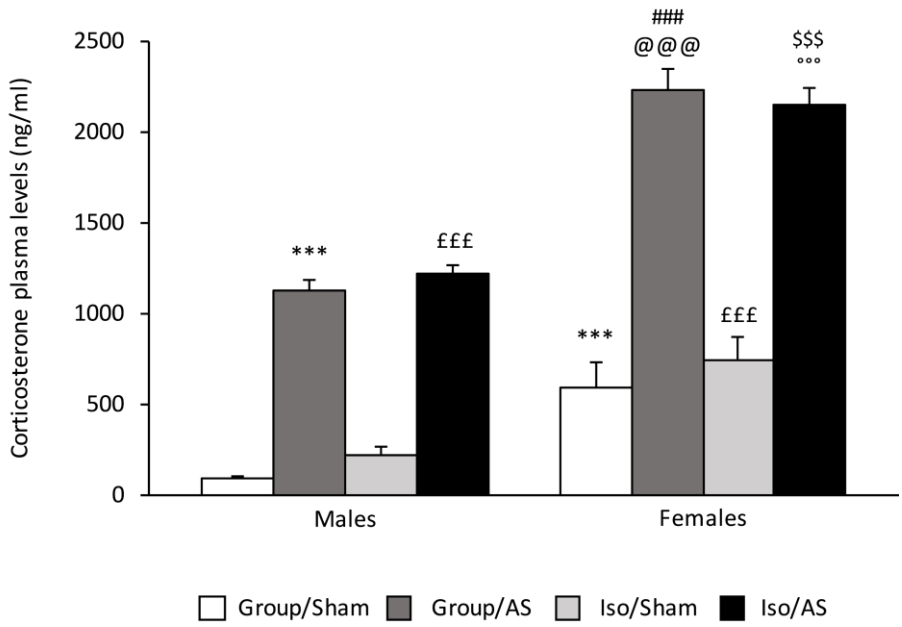

Fig. 4.17 *Circulating corticosterone levels following an acute stress during adult life in animals exposed to isolation rearing during adolescence*

The data, expressed in ng/ml, are the mean ± SEM of at least 11 animals per group. ***$p<0.001$ vs. Group/Sham/Males, $^{£££}p<0.001$ vs ISO/Sham/Males, $^{###}p<0.001$ vs Group/AS/Males, $^{@@@}p<0.001$ vs Group/Sham/Females, $^{°°°}p<0.001$ vs ISO/Sham/Females, $^{\$\$\$}p<0.001$ vs ISO/AS/Males (3-way ANOVA followed by post-hoc test)

4.7.2 Analysis of *Nr3c1*, *Fkbp5* and *Sgk1* mRNA levels in the prefrontal cortex of animals exposed to isolation rearing during adolescence and exposed to an acute stress at adulthood

Circulating corticosterone acts through two receptors: the mineralocorticoid and glucocorticoid receptors (MR and GR). The GR is a ligand-activated transcription factor that translocates from the cytosol to the nucleus after corticosterone binding. This activation and translocation process, and subsequent GR functions on gene transcription, are regulated by a large molecular complex (Binder, 2009). FKBP5 is a co-chaperone able to regulate glucocorticoid receptor sensitivity. Indeed, when FKBP5 is bound to the receptor complex, corticosterone binds with lower affinity and nuclear translocation of the receptor is less efficient. In turn, GR activation induces FKBP5 mRNA and protein expression. The serum- and glucocorticoid-regulated kinase 1 (SGK1) is a target gene of GR and itself directly enhances GR effects.

We thus measured mRNA levels of *Nr3c1* (the coding gene for GR), *Fkbp5* and *Sgk1* in the prefrontal cortex and hippocampus of animals exposed to isolation rearing during adolescence, in response to a challenging condition at adulthood.

As shown in fig. 4.18, we found significant effects of housing condition and acute stress in the expression of *Nr3c1* in the prefrontal cortex of isolated male animals in response to acute stress (Housing: $F_{1,49}$= 8.607, p<0.01; AS: $F_{1,49}$= 21.991, p<0.001). Indeed, under resting condition, we observed a tendency to increase in the mRNA levels of *Nr3c1* in the prefrontal cortex of isolated animals compared to group-housed ones (p=0.072). After the acute restrain procedure, group-housed as well as isolated male rats showed decreased *Nr3c1* mRNA levels, as compared to animals under resting condition (p<0.01). Moreover, isolated males showed significant higher *Nr3c1* expression in response to acute stress than group-housed counterpart (p<0.05). As for female animals, statistical analysis shows a trend for an effect of housing condition in the expression of *Nr3c1* in the prefrontal cortex of isolated female animals in response to acute stress (Housing: $F_{1,52}$= 3.865, p=0.055). In detail, under basal condition social isolation did not produce any significant alterations in the expression of *Nr3c1*, whilst isolated female rats show increased *Nr3c1* mRNA levels after the acute stress, compared to group-housed counterpart (p<0.05).

Regarding *Fkbp5* mRNA levels, we did not detect any effects of housing condition, acute stress or their interaction, for both genders. *Fkbp5* expression seemed not to be altered by either isolation rearing or exposure to acute stress in adult life, both in males and females.

We next examined the expression of one important target gene for GR activation, namely *Sgk1*. Two-way ANOVA revealed a significant effect of acute stress on the expression of *Sgk1* in the prefrontal cortex of single-housed male animals in response to acute stress (AS: $F_{1,55}$= 201.474, p<0.001). In particular, we did not observe any change of *Sgk1* expression as a consequence of isolation rearing, under resting condition (p>0.05). When exposed to the acute stress at adulthood, male rats, regardless of housing condition during adolescence, showed a significant up-regulation of *Sgk1* mRNA levels, as compared to animals under resting condition (p<0.001). As for female rats, we found significant effects of housing condition, acute stress and their interaction on *Sgk1* expression (Housing: $F_{1,53}$= 7.598, p<0.01; AS: $F_{1,53}$= 222.609, p<0.001; Interaction: $F_{1,53}$= 5.157, p<0.05). Indeed, while under resting condition social isolation did not cause any modifications of *Sgk1* expression (p>0.05), both group-housed and isolated female rats showed a significant up-regulation of *Sgk1* mRNA levels in response to acute stress (p<0.001), although such effect appear to be less pronounced in isolated female rats, as compared to group-housed female animals exposed to acute stress (p<0.01).

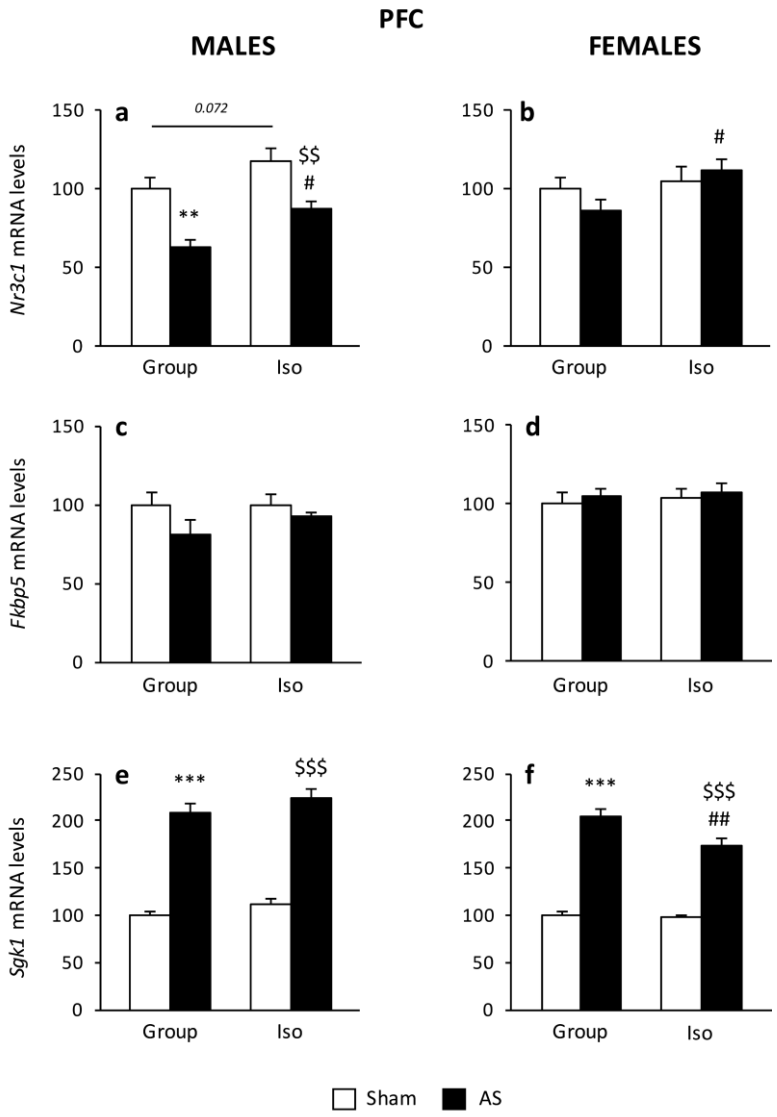

Fig. 4.18 *Analysis of the expression of glucocorticoid-related genes following social isolation during adolescence, under basal condition and after exposure to an acute stress at adulthood*
The mRNA levels of *Nr3c1* (a, b), *Fkbp5* (c, d) and *Sgk1* (e, f) were analysed in the prefrontal cortex of group-housed (Group) or isolated (Iso) adult male (a, c, e) and female (b, d, f) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 10 animals per group.
** $p<0.01$ and *** $p<0.001$ vs Group/Sham, $^{\$\$}$ $p<0.01$ and $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham; $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ vs Group/AS (2-way ANOVA followed by post-hoc test)

4.7.3 Analysis of *Nr3c1*, *Fkbp5* and *Sgk1* mRNA levels in the hippocampus of animals exposed to isolation rearing during adolescence and exposed to an acute stress at adulthood

As shown in fig. 4.19, we found a trend toward significance for the effect of housing*AS interaction in the expression of *Nr3c1* in the dorsal hippocampus of male animals in response to acute stress (Interaction: $F_{1,57}$= 3.146, p=0.082). Indeed, under resting condition, we found a significant increase of *Nr3c1* mRNA levels in males exposed to social isolation during adolescence, as compared to group-housed animals (p<0.05). Moreover, when exposed to acute stress at adulthood, group-housed animals did not show any changes in *Nr3c1* expression levels compared to rats under resting condition (p>0.05), whilst the exposure to acute manipulation at adulthood determined a significant down-regulation of *Nr3c1* mRNA levels in the dorsal hippocampus of single-housed animals, compared to ones under resting condition (p<0.05). Regarding female rats, neither social isolation nor acute stress did not cause any alterations of *Nr3c1* expression within the dorsal hippocampus.

As for *Fkbp5* mRNA levels, we found a statistically significant effect of acute stress in its expression within the dorsal hippocampus of male and female animals (AS (Males): $F_{1,58}$= 4.731, p<0.05; AS (Females): $F_{1,54}$= 6.526, p<0.05). In detail, *Fkbp5* mRNA levels were not affected by the rearing condition during adolescence in both genders (p>0.05). Post-hoc analysis revealed a tendency toward an increase in the expression of *Fkbp5* in response to acute stress, only for group-housed males (p=0.057). On the contrary, acute stress at adulthood produced a significant down-regulation of *Fkbp5* mRNA levels only in the dorsal hippocampus of group-housed female rats (p<0.01).

Two-way ANOVA showed significant effects of housing condition, acute stress and their interaction for the expression of *Sgk1* mRNA levels in the dorsal hippocampus of male and female animals (Housing (Males): $F_{1,55}$= 12.960, p<0.01; AS (Males): $F_{1,55}$= 418.911, p<0.001; Interaction (Males): $F_{1,55}$= 12.401, p<0.01; Housing (Females): $F_{1,52}$= 4.846, p<0.05; AS (Females): $F_{1,52}$= 242.243, p<0.001; Interaction (Females): $F_{1,52}$= 4.420, p<0.05). Under resting conditions, social isolation did not cause any modifications of *Sgk1* expression, for both genders (p>0.05) while, both group-housed and isolated male and female rats showed a significant up-regulation of *Sgk1* mRNA levels in response to acute stress (p<0.001), although such effect appear to be less pronounced in isolated rats, as compared to group-housed animals exposed to acute stress (p<0.001 vs Group/AS/Males; p<0.01 for Group/AS/Females).

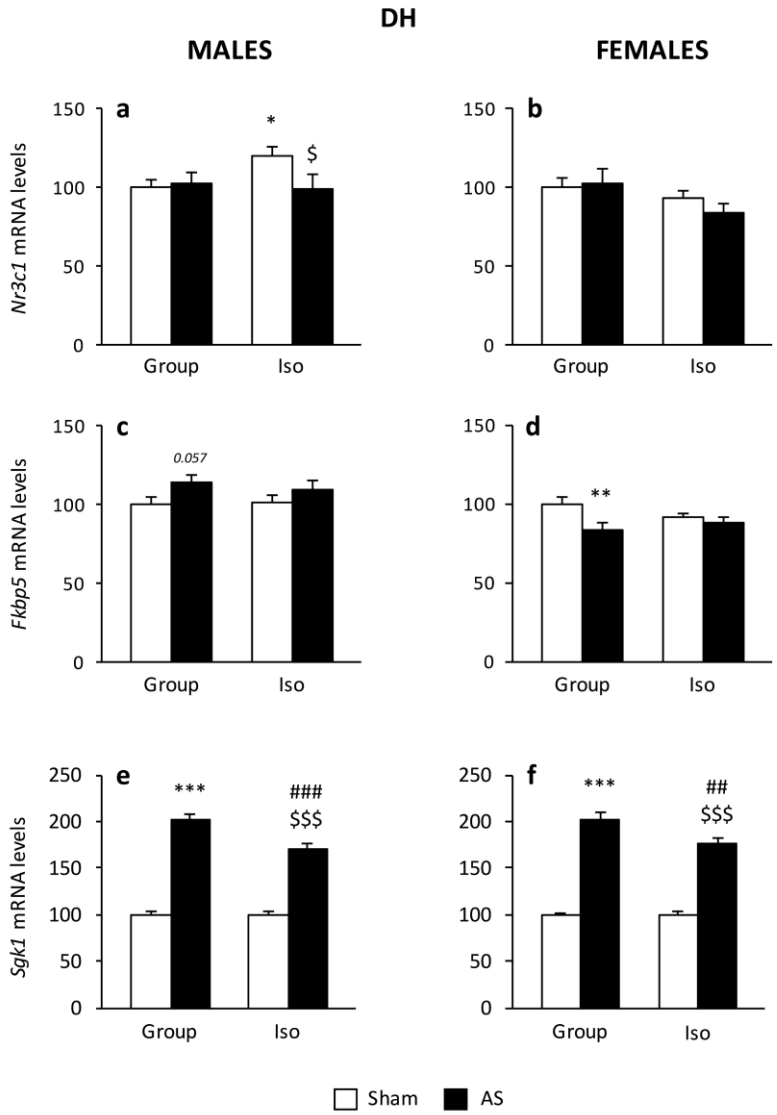

Fig. 4.19 *Analysis of the expression of glucocorticoid-related genes following social isolation during adolescence, under basal condition and after exposure to an acute stress at adulthood*
The mRNA levels of *Nr3c1* (a, b), *Fkbp5* (c, d) and *Sgk1* (e, f) were analysed in the dorsal hippocampus of group-housed (Group) or isolated (Iso) adult male (a, c, e) and female (b, d, f) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 11 animals per group.
* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs Group/Sham, $^{\$}$ $p<0.05$ and $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham; $^{\#\#}$ $p<0.01$ and $^{\#\#\#}$ $p<0.001$ vs Group/AS (2-way ANOVA followed by post-hoc test)

As shown in fig. 4.20, statistical analysis revealed no effect of housing condition, acute stress or their interaction in the expression of *Nr3c1* in the ventral hippocampus of male animals in response to acute stress. Neither under resting condition nor following acute stress, male animals did not show any alterations induced by social isolation and acute stress. As for females, we observed a tendency for the effect of housing condition in the mRNA levels of *Nr3c1* in the ventral hippocampus of female animals (Housing: $F_{1,57}$= 3.260, p=0.077). Indeed, under resting condition exposure to social isolation determined a significant down-regulation of *Nr3c1* mRNA levels compared to group-housed animals (p<0.05). The restrain stress at adulthood did not cause any modifications in both group-housed and isolated female animals (p>0.05).

Contrary to dorsal hippocampus, two-way ANOVA showed no effect of housing condition, acute stress or their interaction in the expression of *Fkbp5* in the ventral hippocampus of male animals. Indeed, neither under resting condition nor following acute stress, male animals did not show any alterations induced by social isolation and acute stress. As for females, we noted a significant effect of housing condition in the mRNA levels of *Fkbp5* within the ventral hippocampus of female animals (Housing: $F_{1,57}$= 5.9.05, p<0.05). In particular, under resting condition social isolation did not determine any alterations of *Fkbp5* mRNA levels in the ventral hippocampus of isolated female rats compared to group-housed animals. However, following acute stress, isolated female rats had significant lower *Fkbp5* mRNA levels than group-housed counterpart (p<0.01). Indeed, after restraint at adulthood, group-housed females showed a tendency for increase compared to animals under resting condition (p=0.05), effect not detected in the ventral hippocampus of isolated animals.

As for *Sgk1* mRNA levels, statistical analysis showed a significant effect of acute stress within the ventral hippocampus of male and female rats (AS (Males): $F_{1,55}$= 213.295, p<0.001; AS (Females): $F_{1,54}$= 236.157, p<0.001). Under resting condition, isolation rearing during adolescence was not able to induce any long-term alterations in *Sgk1* mRNA levels and when exposed to acute stress at adulthood, both group-housed and isolated animals, of both genders, responded to the acute manipulation increasing significantly *Sgk1* mRNA levels, as compared to animals under resting condition (p<0.001).

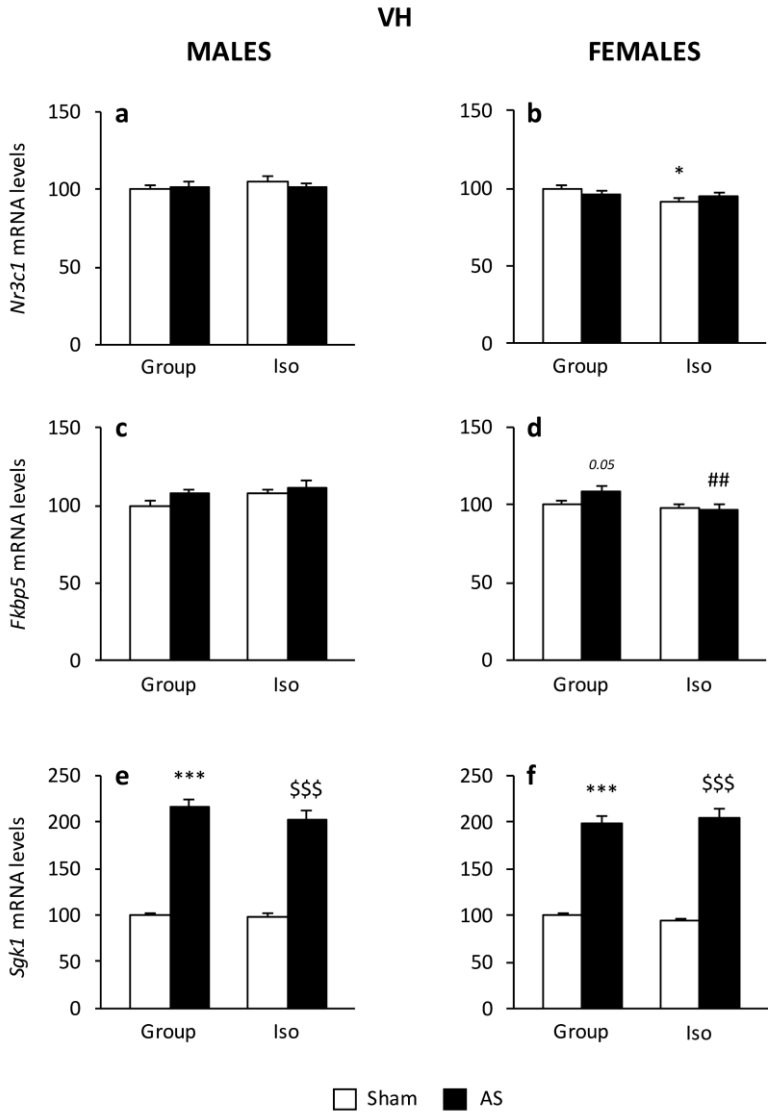

Fig. 4.20 *Analysis of the expression of glucocorticoid-related genes following social isolation during adolescence, under basal condition and after exposure to an acute stress at adulthood*
The mRNA levels of *Nr3c1* (a, b), *Fkbp5* (c, d) and *Sgk1* (e, f) were analysed in the ventral hippocampus of group-housed (Group) or isolated (Iso) adult male (a, c, e) and female (b, d, f) rats under resting condition (Sham) or following exposure to an acute restrain stress (AS) at adulthood. The data, expressed as a percentage of Group/Sham (set at 100%), are the mean ± SEM of at least 13 animals per group.
* $p<0.05$ and *** $p<0.001$ vs Group/Sham, $^{\$\$\$}$ $p<0.001$ vs. Iso/Sham; $^{\#\#}$ $p<0.01$ vs Group/AS (2-way ANOVA followed by post-hoc test)

5. DISCUSSION

During sensitive periods of neural development, the exposure to stressful life events can alter the course of brain development leading to long-lasting functional outcomes. Several studies have shown that adverse environmental situations during these periods may induce re-organizational effects on different systems and increase the vulnerability to develop psychiatric disorders later in life (Burke et al., 2005; Fumagalli et al., 2004; Nishi et al., 2014). With this respect, animal models represent a useful tool to investigate the effects of stress and its long-term consequences. Most research has focused on the reprogramming effects of stress on the developing and perinatal brain taking advantage of well-established prenatal and perinatal experimental paradigms (Fumagalli et al., 2004; Luoni et al., 2014; Roceri et al., 2004; Roceri et al., 2002; van der Doelen et al., 2014). These studies largely showed that exposure to stress during gestation induces the development of anxiety- and depressive-like phenotypes and reduces the ability to cope with stressful situations later in life. Another critical and sensitive period of brain development is adolescence (Blakemore and Mills, 2014; Fuhrmann et al., 2015), a specific time window during maturation characterized by a series of behavioural and structural changes. Adolescents spend more time in social interactions and risk taking, shifting their emphasis from adults to peers. Moreover, adolescent-related changes in brain development show a transition from grey to white matter due to a massive loss of synapses in neocortical brain regions, associated with an increase in specific and less widespread activation of the brain during task performances (Spear, 2000). This evidence indicates that neural networks during adolescence are in a highly transitional state (Andersen and Teicher, 2008) and may therefore represent a critical period for exposure to stressful events.

On these bases, we chose to investigate the model of stress during adolescence. We exposed animals to isolation rearing on the day of weaning (post natal day (PND) 21) for 4 weeks until PND 49. Although there is not a specific time window to define adolescence, animals were isolated specifically during a critical period described by abundant social play, ensuring that any effects may arise from the lack of gaining appropriate social experiences during a particular phase of development (Lukkes et al., 2009c). The age range we used comprises clearly age-specific behavioural discontinuities from younger and older animals (Spear, 2000). Vetter-O'Hagen and Spear (2012) found that females Sprague Dawley had vaginal opening at

PND 32-36 and males Sprague Dawley had balanopreputial separation at PND 40-48 (Vetter-O'Hagen and Spear, 2012). From PND 28 to 48, they also found a gradual increase in estradiol and testosterone (Vetter-O'Hagen and Spear, 2012).

Following social isolation manipulation, all animals, both control and stressed, at PND 49, were reared in group until the end of all experiments at adulthood, mimicking the return to normality. Our approach gave us the opportunity to dissect any mechanisms that can be long-term impaired by stress in adolescence. We found that the exposure to chronic social deprivation during a specific time window of adolescence induced an anhedonic phenotype, without affecting cognitive function later in life, and these changes were associated with an impairment in the expression of brain-derived neurotrophic factor (BDNF). In particular our results showed that these effects occurred with an anatomical specificity and some degree of gender specificity. Adult isolated male animals, undergoing the sucrose preference test, exhibited reduced sucrose preference, as compared to group-housed male animals. Isolated female rats also showed a tendency to reduced sucrose preference compared to adult female rats housed in group during adolescence. Reduced preference to sucrose is considered an index of decreased responsiveness to a normally rewarding substance, namely anhedonia, that is also a major symptom of depression (Hasler et al., 2004). Our results thus suggest that isolation housing during a specific phase of development is able to induce at adulthood a loss of pleasure to enjoyable stimuli that, being a trait marker of depression, can be referred as a depressive-like phenotype. Opposite results were observed by Hong et al., 2012 since social deprivation from 30 to 50 days of age determined an increased sucrose preference at adulthood (Hong et al., 2012). Authors tried to explain this unexpected result suggesting that the increased sucrose intake could reflect less pleasure derived from the normally pleasurable sucrose. It should be noted that in our experimental plan the measurement of sucrose preference was made just once and without food and water deprivation. All rats were also single-housed for the test in order to measure the consumption of water and sucrose solution of individual rats. However, it is unlikely that single housing could influence the effects observed in the test.

Several studies have shown that cognitive dysfunctions are common symptoms of many mental disorders as depression (Iosifescu, 2012). Our results showed that social isolation during adolescence did not produce a long-term impairment of cognitive performance, as adult isolated animals displayed similar cognitive abilities to group-housed animals. Isolated

animals, of both genders, differentiated the familiar and the novel object spending more time exploring the novel one compared to the familiar as they remembered the object previously explored. Thus, this response reflects proper working and recognition memory. These data mirrored results obtained in other studies suggesting that effects of social deprivation on learning and memory were attenuated when tested after a period of social housing (Green and McCormick, 2013). Accordingly, studies on stress during adolescence have found that social deprivation induced learning and memory deficits more clearly than other adolescent stress models (Green and McCormick, 2013). However, most studies investigated the immediate consequences of stress in adolescence, suggesting some performance deficits within the prefrontal cortex and the hippocampus (Lu et al., 2003a; McLean et al., 2010). Chronic stress administered during adolescence emerged in learning and memory modulations and depressive-like phenotypes when tested within days of the end of the stress procedure. Social deprivation from 30 to 60 days of age in male Sprague Dawley rats resulted in development of depressive-like behaviours associated with an improvement of learning processes, when tested at the end of the isolation procedure (Pisu et al., 2011a). When isolation rearing is coupled with chronic unpredictable stress model for three weeks, male Wistar rats showed immediate impairments in reversal learning, directional information flux from thalamus to prefrontal cortex (PFC) and synaptic plasticity in the prefrontal cortex (Quan et al., 2011).

We observed that total *Bdnf* mRNA levels were not altered by social isolation within the prefrontal cortex of adult male rats, while female isolated animals showed a significant reduction in total *Bdnf* mRNA levels compared to group-housed females. 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. Moreover, the coding exon has two polyadenylation sites that lead to the production of a short or a long 3' untranslated region (UTR). The expression of *Bdnf* transcripts is tissue-driven since the pull of transcripts with the short 3'-UTR are concentrated in the soma and the ones with the long 3'-UTR are preferentially targeted to dendrites (An et al., 2008). We found that social isolation did not cause any alterations in the pool of *Bdnf* transcripts with the long 3'-UTR in adult male animals within the prefrontal cortex while in females there is a tendency toward a reduction as a consequence of the isolation procedure during adolescence. Thus, these results suggest that the reduction of total *Bdnf* mRNA levels found in the prefrontal cortex of adult stressed female

rats may be driven only in part by the reduction of *Bdnf* transcripts with the long 3'-UTR, leading to possible effects at dendritic levels. However, these results are not in line with previous observations on the effects of early life stress on *Bdnf* transcription. Indeed, we have previously showed that exposure to prenatal stress leads to a significant down-regulation of the pool of *Bdnf* transcripts with long 3'UTR in the prefrontal cortex of male and female rats, and not of total *Bdnf* mRNA levels (Luoni et al., 2014). These results suggested that this selective decrease of long 3'UTR *Bdnf* mRNA levels may be linked to a selective impairment of transcripts undergoing dendritic targeting.

When investigating exon IV and VI-containing transcripts, we found that isolation rearing produced a significant reduction of exon IV-containing variants in the prefrontal cortex of adult female isolated rats, while isolated male animals did not show any changes in the expression of *Bdnf* exon IV-containing transcripts. Concerning exon VI-containing variants, we observed no changes in gene expression levels in prefrontal cortex of both male and female animals. In the brain, *Bdnf* splice variants are localized in different neuronal compartments so that they mediate synaptic plasticity within each area (Baj et al., 2011). Exon IV-containing *Bdnf* transcripts are localized to the cell body and proximal dendrites, while exon VI-containing transcripts are found in distal dendrites (Baj et al., 2011). Moreover, up-regulation of specific *Bdnf* transcripts determined a spatially restricted activation of TrkB receptors in distinct dendritic compartments (Baj et al., 2011), suggesting that splice variants containing the coding region of *Bdnf* are transported in proximal or distal dendrites and the protein localizes accordingly. Thus, it is becoming clear that, besides the known localisation of *Bdnf* transcripts with the long 3'UTR region in distal dendrites and the ones with the short 3'UTR in the soma, it must be also taken into account the specific 5'UTR sequence for dendritic targeting. Considering that GABAergic networks are localized in the soma while glutamatergic networks in the distal compartments, production of selective *Bdnf* slice variants may modulate the activation of specific networks. Lastly, our results suggested a gender specific effect of social deprivation during adolescence on *Bdnf* expression at adulthood.

Our analyses revealed that total *Bdnf* mRNA levels were not affected by social isolation in the dorsal hippocampus of male stressed animals, with a tendency to reduction in isolated female rats. Within the ventral hippocampus, isolation rearing did not induce any changes in total *Bdnf* mRNA levels in adult isolated animals, of both genders. We found that the stressful experience during adolescence induced a significant reduction of *Bdnf* long 3'UTR mRNA levels

in the dorsal hippocampus of female isolated animals, but not of male rats. As for the ventral hippocampus, *Bdnf* long 3'UTR mRNA levels were not afflicted by social isolation. These results confirm the gender specificity observed also within the prefrontal cortex and suggest that single housing condition during adolescence may impact the expression of total *Bdnf* and the pool of transcripts with the long 3'UTR in selected brain region. Our data on *Bdnf* expression adduced most deterioration within the prefrontal cortex and the dorsal hippocampus, pivotal brain areas related to cognition, although the cognitive output observed in the object recognition test seemed not affected by social isolation. Several studies suggested that the brain-derived neurotrophic factor BDNF, neurogenesis and synaptic plasticity have an important function in the development of depression (Monteggia et al., 2007; Shirayama et al., 2002). Hence, BDNF expression is reduced in major depression, both in central regions as cerebral cortex and hippocampus, and peripherally in serum. Moreover, antidepressant treatments ameliorate depressive symptoms and increase BDNF expression (Duman and Monteggia, 2006). In line, preclinical studies involving animal models of depression found that early life adversities produced a significant reduction of BDNF expression in the prefrontal cortex and hippocampus at adult life (Cirulli et al., 2009; Fumagalli et al., 2004). Very few studies modelling chronic stress exposure during adolescence investigated the effects on *Bdnf* transcription. Social isolation procedure for two weeks, followed by resocialization for three weeks produced increased *Bdnf* mRNA levels in the prefrontal cortex associated with reduced *Bdnf* mRNA levels in the hippocampus (Li et al., 2016). However, in this paradigm of adolescent stress, animals underwent behavioural investigations at early adulthood (PND 56) just 24h before the killing and dissection of brain areas. Thus there is the possibility that the subsequent molecular analyses could be influenced by the previous behavioural examination as animals were not allowed to recover from the test (Li et al., 2016). Female Sprague Dawley rats exposed to social isolation from PND 30 to 50 and then re-grouped until PND 70 showed lower *Bdnf* mRNA levels in CA3 but not in CA1, dentate gyrus, piriform cortex or basolateral amygdala, as compared to control animals (Weintraub et al., 2010). Also in this study, molecular analyses were conducted on brain areas dissected few hours after behavioural tests. On the contrary, in our experimental model, the molecular analyses were conducted two weeks after the end of all behavioural tests in order to prevent any potential influence of the behavioural examination.

It is known that exposure to an initial stressor can alter the response to subsequent stressors, which may lead to a precipitation of the psychiatric condition (Maccari et al., 2003). Indeed, the effects of chronic stress may be masked under resting (basal) conditions and may become apparent only after the exposure to a challenging condition later in life. The results of our study are in line with this possibility. Indeed, we found that exposure to an acute stress at adulthood produced a significant up-regulation of total *Bdnf* mRNA levels only in the prefrontal cortex of isolated animals, of both genders, suggesting that exposure to a challenging condition at adulthood induced the activation of *Bdnf* only if preceded by earlier chronic stress. This acute stress-induced increase in total *Bdnf* levels was mirrored also in the pool of *Bdnf* transcripts with the long 3'UTR in the prefrontal cortex of female isolated animals but not of males. As for exon IV and VI-containing transcripts, we found that the acute manipulation induced a selective activation of exon IV-containing variants, although regardless of the housing condition. The fact that the modulation of some *Bdnf* transcripts does not match the changes observed for total *Bdnf* mRNA levels suggests the possible regulation of other *Bndf* splice variants. As for the hippocampus, we observed again a gender specificity in the response to the acute stress. We found that acute stress did not induce any changes in the dorsal hippocampus of male animals, housed both in group and isolated as adolescents. Female animals showed a down-regulation of total *Bndf* and of the pool of *Bndf* transcripts with the long 3'UTR only in the dorsal hippocampus of control animals following acute stress, effect not observed in isolated animals. Regarding the ventral hippocampus, we did not observe any significant alterations produced by the exposure to the acute stress. All in all, these data reveal a gender and anatomical specificity in response to a challenging condition at adulthood suggesting that the exposure to an adverse condition during adolescence may lead to persistent modification of the mechanisms controlling BDNF expression.

The gene expression changes observed in rats exposed to social isolation were not paralleled by modification of BDNF protein levels. Indeed, isolated male animals showed increased mature BDNF protein levels within the crude membrane fraction from the prefrontal cortex associated with a tendency toward a reduction following acute stress only in isolated animals. All *Bdnf* transcripts are translated into a precursor protein, proBDNF, which is then cleaved to produce the mature form of the neurotrophin. Both proBDNF and mBDNF exert distinctly different biological actions; proBDNF, binding to the p75 neurotrophin receptor (p75 NTR),

drives pro-apoptotic processes whilst mature BDNF leads to cell survival, growth and differentiation and synaptic plasticity (Begni et al., 2017). We found that social isolation induced a significant increase in proBDNF levels within the crude membrane fraction of prefrontal cortex with no changes brought by acute stress. Isolation rearing for 4 weeks after weaning resulted in no modulation of BDNF protein levels within the prefrontal cortex when animals were tested immediately after the end of isolation procedure (Wall et al., 2012). Similarly, social deprivation for 5 weeks after weaning determined no differences of BDNF protein levels in frontal cortex, striatum, hippocampus and cerebellum (Simpson et al., 2012). These studies are in contrast with the investigation of Pisu et al., (2011) in which isolation housing from PND 30 to 50 produced significant reduction of mBDNF in the hippocampus of male animals, tested immediately after the end of social deprivation (Pisu et al., 2011a; Pisu et al., 2011b). The same group observed that this modulation was gender-specific as female animals did not show any significant modulation of mBDNF protein levels (Pisu et al., 2016). Our data on BDNF protein levels are in line with other two studies that applied the social manipulation for two weeks from 21 to 35 days of age and then animals were re-grouped for 3 weeks until PND 56. Return to social housing conditions after two weeks of isolation rearing resulted in increased mBDNF protein levels in the prefrontal cortex and reduced mBDNF protein levels in the nucleus accumbens, CA1 and dentate gyrus (Han et al., 2011). The same results were observed also in the study of Li et al., (2016) in which isolation housing as adolescents caused a significant up-regulation of BDNF protein levels in the prefrontal cortex coupled with a significant down-regulation in the hippocampus (Li et al., 2016).

Once secreted, mBDNF can bind two receptors: the high-affinity TrkB receptor or the low-affinity p75 neurotrophin receptor (p75 NTR) (Chao, 2003). BDNF binding to TrkB is followed by receptor dimerization and auto-phosphorylation leading to the activation of three signalling pathways: the phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospholipase C-γ (PLCγ) pathways. All these pathways lead to the phosphorylation and activation of the transcription factor cAMP-response element binding protein (CREB) which induces the transcription of genes essential for cell survival, growth and differentiation and synaptic plasticity (Begni et al., 2017). Our results revealed that, although social isolation produced only a marginal, non-significant reduction in the ratio of phospho TrkB/TrkB protein levels under resting condition, when exposed to acute stress at adult life, isolated animals showed increased ratio of pTrkB/TrkB protein levels as compared to group-

housed animals. TrkB phosphorylation occurs at several tyrosines present in the cytoplasmic domains of each receptor. Phosphorylated TrkB at tyrosine 515 activates the MAPK signalling pathway while phosphorylation at tyrosine 816 recruits and phosphorylates PLCγ. Our analysis focused on phosphorylated TrkB at tyrosine 816 as the data on BDNF-TrkB related downstream signalling pathways showed significant alterations in the PLCγ mediated pathway. We found that under resting condition, social isolation caused a significant reduction of PLCγ phosphorylation levels at Tyr783 and, when exposed to acute stress, isolated rats showed a significant increase of phosphorylation levels at Tyr783, as compared to animals under resting condition, an effect that was not present in group-housed animals. As for PLCγ protein levels, we did not detect any effects of social isolation and the exposure to acute stress did not alter basal protein levels. Once activated by TrkB, phosphorylated PLCγ at Tyr783 hydrolyses phosphatidylinositol 4,5-bisphosphate, to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates PKC and IP3 releases Ca^{2+} from intracellular stores, leading to activation and phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII). Through these events, PLCγ is implicated in several processes including synaptic plasticity, proliferation, differentiation, migration and survival of neuronal cells. Moreover, increasing evidence has suggested a close relationship between PLCγ and depression. In a stress-induced model of depression, a single event decreases PLCγ activity and repeated stress events inhibit the expression of PLCγ (Dwivedi et al., 2005). Furthermore, antidepressant treatments enhance TrkB/ PLCγ signalling (Rantamaki et al., 2007). Our data suggested that exposure to social isolation during adolescence did not determine a long-term dysfunction in the basal expression of PLCγ but it could impair the phosphorylation response of the protein under resting condition. Moreover, when an acute challenge was administered during adult life, control and isolated animals showed differential traits of response on phosphorylation levels. In addition, related changes in TrkB phosphorylation at tyrosine 816 were observed. These TrkB-mediated effects on downstream signalling pathways seemed to be PLCγ -selective as we did not observe any alteration in PI3K- and MAPK-related cascades. Our data on TrkB basal expression are quite consistent with another very recent study of Mikics et al., (2017), which observed no effects of social isolation on basal TrkB protein levels in the infralimbic cortex (Mikics et al., 2017). To our knowledge, no study has previously

investigated the long-lasting effects of social isolation and its influence on adult responsivity to acute stress on BDNF/TrkB mediated pathways.

Our data showed that social isolation did not induce any long-lasting alterations in the expression of glutamatergic receptors in the PFC. Unlikely, the exposure to acute stress at adulthood did not cause any modulations in the expression levels of both phosphorylated and total selected glutamatergic receptor subunits. These results are not in line with the common hypothesis that acute stress prompts potentiation of glutamatergic receptor-mediated synaptic currents in PFC and assists working memory performances (Yuen et al., 2009). Different researchers found that the exposure to chronic stress during adult life can impact the expression of glutamatergic receptors, leading to deficits of PFC-mediated cognitive processes (Yuen et al., 2012). As well, exposure to prenatal stress also induced long-term alterations in glutamate transmission reducing glutamate release (Marrocco et al., 2012). Isolation for 8 weeks after weaning resulted in decreased glutamate levels in the dorsal hippocampus but not in the cerebral cortex (Shao et al., 2015). Similarly, the same experimental model showed an up-regulation of NR2A and NR2B mRNA levels in the hippocampus and a down-regulation of NR2A in the prefrontal cortex (Zhao et al., 2009). To date, we investigated stress-driven glutamatergic modulation only within the prefrontal cortex and it may be possible that other brain areas could show impaired expression of glutamatergic receptors.

The differential modulation of *Bdnf* and its signalling pathways between animals exposed to social isolation and their sham counterpart does not seem to be the consequence of altered activation of the specific brain region, at least for prefrontal cortex. Indeed, we observed an activation of immediate early genes following acute stress, regardless of housing condition, suggesting that social isolation does not impair long term activity-induced genes stimulation. Research on the influence of adolescent social isolation on subsequent acute social interaction focusing on the mRNA levels of immediate early genes found, in line with our results, an up-regulation in both control and isolated animals exposed to the challenging situation compared to animals under resting conditions (Wall et al., 2012).

We have also explored the possibility that social isolation during adolescence could alter the function and responsiveness of the HPA axis. Our analyses showed that isolated animals under resting condition did not exhibit altered levels of circulating corticosterone (CORT), although female rats displayed higher CORT levels, compared to males. When exposed to stress at

adulthood, we observed, as expected, an increase in circulating CORT levels in control animals as well as in isolated animals, suggesting that the adverse experience during adolescence did not affect the ability to activate the HPA axis in response to an acute stress. Nevertheless, since our analysis was conducted at a single time point, we do not know if there is any difference in the time required to turn off the system. Indeed, although we observed no differences in the activation peak following acute stress between control and isolated animals, social deprivation during adolescence could determine longer high levels of circulating corticosterone after the end of the acute manipulation than group housing condition. When animals were tested immediately after the isolation period, Pisu et al., (2016) observed reduced basal plasma corticosterone in isolated animals of both genders coupled with a significant increase following the exposure to an adult acute stress, regardless of housing condition as adolescents (Pisu et al., 2016).

As a consequence of stress exposure, different brain structures, including hypothalamus, hippocampus and medial prefrontal cortex PFC efficiently activate a negative feedback, aimed to limit the exposure to glucocorticoids (McEwen, 2012). We found that group-housed as well as isolated male rats showed a downregulation of *Nr3c1* expression in the PFC in response to acute stress. Moreover, both male and female isolated animals showed higher *Nr3c1* levels, when compared to group-housed rats exposed to acute stress. Within the dorsal hippocampus, we observed a distinct trait of response to acute stress in isolated animals, since we found a reduction of *Nr3c1* levels in isolated male animals exposed to acute challenge versus males under resting condition, an effect that was not observed in control animals.

Our results showed selective effects on the expression levels of two glucocorticoid receptor (GR) target genes, *Fkbp5* and *Sgk1*, in both PFC and hippocampus. FKBP5 is a co-chaperone able to regulate glucocorticoid receptor sensitivity. Indeed, when FKBP5 is bound to the receptor complex, corticosterone binds with lower affinity and nuclear translocation of the receptor is less efficient. In turn, GR activation induces FKBP5 mRNA and protein expression (Binder, 2009). We found minor changes in the expression of *Fkbp5* as a consequence of social isolation during adolescence or exposure to acute stress suggesting that, at least with respect to mRNA changes, its modulation may not account for altered GR sensitivity and responsiveness. As for *Sgk1* we observed sustained upregulation in response to acute stress in both group-housed and isolated animals, for all the areas analysed. Based on these results

we cannot forecast a global and sustained alteration of HPA axis function, although subtle and more specific changes in the transcriptional activity of GR cannot be excluded.

In summary, considering the present results from a more general point of view, the paradigm of chronic social manipulation in rats we adopted leads to the development of a depressive like phenotype associated with some degree of gender specificity. Moreover, our data suggest that there are anatomical selective molecular alterations that may mediate the enduring effects of adolescent social deprivation as well as the reactivity to acute challenges. The plethora of consequences brought on by social deprivation on adult behaviours can be the result of epigenetic alterations, as previously demonstrated for other model of stress during development (Blaze and Roth, 2013; Luoni et al., 2016; Maccari et al., 2014). Thus, future and on-going analyses will try to establish the pattern of epigenetic changes that may sustain the behavioural and functional impairment resulting from exposure to adverse experience during adolescence.

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