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BEHAVIORAL, MOLECULAR AND EPIGENETIC CONSEQUENCES OF EARLY LIFE STRESS EXPOSURE AND THEIR IMPACT ON ADULT PSYCHOPATHOLOGY

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1. INTRODUCTION

Growing evidence suggests a pivotal role of gestational and early life stressors in the susceptibility to mental disorders and this role has been increasingly investigated in recent years by multiple epidemiological, risk factor and birth cohort studies. Early life is a period of great sensitivity, when a wide range of exogenous adverse events can alter the course of normal brain development thereby compromising the integrity and function of various brain systems with permanent consequences. It is thus clear that specific environmental factors acting during sensitive prenatal or early postnatal developmental periods can program adult disease, by inducing persistent changes in physiological, emotional and behavioural functions throughout life (Bale et al., 2010). Epidemiological studies conducted in humans also inspired preclinical research using various animal models of exposure to gestational and early life stressors. These studies have been of pivotal importance in trying to elucidate at least some of the molecular mechanisms underlying long term programming of adult disease.

1.1 EPIDEMIOLOGICAL STUDIES

In order to study the association between early life adverse events and mental disorders, different research designs have been implemented. The first studies were usually retrospective studies, where mental disease cases were collected from hospital records and information on potential risk factors was obtained from archived resources (Gordis, 2000). While these studies provided important initial data linking environmental exposure to adverse events and mental disease, they were also compromised by various limitations arising from the method of sample selection, which can create bias, limit generalizability and information collection. Thus, investigators have developed a more robust design to examine early life determinants of psychiatric illnesses based on birth cohorts. These are comprised of a group of subjects born during a specific period in a specific geographic region, and representative samples of the pregnancies and first years of life of newborns are collected to assess various factors, as for example demographics, health habits, infection during pregnancy, maternal conditions during pregnancy and in the first years of motherhood. The samples are collected and stored specifically for research purposes and allow an accurate follow up of each participant. A variation of cohort studies are retrospective studies based on ecologic data that rely on documented population-based exposure. In these studies, investigators use epidemics or famine in populations to define exposure status, and these studies are thus limited by the fact that there is no actual confirmation of maternal infection or famine exposure during pregnancy, because they are based exclusively on whether the mother was pregnant during an epidemic or famine. All in all, these different epidemiological studies demonstrate that a variety of early life adverse events participate in the origins of mental disorders, as for example schizophrenia, bipolar disorder, autism and major depression.

Prenatal factors

Fetal brain development is a delicate process that takes place in the mother's body protected by the placental barrier. As discussed below, a variety of exogenous agents can alter the course of brain development in stressful conditions increasing vulnerability to the development of psychiatric disease later in life. Various studies, for example, demonstrated an increased risk of schizophrenia and autism among individuals born and raised in an urban area compared to a rural area (Lauritsen et al., 2013; March et al., 2008; Mortensen et al., 1999), which per se does not represent an early life adversity but could determine fetal exposure to environmental stressors like pollution and infectious microbes. Prenatal exposure to infectious agents has, in fact, been

identified as a risk factor for many mental disorders, and both retrospective and birth cohort studies have investigated this link. Indeed, initial studies focusing on the influenza type 2 pandemic of 1957 reported associations between influenza and schizophrenia (for a detailed review see Brown and Derkits, 2010), and major affective disorder (Machon et al., 1997). Subsequently, many birth cohort studies have confirmed the association between influenza and schizophrenia (Brown et al., 2004), and have broadened these results by encompassing other infectious agents, as rubella (Brown et al., 2001), Toxoplasma Gondii (Brown et al., 2005; Mortensen et al., 2007) and HSV2 (Buka et al., 2008; Mortensen et al., 2010). Recent work by Parboosing and colleagues, moreover, demonstrates a 4-fold increase in bipolar disorder risk after exposure to maternal influenza (Parboosing et al., 2013) and there is also some evidence that prenatal immune challenge and disruption of various maternal cytokines are associated with increased risk of developing autism (Goines et al., 2011).

Maternal nutrition has also been identified as a possible factor involved in fetal programming of adult disease, as shown by epidemiological studies from the Dutch Hunger Winter and the Chinese Famines (Brown and Susser, 2008; Brown et al., 2000; Xu et al., 2009) that found a statistically significant increase in the risk of developing schizophrenia and major affective disorder as a consequence of prenatal malnutrition. Prenatal deficiency of specific micronutrients, as for example folate, iron and vitamin D, has also been associated with adult mental disease (Eyles et al., 2013; McGrath et al., 2011). Apart from these environmental factors, maternal psychological stress during pregnancy also plays a critical detrimental role in fetal development. Exposure to bereavement stress, natural disaster, war, serious injury or death of a close relative and also unwantedness of pregnancy have all been associated with a higher risk of psychiatric disease, as for example schizophrenia (Herman et al., 2006; Khashan et al., 2008; van Os and Selten, 1998), affective disorders (Kleinhaus et al., 2013) and autism (Class et al., 2014). Maternal stress, and in particular elevated levels of fetal glucocorticoids, detrimentally reprograms the fetal HPA axis and this, in turn, could increase long-term mental disease risk (Harris and Seckl, 2011). Moreover, higher basal HPA axis activity coupled with greater stress reactivity is associated with the development of depressive and anxiety-related disorders. Therefore, it has been proposed that children born to stressed mothers during pregnancy could have a higher risk of developing affective and depressive disorders (Koenig, 2006; Weinstock, 2005, 2008). Lastly, obstetric complications during delivery, as for example asphyxia, uterine atony, hypoxia and emergency C-section, are among the first epidemiological findings shown to contribute to susceptibility for

schizophrenia (for detailed reviews see Cannon et al., 2002; Matheson et al., 2011), and also seem to be associated with an increased risk for autism (Kolevzon et al., 2007) and cerebral palsy (Rennie et al., 2007).

Postnatal factors

As the human brain continues to mature well into adolescence, the postnatal environment also has a critical influence on programming disease risk. Increasing evidences unequivocally reveal that adults exposed to child abuse, neglect, trauma, and infection, are at greater risk of developing psychiatric disorders. Results from the Adverse Childhood Exposure study, in fact, demonstrate that childhood exposure to a variety of adverse experiences (eg. psychological maltreatment, physical abuse, sexual abuse, child neglect, caregiver's substance/alcohol use, caregiver's depressive symptoms, caregiver's being treated violently, and criminal behaviour in the household) is associated with an increased risk of general health complaint and, in particular, with the development of affective disorders (Chapman et al., 2004; Felitti, 2009; Felitti et al., 1998). A recent review by Fryers and Brugha assesses the current evidence arising from longitudinal studies for childhood determinants of adult psychiatric disease (Fryers and Brugha, 2013). Physical illness during childhood, for example, can be considered as an early life adversity and was found to be associated with later depression (Pless et al., 1989). Being bullied in school also increases susceptibility to depression and difficult peer relationships in adulthood (Lund et al., 2009; Sourander et al., 2009), as well as divorce of parents and inter-parental conflict, even if the overall increase of risk is somewhat low (Fryers and Brugha, 2013). Childhood abuse, and in particular sexual abuse, stands out as one of the main postnatal risk factors for developing adult mental disorders and can lead to various pathological outcomes. Increased risk following sexual and physical abuse has in fact been demonstrated for depression, anxiety, personality disorders and, to a lesser extent, schizophrenia (for a detailed review see Fryers and Brugha, 2013).

1.2 PRECLINICAL STUDIES

The epidemiological evidence obtained in human studies has stimulated a variety of preclinical studies conducted in animals aimed at identifying the role of early life programming in the alterations of brain structure and function that may lead to psychiatric disease. Animal models have helped to partially elucidate how early life experiences such as stress, infection, malnutrition and maternal care are able to confer vulnerability to the development of mental disease. Moreover, these studies have pointed out how the nervous system shows a temporal specificity in programming of long term effects, in the sense that the timing of the stressful event is important for determining the functional and pathological outcome.

Prenatal factors

The role of maternal immune activation in the development of psychiatric disorders has been investigated by administration of different substances: polyinosinic:polycytidylic acid (polyI:C), a synthetic analogue of a double stranded RNA that mimics viral infection; lipopolysaccharide (LPS), a bacterial cell wall endotoxin; human influenza virus and selected inflammatory cytokines. These studies are based on the rationale that exposure to an excess of inflammatory maternal cytokines could disrupt fetal neurodevelopment and determine long-lasting consequences which would ultimately lead to the emergence of postnatal brain dysfunctions and pathologies. Prenatal exposure to human influenza, Poly(I:C) or LPS determines the insurgence of many brain and behavioural abnormalities in the offspring that bare relevance for various psychiatric diseases, *in primis* schizophrenia and autism, as for example impaired sensorimotor gating, impaired selective attention, reduced social and exploratory behaviour, sensitivity to psychotomomimetic drugs, impaired working memory and a broad range of neurochemical and brain morphological abnormalities (for detailed reviews on the subject see Harvey et al., 2012; Patterson, 2011). It must also be of note that the precise timing of the infection influences the specific spectrum of neuropathological abnormalities observed in the offspring (Meyer et al., 2007).

As suggested by epidemiological evidence in humans, exposure of the developing brain to severe and/or prolonged stress may result in hyper-activity of the stress system, dysfunction of the HPA axis, defective glucocorticoids-negative feedback, altered cognition and mood-related disorders (Maccari et al., 2003; Weinstock, 2008). Thus, stressful challenges that occur in the prenatal period can permanently influence brain and behaviour of the developing individual and increase the risk of developing mental illness. Many animal models have thus investigated the consequences of

prenatal exposure to stress in order to study the neurobiological basis of the association between early life stress and mental illness. In order to model prenatal stress in animals, investigators expose pregnant subjects to a variety of psychological stressors during specific periods of gestation, as for example repeated restraint stress (Maccari and Morley-Fletcher, 2007), repeated exposure to variable unpredictable stress (Markham et al., 2010; Mueller and Bale, 2008) and exposure to repeated foot shocks (Shalev and Weiner, 2001). Prenatal exposure to stressors has been extensively used as a model of major depression (Kaufman et al., 2000; Nestler et al., 2002), as several lines of evidence show that animals exposed to stress in utero reproduced behavioural characteristics that are close to a depressive-like phenotype, as for example a higher degree of immobility in the forced swim test compared to controls (Morley-Fletcher et al., 2003), reduced exploratory behaviour (Vallee et al., 1997; Van den Hove et al., 2005), increased stress response and abnormal circadian and sleep function (Maccari et al., 2003) and sex-specific reduced hippocampal neurogenesis (Lemaire et al., 2006).

In utero stress exposure also bears relevance for schizophrenia, as both predictable and unpredictable forms of prenatal stress exert a long-term impact on responsivity to dopamine-stimulating drugs or environments (Deminiere et al., 1992; Koenig et al., 2005), while only prenatal exposure to variable stress disrupts sensorimotor gating (Koenig et al., 2005). Moreover, numerous experiments have demonstrated that prenatal exposure to stress also leads to cognitive deficits (Kapoor et al., 2009; Son et al., 2006; Wu et al., 2007; Yang et al., 2006) and reduced social interaction (Lee et al., 2007), both critical aspects of the negative symptoms of schizophrenia.

Last, to model the epidemiological link between prenatal undernutrition and higher risk of mental illness, investigators have subjected pregnant animals to protein deficient diets (PDD) throughout or at restricted stages of pregnancy. These studies have demonstrated that prenatal protein deprivation has long-term negative influences on the integrity of hippocampal structures and functions (Diaz-Cintra et al., 1991; Diaz-Cintra et al., 2007; Lister et al., 2011; Morgane et al., 2002), which in turn are crucially involved in the neuropathology of various mental disorders, as for example depression and schizophrenia. Moreover, protein deprivation also induces neurochemical (Mokler et al., 2003; Palmer et al., 2008) and behavioural abnormalities relevant for schizophrenia, such as impaired sensorimotor gating (Palmer et al., 2004), enhanced behavioural sensitivity to psychostimulants (Palmer et al., 2008) and working memory impairments (Ranade et al., 2008), some of which are sex specific. Based on the possible epidemiological contribution of developmental vitamin D deficiency to schizophrenia risk

(McGrath, 1999), various studies have implemented models of maternal vitamin D deficiency in mice and rats, and recent evidence associates vitamin D deficiency with various psychiatric disorders other than schizophrenia, as for example autism, depression and Alzheimer (for a detailed review see Eyles et al., 2013).

Postnatal factors

Disruption of the delicate mother-infant relationship has a profound impact on brain development and HPA axis programming, as demonstrated by its effects on the endocrine response to stress and numerous behavioural domains. Various preclinical studies have thus been implemented to model early life parental separation or childhood neglect, and these usually refer to the paradigms of maternal separation and maternal deprivation.

The maternal separation paradigm consists in removing the pups from the mother usually for 3 h a day during postnatal day 1-14, while maternal deprivation usually entails a single separation from the mother for 24h, and the effects vary depending on the precise timing of the separation. Maternal separation studies often report contradictory results, with some reporting no clear influence on anxiety and depressive-like behaviour (Hulshof et al., 2011; Millstein and Holmes, 2007), while others show the opposite (Matthews and Robbins, 2003; Reus et al., 2011; Romeo et al., 2003), and the effects are often sex- and strain- specific. Maternal separation is also associated with PPI deficits, but, once again, studies yield mixed results: Millstein et al. found that MS produced no effect on PPI in various mice strains (Millstein et al., 2006), while other studies demonstrate opposite findings in both adolescent and adult rats (Li et al., 2013; Van den Buuse et al., 2003). Maternal deprivation has also been shown to model many behavioural alterations relevant for schizophrenia, as PPI deficits (Ellenbroek et al., 2004; Ellenbroek et al., 1998; Garner et al., 2007) and sensitivity to dopaminergic drugs (Rentesi et al., 2013). All in all, both these experimental paradigms offer the opportunity to study the neurochemical and molecular mechanisms underlying the detrimental effects of early postnatal stress, even if a variety of factors, as timing and duration of the separation, rodent strain and sex differentially influence the results.

1.3 IMPACT OF EARLY-LIFE EXPOSURE TO STRESS ON THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The hypothalamic-pituitary-adrenal (HPA) axis is a system designated to control the body's responses to stress and the regulation of circadian circuits.

Under conditions of stress, activation of the HPA axis results in increased production and release of corticotrophin-releasing hormone (CRH) from the paraventricular nuclei (PVN). This hormone, once is released into the portal blood system, acts on cells of the anterior pituitary to stimulate adrenocorticotropic hormone (ACTH) expression and release, which in turn increases glucocorticoid production at the adrenals. Subsequently, increased levels of circulating glucocorticoids feed back to inhibit HPA axis activity and prevent excessive production of stress hormones. Negative feedback occurs at the level of the brain and at the pituitary, and in particular inhibitory inputs derive from hippocampal GR-expressing neurons to inhibit PVN hormone release (Fig.1).

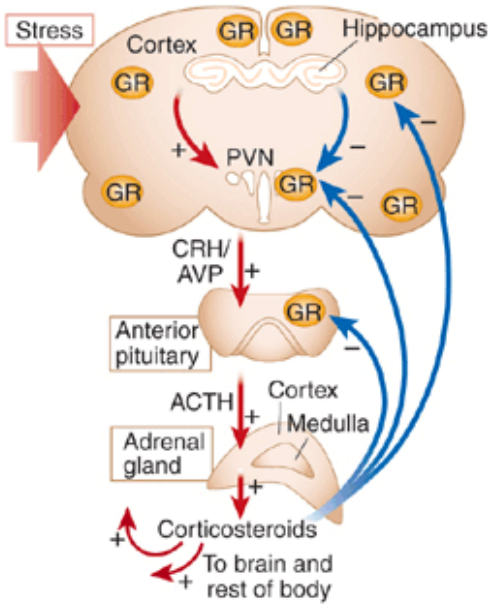

Figure 1 *Activation of the hypothalamic -pituitary- adrenal axis by stress (in red) and negative feedback inhibition (in blue) (adapted from Akil, 2005)*

It has been shown that prolonged prenatal restraint stress can produce persistent effects on the HPA axis functionality (Maccari et al., 2003; Owen et al., 2005; Seckl, 2004; Weinstock, 2005).

Prenatally stressed rats exhibit elevated plasma levels of glucocorticoids for a more prolonged period of time compared to controls (Maccari et al., 1995; Vallee et al., 1997), which results in a

higher stress-responsiveness. The high stress response is indicative of a reduced negative feedback of glucocorticoids, which may be related to the reduction of the number of glucocorticoid receptors observed in the hippocampus and hypothalamus of rats whose mothers were exposed to stress during gestation (Henry et al., 1994; Maccari et al., 1995; Seckl, 2004). Moreover, it has been shown that the reduction in the expression of hippocampal glucocorticoid receptor occurs through changes in the methylation of one of its promoter (Cottrell and Seckl, 2009; Meaney, 2001; Weaver et al., 2004). Interestingly, these results are in line with other studies based on the administration of synthetic glucocorticoids during pregnancy, such as dexamethasone, or pharmacological inhibition of 11beta-hydroxysteroid dehydrogenase type 2, a placental enzyme which converts glucocorticoids (cortisol/corticosterone) into their inactive metabolites (cortisone/11beta-deidrocorticosterone), limiting the exposure of the fetus to maternal glucocorticoids (Shoener et al., 2006; Welberg et al., 2000).

It is believed that one of the causes of the long-term effects of prenatal stress may be excessive exposure of the fetus to maternal corticosterone, since it has been shown that surgical removal of the adrenal glands of the mother can reduce or abolish the effects on the fetus (Barbazanges et al., 1996; Maccari et al., 2003).

1.4 IMPACT OF EARLY-LIFE EXPOSURE TO STRESS ON NEURONAL PLASTICITY: A FOCUS ON BDNF

Specific proteins, known as neurotrophic factors, are essential for the development, survival, function and plasticity of neuronal cells, both in the Central Nervous System (CNS) and in the Peripheral Nervous System (PNS). Over the past twenty years, in accordance with nucleotide similarities with the founder Nerve Growth Factor (NGF) discovered over fifty years ago (Levi-Montalcini and Hamburger, 1951), other proteins NGF-related have been identified such as Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and NT-4/5, which are called neurotrophins.

In the last decade the research in the field of neurotrophic factors has led to the discovery of numerous functions of these proteins. They counteract the degeneration of the cells of the CNS promoting neuronal survival, they play an important role in the mechanisms of cellular plasticity and they are able to induce neuronal differentiation by increasing the synthesis of neurotransmitters or by promoting neurite outgrowth. These actions are carried out throughout the whole lifespan, from the first period of life (prenatal and immediately postnatal), influencing both the maturation and the development of the CNS, to the adulthood, affecting its functionality and plasticity (Cowansage et al., 2010; Lu et al., 2005).

It is thus expected that changes of the regulation of the neurotrophin system at different levels (transcriptional, translational, epigenetic) may represent one of the susceptibility mechanisms through which manipulations during early perinatal development, such as stress exposure or adverse environmental conditions, may alter the cellular resilience and affect the proper brain structure maturation (Branchi et al., 2004). A reduction in the functionality of these elements could in fact be detrimental for specific cellular phenotypes and may increase the vulnerability of neurons rendering the system powerlessness to cope with adverse events (Duman et al., 1997). In particular, the neurotrophin BDNF has emerged as a crucial mediator of neuronal plasticity, not only because it is abundant in brain regions that are particularly relevant for plasticity, but because it shows a remarkable activity-dependent regulation of it expression and secretion (Bramham and Messaoudi, 2005), suggesting that it might indeed bridge experience with enduring change in neuronal function.

BDNF has a sophisticated organization in terms of transcriptional, translational and post translational regulatory mechanisms. With regards to *Bdnf* transcription, its gene consists of nine 5’ untranslated exons, each linked to individual promoter regions, a 3’ coding exon (IX), which

codes for the BDNF pre-protein aminoacid sequence, and two polyadenylation sites at the 3' untranslated region (UTR) (Aid et al., 2007) (Fig.2). The transcription of each exons is driven by separate promoters in turn controlled by an array of signalling mechanisms, including Ca2+, CREB, CaRF, Npas4, USF, MeCP2, CaMKII and hormones (Molteni et al., 2010a; Pruunsild et al., 2011; Zhou et al., 2006). Furthermore, it has been demonstrated that the transcription of specific splice variants is controlled by a variety of epigenetic mechanisms, including DNA methylation and histone posttranslational modifications (Lubin et al., 2008; Molteni et al., 2010b; Roth et al., 2009). The regulation of specific promoters causes the temporal and spatial expression of specific *Bdnf* transcripts (Lauterborn et al., 1996), some of which can undergo trafficking and targeting to dendrites (Chiaruttini et al., 2008), a process that may also be influenced by the 3'-UTR of the neurotrophin (An et al., 2008; Ghosh et al., 1994).

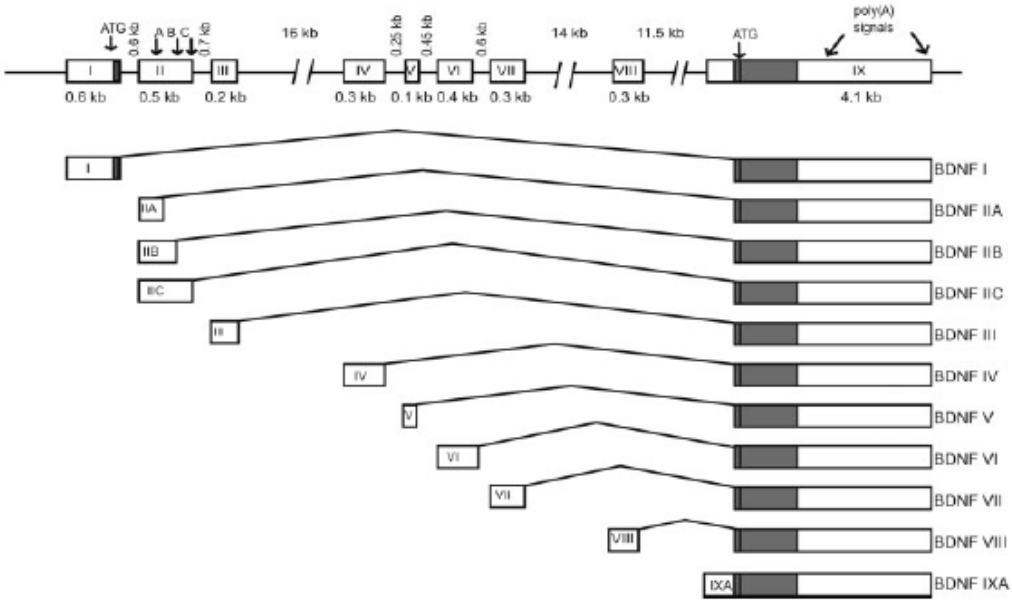

Figure 2 *Gene structure of Bdnf and its transcripts (adapted from Aid et al., 2007)*

The BDNF protein is initially synthesized as preform that can either be cleaved into the mature neurotrophin or transported to the plasma membrane and released in an unprocessed manner. Several matrix metalloproteases (MMP) as well as plasmin are responsible for extracellular cleavage of the neurotrophin, whereas furin and specific proconvertases control its intracellular processing (Schweigreiter, 2006). Differentially from other neurotrophins, BDNF could be secreted though a constitutive as well as a regulated pathway. Upon release, proBDNF and mBDNF have

divergent activities, since the former binds with high affinity to p75NTR leading to apoptosis, whereas mBDNF binds to TrkB receptors promoting cell survival (Lu et al., 2005).

Since the expression of neurotrophic factors is reduced following the exposure to early stressful events, and this effect strictly depends on the timing and duration of the stress exposure, several authors have addressed this issue using different perinatal exposure to stress paradigms, to investigate the consequences of perinatal manipulations on such proteins involved in the regulation of neuronal plasticity.

It has been shown that a single episode of maternal separation of 24h at PND9 in rats selectively reduces the expression of *Bdnf* in the adult hippocampus, both at mRNA and protein level, and also its modulation after a challenge exposure (Roceri et al., 2002). Following repeated maternal separation (PND2-14), however, the same group reported a short-term upregulation of *Bdnf* (at PND17), probably an attempt to counteract the adverse stimuli, which is followed, at adulthood, by a reduced expression of the neurotrophin in the prefrontal cortex (Roceri et al., 2004). Interestingly, MacQueen and collaborators, using a longer protocol in mice (PND4-22), observed a reduced *Bdnf* expression also in the adult hippocampus of maternal-separated animals (MacQueen et al., 2003b).

The mother-pup interaction seemed also important during the early stages of postnatal life. In fact, rats grown up by "high care" mothers had higher hippocampal *Bdnf* levels when compared to rats grown up by "low care" mothers (Liu et al., 2000).

Regarding the gestational manipulation, we have previously shown that prenatal immobilization stress markedly reduces *Bdnf* expression in the adult prefrontal cortex of rats (Fumagalli et al., 2004), a finding that has been recently confirmed in mice exposed to restraint stress during pregnancy, which show, at adulthood, a decreased expression of this neurotrophin in the frontal cortex, besides several schizophrenia-like phenotypes (Matrisciano et al., 2012). Moreover, the group of Burton has reported that the combination of prenatal exposure to stress together with the artificial postnatal rearing paradigm decreases, at adulthood, the expression of *Bdnf* in the medial prefrontal cortex (Burton et al., 2007). Recently, Yeh and co-workers have shown that rats exposed to repetitive immobilization stress during gestation showed impaired hippocampal synaptic plasticity, an effect observed until adolescence. They demonstrated that the mechanism underlying this effect is a reduced processing of the proBDNF into the mature form of the neurotrophin (Yeh et al., 2012).

All these findings show that the neuronal circuits deputies to neurotrophic response could be reprogrammed by experiences that occur during the last week of gestation or the first few weeks of life, and could have long-term consequences resulting in an increased vulnerability to the development of psychiatric diseases at adulthood.

1.5 THE EARLY-LIFE ENVIRONMENT AND EPIGENETIC PROGRAMMING

The long-lasting nature of the behavioural and molecular changes following the exposure to early adversities suggests that such modifications are not a direct consequence of the stressful experience, but may indeed represent a relevant component for functional disability.

The critical question relates to the mechanisms involved in mediating the effects of early life adversities and memorizing the exposure in the genome long after the exposure itself is gone. Although genetic mechanisms are obviously involved in a certain fraction of such transmission of vulnerabilities from mother to offspring, a new understanding of genome-function is emerging. Genome functionality is determined not only by the DNA sequence but also by a set of markings of the genome termed "epigenome". The epigenome encompasses the molecular mechanisms that govern gene expression in a time- and cell-type dependent fashion. Specifically, this involves DNA methylation (a chemical coating of the DNA molecule by addition of methyl groups), packaging of DNA by proteins called histones as well as binding of specific transcription factors to regulatory regions of the genome (Szyf, 2009) (Fig.3). Both chromatin modification and DNA methylation are catalysed by highly regulated and programmed enzymatic reactions. DNA methylation and histone modification act in concert to regulate gene expression and there is extensive crosstalk between these two components of the epigenome. DNA methylation reactions are catalysed by enzymes termed DNA methyltransferases that transfer a methyl moiety from the donor molecule S-adenosyl methionine (SAM) onto the 5 position of the cytosine ring. Three distinct DNA methyltransferases were identified in mammals (Okano et al., 1998), namely DNMT1, DNMT2 and DNMT3(a,b) characterized by different activities, regulating maintenance as well as *de novo* methylation (Okano et al., 1999). The classic accepted model has been that DNA methylation patterns change in a highly organized way during early development and differentiation, but that the pattern of methylation remains fixed thereafter (Razin and Riggs, 1980). This model was founded on the belief that DNA methylation patterns are irreversible. It was believed that the only DNA methylation reaction that exists in somatic cells is maintenance DNA methylation, a semiconservative replication of DNA methylation by DNMT1, a DNA methyltransferase that shows high preference to hemimethylated DNA and preserves the DNA methylation pattern during cell division (Gruenbaum et al., 1982).

Almost a decade ago the Szyf's laboratory proposed that DNA methylation was a reversible biological signal and that the pattern of DNA methylation was dynamically maintained during life by equilibrium of enzymatic DNA methylation and DNA demethylation (Ramchandani et al., 1999).

This raised for the first time the prospect that DNA methylation plasticity might play a role in physiological responses as well as adaptive programming of the genome to changing environments early in life and perhaps throughout life.

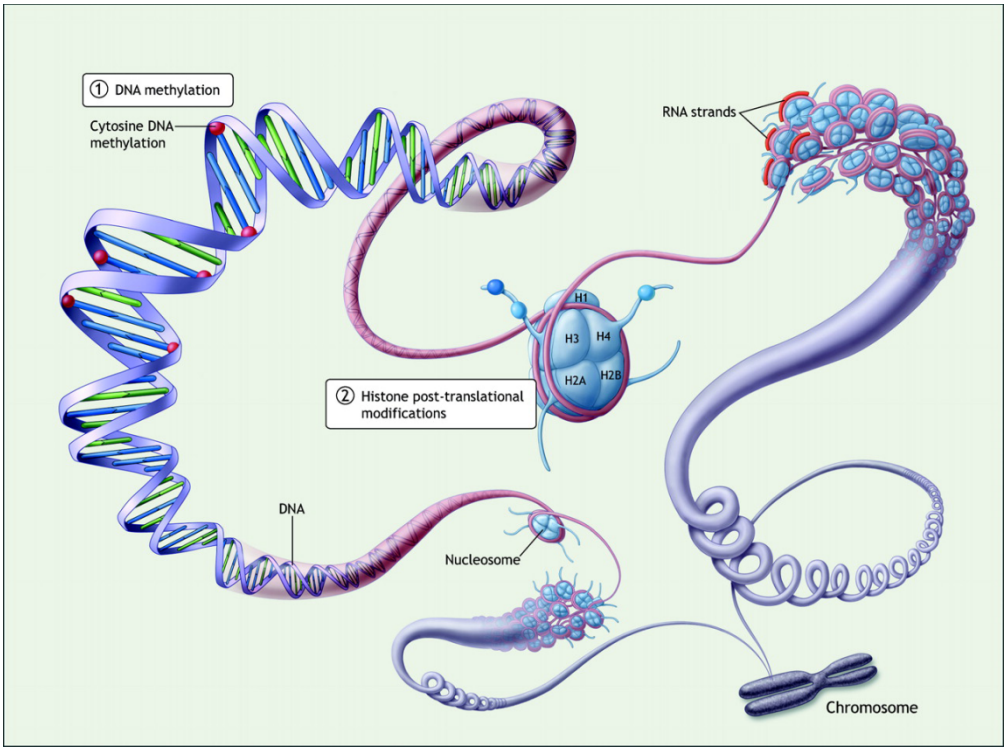

Figure 3 *Epigenetic mechanisms of gene regulation (adapted from Matouk and Marsden, 2008).* 1) DNA methylation (depicted as red balls) and 2) posttranslational modifications of the histone amino terminal tails (depicted as light and dark blue balls).

Animal models have already suggested the implication for epigenetic regulation of gene expression to mediate the effects of early life stress (ELS) on adult behaviour and health. For example, variations in maternal care in rats were reported to trigger life long differences in stress responsivity involving differences in DNA methylation and histone acetylation of the glucocorticoid receptor (GR) in the hippocampus of the offspring (Weaver et al., 2004). In fact, offspring of good mothers (high care) show, when compared to 'low care' mothers, reduced DNA methylation at GR promoter in line with increased gene expression of hippocampal GR gene. Such differences emerged over the first week of life, persisted into adulthood and altered histone acetylation and transcription factor binding to the GR promoter (Weaver et al., 2004). Similarly, Szyf & McGowan have demonstrated that, in humans, early life abuse was associated with increased methylation of the GR exon1f promoter in the hippocampus of suicide (depressed) subjects (McGowan et al.,

2009), in support of 'translational' implications for such findings. More recently maternal separation, which leads to hypersecretion of corticosterone and alteration in passive stress coping and memory, was shown to be associated with a persistent increase of hypothalamic arginine vasopressin (AVP) expression, due to sustained and persistent DNA hypomethylation (Murgatroyd et al., 2009). In this regard, it is worth mentioning that in rats subjected to restraint stress during the last week of gestation, the reduction of the expression of 11beta-HSD2 (at the end of pregnancy) is associated with epigenetic mechanisms, including an increased methylation of the promoter of its gene (Jensen Pena et al., 2012).

In addition to the stress-responsive systems, the regulation of other genes may be relevant for long-term changes in brain function in the context of psychiatric disorders. To this respect, neurotrophic factors represent an interesting class of molecules, which regulate several cellular functions and, in many instances, may couple neuronal activity with long-term structure and function of brain circuitry (Duman and Monteggia, 2006; Pittenger and Duman, 2008). Interestingly, the expression of some neurotrophic molecules may be regulated epigenetically in the context of ELS or differences in maternal care (Cirulli et al., 2009; Roceri et al., 2004; Roth et al., 2009). As an example, the persistent reduction of the neurotrophin expression in the social defeat stress paradigm is due to epigenetic changes in the promoter region of two of its transcripts (Tsankova et al., 2006). Similarly, we have recently demonstrated that in the prefrontal cortex of serotonin transporter knockout rats, a rodent model characterized by anxiety and depressive behaviour (Olivier et al., 2008), the expression of *Bdnf* is significantly reduced through an increased methylation in the promoter region of exons IV and reduced H3 acetylation at exon VI (Molteni et al., 2010b). Interestingly, early maltreatment in rodents produces persistent changes in the methylation of BDNF that caused alteration in its expression in the adult prefrontal cortex (Roth et al., 2009).

Since epigenetic effects on the transcriptome are potentially reversible in adulthood, all these studies have a prominent role in the development of new diagnostic and, potentially, therapeutic approaches.

2. AIM OF THE PROJECT

There is now consistent evidence that psychiatric diseases may often represent the consequence of exposure to adverse events early in life, which may disrupt the correct program of brain maturation thus leading to long-lasting changes in brain function.

Accordingly, exposure to stress during gestation in rats has a strong impact on brain development and can cause long-term abnormalities in adult behavior (Fumagalli et al., 2007; Seckl, 1998). In this context, the study of environmental manipulations in animal models offers the possibility to investigate the mechanisms that may be responsible for functional deterioration, with the advantage of keeping the influence of various factors such as the timing and intensity of the adverse condition, the growth environment and the genetic background under control.

Given all these premises, in this study we first set up and employed a paradigm of prenatal stress in rodents in order to reproduce early life adversities that may encompass pregnancy and early postnatal life. Indeed, gestational stress has long-lasting effects on the hypothalamic-pituitary-adrenal (HPA) axis and on the behavior of the dams, suggesting that alterations in maternal behavior following exposure to prenatal stress could also contribute to the long-term effects (Maccari et al., 2003; Maccari and Morley-Fletcher, 2007) of this environmental stressor. In particular, the paradigm we employed consisted in restraining the dams during the last week of gestation for 45 minutes three times a day under bright light, from gestation day 14 until delivery. We next sacrificed the pups, both males and females, at different postnatal time points, in order to create a time profile of the modifications under investigation.

First, we tested the cognitive functionality of adult animals with the object recognition test, since cognitive disabilities are one of the common symptoms that characterize different psychiatric conditions (Disner et al., 2011; Lapiz-Bluhm et al., 2008; Lesh et al., 2011; Lewis et al., 2012).

Next, we performed a detailed analysis of two candidate systems whose deterioration could contribute to the development of the diseased phenotype, namely the glucocorticoid receptor (GR) and the neurotrophin brain-derived neurotrophic factor (BDNF), through basal and functional analyses at gene and protein levels. These two systems have emerged as the most vulnerable elements of exposure to stress during development and can be considered markers of the dysfunctions associated with psychiatric disorders. The HPA axis is involved in the response to stressful events (Maccari et al., 2003), whereas neuronal plasticity represents an array of

mechanisms involved in the adaptive capacity to environmental changes (Calabrese et al., 2009; Duman and Monteggia, 2006).

We performed the analyses at various stages of development, trying to establish how early the molecular alterations become manifest and their persistence in time. Notably this bears the possibility to evaluate the potential of early pharmacological interventions that may prove effective in preventing the molecular and functional alterations set in motion by prenatal stress exposure, leading to long-term beneficial effects on the brain function.

Several animal models and human studies suggest that the effect of exposure to stress early in life on lifelong phenotypes is mediated by epigenetic regulation of gene expression involving changes in DNA methylation (McGowan and Szyf, 2010; Weaver, 2007). A further aspect of this experimental work was thus to determine the methylome profile of the hippocampus and the prefrontal cortex of adult rats exposed to prenatal stress. In order to do this, we combined methylated DNA immunoprecipitation (MeDIP) followed by the hybridization on a custom designed high-density oligonucleotide arrays, in order to identify, with an unbiased approach, the genes that are persistently affected by gestational exposure to stress at expression level through changes in the methylation of their promoters.

Last, we aimed at identifying novel candidate markers in a translational approach, by comparing the methylome results obtained in the rat model with a non-human primate model based on different rearing condition, and with a human model of maternal adversities. The identification of genes that show a similar response to early adversities in the brain and in peripheral tissues, in three different species and across the lifespan, is critical for the development of novel diagnostic tools and for therapeutic interventions.

3. MATERIALS AND METHODS

3.1 RATS

3.1.1 Animals and prenatal stress procedure

Nulliparous adult female (body weight 230–260 g) and male (400 g) Sprague-Dawley rats were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, they were pair-housed with a same-sex conspecific with food and water available *ad libitum* (21±1 °C, 60±10% relative humidity, reversed 12/12 h light/dark cycle). After 10 days of habituation in the facility, rats were mated for 24 h and individually housed immediately thereafter. Pregnant females were randomly assigned to control (Ctrl) or prenatal stress (PNS) conditions. PNS consisted of restraining pregnant dams in a transparent Plexiglas cylinder (7.5 cm diameter, 19 cm length) under bright light for 45 min three times daily during the last week of gestation until delivery (Anacker et al., 2013; Maccari et al., 1995). PNS sessions were separated by 2–3 h intervals and conducted at varying periods of the day to reduce habituation. Control rats were left undisturbed. While both male and female pups belonging from both Ctrl and PNS dams were used in experiment 1, we used only male pups in experiment 2.

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

3.1.2 Experiment 1

5 dams belonging from the Ctrl group and 5 from the PNS group were sacrificed at the end of gestation (gestation day 21) in order to collect and weight placentas. Of the remaining dams, on postnatal day 1 (PND1), litters culled to 5 male and 5 females. Weaning occurred on PND21 and same sex rats were housed in groups of 3 per cage. One female and one male from each litter pertaining to Ctrl and PNS groups were sacrificed on PND1, 7, 21, 40 and 62, in order to create a time-profile of the changes under investigation. On PND62, rats pertaining the Ctrl and PNS groups were divided in two. Of each, one half (one male and one female from each litter) was sacrificed (Sham group) and trunk blood was collected for the analysis of plasma corticosterone levels. The other half underwent an acute Forced Swim Stress (AS group). Briefly: in a quiet room, with a dim

light, each AS subject was gently placed into a cylindrical plastic container (height = 59 cm; diameter = 25 cm) filled up with water (26 ± 1 °C) to a level of 36 cm. The session lasted 5 minutes. All AS animals were sacrificed 1 hour after the end of the acute stress procedure.

Furthermore, a group of Ctrl and PNS male and female rats was left undisturbed in their home cage until PND80, when they underwent the object recognition test.

3.1.3 Analysis of plasma corticosterone levels

Samples of blood from each rat were collected in heparinized tubes. Plasma was separated by centrifugation (6500 *g* for 10 min) and corticosterone was determined using a commercially available radioimmunoassay (RIA) kit containing ^{125}iodine labeled CORT; 5 µl of plasma were sufficient to carry out CORT measurement. Sensitivity of the assay was 0.125 mg/dl, inter- and intra-assay variation was less than 10% and 5%, respectively (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

3.1.4 Object Recognition Test

Animals belonging to each experimental group (Ctrl or PNS), both males and females, were submitted to the object recognition test (ORT) at PND80. This test exploits the natural tendency of rodents to explore the novel things more than familiar ones (Ennaceur and Delacour, 1988).

The experimental apparatus used for the test was an open-field box (43 X 43 X 32 cm) made of Plexiglas, placed in a quiet room dimly illuminated. On the day of testing, animals were habituated in the room for 30 min period, before experimental procedure began. As previously reported for Sprague-Dawley rats (Dardou et al., 2008), the experiment comprised two sessions of 300s each. During the first session (familiarization phase) two identical objects were presented to the animal. After an inter-trial interval of 3 min, one of the two familiar objects was replaced by a novel, previously unseen object (with distinctive different shape, color and texture), as presented to the animal during the second session (test phase). For both sessions object exploration time was measured and a discrimination index was calculated for each animal and expressed as follows:

[(time novel object - time familiar object)/(time novel object + time familiar object)] X 100

Testing cage was wiped clean with 0.1% acetic acid and dried after each trial.

3.1.5 Experiment 2

This study was conducted in adult male rats. These animals derived from a second cohort of breeding for which animals were mated and dams were stressed as described for experiment 1. The litters were composed of 5 males and 5 females and only part of the animals were used for this experiment. Moreover, in order to avoid litter effects we used 4 male rats from each brood (11 Ctrl dams and 10 PNS dams), each devoted to a specific treatment or time point: 1 animal treated with vehicle and sacrificed 24 h after the end of the treatment; 1 animal treated with lurasidone and sacrificed 24 h after the end of the treatment; 1 animal treated with vehicle and sacrificed 2 weeks after the end of the treatment; 1 animal treated with lurasidone and sacrificed 2 weeks after the end of the treatment.

In summary the following experimental groups were present:

- control male rats treated with vehicle (n=11) and sacrificed 24 h or 2 weeks after the end of the treatment (Ctrl/Veh);
- control male rats treated with lurasidone (n=11) and sacrificed 24 h or 2 weeks after the end of the treatment (Ctrl/Lur);
- PNS male rats treated with vehicle (n=10) and sacrificed 24 h or 2 weeks after the end of the treatment (PNS/Veh);
- PNS male rats treated with lurasidone (n=9) and sacrificed 24 h or 2 weeks after the end of the treatment (PNS/Lur);

For both experiments (1 and 2), hippocampus and prefrontal cortex were dissected, frozen on dry ice and stored for later analyses. Dissections were performed according to the atlas of Paxinos and Watson (Paxinos and Watson, 1996). In detail, the hippocampus was dissected from the whole brain, while the prefrontal cortex was dissected from 2-mm thick slices (prefrontal cortex defined as Cg1, Cg3, and IL subregions corresponding to the plates 6–9).

3.1.6 Drug administration

Lurasidone used in experiment 2 was prepared by suspending the drug at a concentration of 3 mg/ml in a 1% hydroxyethylcellulose solution. The drug or vehicle was administered via oral gavage (*per os*) in the amount of 1 ml/kg body weight. We selected the dose and route of delivery based on previous studies demonstrating the antidepressant and procognitive actions of lurasidone (Ishibashi et al., 2010; Ishiyama et al., 2007; Tarazi and Riva, 2013). Starting at PND35, a

group of Ctrl and PNS male rats were treated with vehicle or lurasidone at the dose of 3 mg/kg for 14 days (until PND49).

3.1.7 RNA Preparation for qRT-PCR and analysis of mRNA levels

Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy), according with the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (qPCR) to assess mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination.

Table 1: Sequences of forward and reverse primers and probes used in qRT-PCR analysis and purchased from Eurofins MWG Operon (Germany)

Gene	Forward Primer	Reverse Primer	Probe
Total Bdnf	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Bdnf exon I	GGGAGACGAGATTTTAAGACACTG	GTCATCACTCTTCTCACCTGG	TTGTGGCTTTGCTGTCCTGGAGA
Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGAGA	AGCTTTGTCAGTTGGTAAAACCGTTGC
D1	GTCTGTCCTTATATCCTTCATCCC	ATACGTCCTGCTCAACCTTG	ACAGTTGTCATCCTCGGTGTCCTC
D2	ACCACTCAAGGGCAACTG	TGACAGCATCTCCATTTCCAG	AGCATCCATTCTCCGCCTGTTCA
5HT1a	CTACACCATCTACTCCACTTTCG	TCTTTTCCACCTTCCTGACAG	CGCTTTCTATATCCCGCTGTTGCTCA
5HT1b	GTCACCTCCATTAACTCCCG	AGACTCGCACTTTGACTTGG	ACGTACACAGGAGACCCGGACT
5HT2a	TCAACTCCAGAACCAAAGCC	CAAAGTTGTCATCGGCAAGC	CCTACAGATATGGTCCACACGGCAA
5HT2b	CCAGCCCAGACCATTTCTAA	TTGCAGGAATCACACAGAGC	TGAACAGAGAGCCTCAAAGGTCCT
5HT7	ACCTGCTGAGTGGCTTCCTA	GATGGAGCCGATCACAACTT	AGATCAACTATGGCAGAGTGGAGA
36B4	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC	AAGGCCTTCCTGGCCGATCCATC
Dnmt3a	CCGAAACATCGAGGACATTT	TGGTACCCATCGTCATCGTA	CTGTAAGAACTGCTTCTTGGAGTG
Dnmt1	GAAAGGTGGCAAGGTCAATG	CAGTAGAGCAGGTTGATGTCG	CCTGAGAACACCCACAAGTCCATCC
Hdac1	GGCTATACCATCCGTAATGTCG	TTGGAAGGGCTGATGTGAAG	TGGACACAGAGATCCCTAATGAGCTACC
Hdac2	TGCTCGATGTTGGACGTATG	TCTGGAGTGTTCTGGTTTGTC	TGCAGTTGCCCTTGATTGTGAAATTCC
Hdac5	TGAGAACGGCTTTACTGGC	GATGTTAGGCAGAGAAGGAGAC	CGTGTAGAGGCTGAACTGGTTTGGG

RNA was analyzed 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 (36B4). We choose 36B4 as internal standard for gene expression analyses since its expression was not affected by developmental changes and it was not altered by prenatal stress.

Probe and primer sequences of long 3'-UTR *Bdnf* (Assay id: Rn02531967_s1), *Bdnf* exon IV (Assay id: Rn01484927_m1), *Bdnf* exon VI (Assay id: Rn01484928_m1) and *Gadd45β* (Fwd primer:

GCTGCGACAATGACATTGACATC; Rev primer: CTCGTTTGTGCCTAGAGTCTCT) were purchased from Life Technologies (Monza, Italy) and are available on request, while the other TaqMan gene expression assays were purchased from Eurofins MWG-Operon (Germany) and are summarized in Table 1.

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Relative target gene expression was calculated according to the 2(-Delta Delta C(T)) method.

3.1.8 Protein extraction and western blot analysis

Western blot analysis was used to investigate BDNF protein levels in the prefrontal cortex tissue of animals that underwent gestational stress and controls. Protein analyses were performed on the crude membrane fraction (P2), in order to investigate changes in BDNF levels within a cellular compartment where they represent a ready-to-use pool of the neurotrophin that may be critically involved in synaptic function and plasticity (Poo, 2001).

Brain samples from the different experimental groups were manually homogenized in a glass-glass potter in ice-cold 0.32 M sucrose buffer (pH 7.4) containing 1 mM HEPES, 0,1 mM EGTA and 0,1 mM phenylmethylsulfonyl fluoride (PMSF), in the presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors. The homogenate was clarified at 1000g for 10 min. The resulting supernatant was then centrifuged at 9000g for 15 min. The supernatant was discarded while the pellet (P2), corresponding to the crude membrane fraction, was resuspended in a buffer (20 mM HEPES, 0,1 mM dithiothreitol, 0,1 mM EGTA) supplemented with protease and phosphatase inhibitors. This fraction is enriched in synaptic proteins as previously demonstrated (Fumagalli et al., 2008). Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Equal amounts of protein (10 mg) were run under reducing conditions on Any Kd Criterion TGX precast gels (Bio-rad Laboratories) and then electrophoretically transferred onto Polyvinylidene fluoride (PVDF) membranes. Unspecific binding sites were blocked for 2 h in 10% nonfat dry milk in Tris-buffered saline, and membranes were then incubated with two different primary antibodies which recognize the mature form of the neurotrophin (mBDNF: 14 kDa; 1:500, Santa

Cruz Biotechnology) and its precursor (proBDNF: 32 kDa; 1:2500, GeneTex) in blocking solution at 4°C over night. Membranes were washed for 2 h with Tris-buffered saline and incubated for 1 h at room temperature with a peroxidase-conjugated antirabbit IgG (1:1000 for mBDNF; 1:5000 for proBDNF) in Tris-buffered saline and immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (PerkinElmer) and the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were standardized using b-actin as the control protein, which was detected by evaluating the band density at 43 kDa after blocking the membranes with 10% nonfat dry milk and probing them with a polyclonal antibody (1:10000, Sigma-Aldrich) followed by a 1:10000 dilution of peroxidase-conjugated antimouse IgG (Sigma-Aldrich). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used. b-Actin was employed as an internal standard because its expression is not regulated by the experimental paradigm used.

3.1.9 Extraction of DNA

Isolation of DNA from rat brain samples was performed using the DNeasy Tissue kit (Qiagen; Hilden; Germany). Genomic DNA was shared by sonication and quantified by fluorometric analysis (Qubit® 2.0 Fluorometer, life technologies; Darmstadt; Germany). The procedure was performed in accordance with the instructions of the manufacturer.

3.1.10 Statistical analysis

Changes produced by ELS (PNS in rats, SPR in monkeys or high ELS in humans) were analyzed with Student's *t* test (Figs. 4, 5, 10, 12, 13, 14 and 29).

Changes produced by prenatal exposure to stress and age, acute stress or lurasidone treatment were analyzed using a two-way ANalysis Of VAriance (ANOVA), followed by Fisher's LSD post-hoc comparisons. SPSS for Mac OS X (Release 19.0.0) was used to statistically analyze the data.

Person product moment correlation (r) between corticosterone plasma levels and brain *Nr3c1* mRNA levels was performed to evaluate the correlation between the two variables (Fig.16).

The significance of the overlap presented in Fig. 27 was calculated with a hypergeometric test using R (version 2.14.2).

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

3.2 RHESUS MONKEYS

Male rhesus monkeys (*Macaca mulatta*) were reared at the breeding facility of the Animal Center of the Laboratory of Comparative Ethology, National Institute of Child Health and Human Development (NICHD; Head: Stephen Suomi, Poolsville, MD). At birth, the animals were randomly divided into two groups resulting in different early-life social and rearing experience: "mother-reared" (MR) monkeys were raised by their biological mother in a social group, while "surrogate peer-reared" (SPR) monkeys were reared with an inanimate surrogate. For the first month of life, the SPR monkeys were placed in a nursery until they were able to drink milk from a bottle by themselves, at which point they were transferred to a cage with their surrogate mother. After 37 days of age, the individual housing with the surrogate mother was supplemented by 2h/day of social interaction in a playroom with age-matched peers. Both rearing conditions have been described in detail previously (Shannon et al., 1998). At 7 months of age, the SPR animals were socially housed in a large, mixed-sex peer group and were maintained under identical physical and social conditions. All environmental conditions, procedures and handling of animals were in strict compliance with the requirements of the Institutional Animal Care and Use Committee, and all experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Venous blood samples were obtained from 14-30 days old (born between 2010 and 2011), and from 2 years old (born between 2006 and 2009) monkeys. All samples were processed and analyzed by experimenters who were blind to rearing conditions.

3.2.1 Separation of CD3+ T cells from monkey peripheral blood

For the 2-year old monkeys, 20 ml of blood was drawn into EDTA-coated tubes and stored at 4°C overnight. For the 14- to 30-day old monkeys, 3 ml of blood was perfused in EDTA-coated tubes. Briefly, PBMCs were isolated through centrifugation with Ficoll-Paque (GE Healthcare; Munich; Germany). The PBMCs were than washed twice with HBSS (Invitrogen; Darmstadt; Germany). T cells were isolated from the PBMCs by immunomagnetic isolation using CD3+ Dynabeads (life technologies; Darmstadt; Germany). The beads were washed three times and incubated with the PBMCs for 45 min on a rotator at 4°C followed by a washing step (five times) with PBS/FBS. CD3+ T cells were then stored at −80°C until DNA extraction.

3.2.2 Extraction of DNA

CD3+ T cell DNA was extracted using the Wizard Genomic DNA Purification kit (Promega; Mannheim; Germany). Genomic DNA was shared by sonication and quantified by fluorometric analysis (Qubit® 2.0 Fluorometer, life technologies; Darmstadt; Germany). The above procedure was performed in accordance with the instructions of the manufacturer.

3.3 HUMAN STUDY

3.3.1 Participants

Data were obtained from a cohort of mothers and their infants (n = 180) recruited between 03/2011 and 03/2012 from two obstetric hospitals in the Rhine-Neckar Region of Germany (Mannheim, Ludwigshafen). The mothers were recruited during the third trimester of pregnancy. The study protocol was approved by the Ethics Committee of the Medical Faculty Mannheim of the University of Heidelberg and was conducted in accordance with the Declaration of Helsinki. All mothers provided written informed consent prior to participation.

Inclusion criteria for mothers were: Caucasian descent; main caregiver; German-speaking; age 16 – 40 years. Exclusion criteria were: maternal hepatitis B, hepatitis C or HIV-infection; any current psychiatric disorders requiring inpatient treatment; a history, current diagnosis, of schizophrenia / psychotic disorder, or any substance dependency other than nicotine during pregnancy. Exclusion criteria for infants were: birth weight < 1.500 grams; gestational age at birth < 32 weeks; multiples; any congenital diseases; malformations; deformations or chromosomal abnormality.

3.3.2 Assessment of ELS and selection of extreme groups for the genome-wide analysis

The mothers were assessed using a structured interview and a series of questionnaires in order to collect information concerning a broad range of environmental and sociodemographic risk factors, prenatal medical risk factors, general medical characteristics, and psychosocial risk factors. Cord blood was collected immediately after the birth.

Eight main stressor variables derived from eight different questionnaires were selected to represent a variety of prenatal adversities, and to take three different dimensions of stress into account: maternal psychopathology (primarily depressive and anxiety symptoms), perceived stress and socioeconomic and psychosocial stress. Stressful prenatal adverse conditions were also considered in order to define 10 infants with extremely high and 10 infants with extremely low levels of prenatal ELS, respectively. The sociodemographic and medical characteristics of the mothers and infants in the extreme groups are shown in Table 2. The psychopathology, perceived stress and psychosocial and socioeconomic stress status of the mothers are shown in Table 3. For the comparison of the extreme groups, two-tailed *t*-tests for independent samples were used (SPSS® Statistics 20). The nominal level of significance was set at p=0.05. All data and results are expressed as means ± standard deviation or as a percentage, as appropriate.

3.3.3 Separation of CD34+ cells from human cord blood

Human cord blood was drawn into ethylenediaminetetraacetic (EDTA) coated tubes immediately after birth. CD34+ cells were extracted within 24 h following delivery. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by centrifuging the cord blood with Ficoll-Pague PLUS (GE Healthcare; Munich; Germany) in Leucosep tubes (Greiner Bio-One; Frickenhausen; Germany). CD34+ cells were then isolated from the PBMCs by immunomagnetic isolation using the Dynal CD34 Progenitor Cell Selection System (life technologies, Darmstadt, Germany) in accordance with the manufacturer´s instructions. The CD34+ cells were then stored at −80°C until DNA extraction.

3.3.4 Extraction of DNA

Genomic DNA was extracted from CD34+ cells using the Qiagen Blood Mini Kit (Qiagen; Hilden; Germany). Genomic DNA was shared by sonication and quantified by fluorometric analysis (Qubit® 2.0 Fluorometer, life technologies; Darmstadt; Germany). The above procedure was performed in accordance with the instructions of the manufacturer.

Table 2: Demographic characteristics and general medical status of mothers and infants in the methylome analysis.
wpma = weeks postmenstrual age; BMI = body mass index; ns = not significant; g = gram

Variable	High prenatal ELS (n = 10)	Low prenatal ELS (n = 10)	p value
Maternal Age (in years)	24.10 ± 5.43	34.00 ± 3.30	0.000
Smoking during early pregnancy (4^{th} to 12^{th} wpma; %)	70 %	12.5 %	0.013
Cigarettes in total (4^{th} to 12^{th} wpma) Range of cigarettes smoked	297.70 ± 412.68 0-1092	3.00 ± 8.50 0-24	0.05
Smoking during late pregnancy (3^{rd} trimenon, %)	40 %	0 %	0.037
Cigarettes per day (3^{rd} trimenon) Range of cigarettes smoked	3.30 ± 6.31 0-17	0 0	ns
Alcohol intake during early pregnancy (4^{th} to 12^{th} wpma; %)	30 %	75 %	ns
Total alcohol intake (4^{th} to 12^{th} wpma in g)	75.50 ± 187.18	23.50 ± 22.62	ns
Alcohol during late pregnancy (3^{rd} trimenon; %)	0%	0 %	ns
Primiparous	30 %	37.5 %	ns
Number of risk factors in the maternity log	4.60 ± 2.38	3.17 ± 01,51	ns
Pre-Pregnancy BMI	25.53 ± 7.08	21.69 ± 4.57	ns
Gestational diabetes (%)	20 %	0%	ns
Gestational age at birth (wpma)	38.70 ± 2.003	39.63 ± 1.60	ns
Infant's Gender (%), male	50 %	37.5 %	ns

Table 3: Psychopathology, socioeconomic, psychosocial and perceived stress of the extreme group mothers.
[1] the first eight main variables of the principal component analysis (PCA)
[2] the twelve prenatal stressors that generate the adversity score as the ninth main variable of the PCA

Variable	High prenatal ELS (n = 10)	Low prenatal ELS (n = 10)	p value
Maternal psychopathology			
EPDS Score[1]	15.40 ± 4.95	2.13 ± 1.25	0.000
STAI-S Score[1]	52.60 ± 13.32	32.62 ± 4.81	0.001
STAI-T Score[1]	50.90 ± 10.50	28.75 ± 4.53	0.000
ASQ Score[1]	5.40 ± 1.84	0.25 ± 0.46	0.000
M.I.N.I. Diagnosis[2]			
none	20 %	100%	0.000
depressive disorders	50 %	0%	0.015
anxiety disorder	10 %	0%	ns
Current psychiatric disorder[2] (%)			
none	30%	100%	0.001
depressive disorder	50%	0%	0.015
anxiety disorder	10 %	0%	ns
Perceived stress			
PSS Score[1]	32.70 ± 6.93	15.25 ± 3.92	0.000
PDQ Score[1]	23.70 ± 8.06	8.63 ± 4.37	0.000
Socioeconomic and psychosocial stress			
LES-negative events Score[1]	8.50 ± 6.98	1.63 ± 1.06	0.013
Soz-U Score[1]	37.80 ± 10.68	48.00. ± 6.78	0.026
Living without a partner[2] (%)	40 %	0%	0.037
Encouragement (Partner) [2] (%)	70 %	100 %	ns
Separation(s) in the last year[2]	50 %	0%	0.015
Daily arguments[2]	20 %	0%	ns
Physical conflicts within the preceeding 12 months[2]	60 %	0%	0.005
Composition of household >one person /room[2]	30 %	0%	0.037
No graduation[2] (%)	20 %	0%	ns
No professional education[2] (%)	40 %	0%	0.037
Monthly income per household ≤ 1750 Euro[2] (%)	70 %	0%	0.001
Financial debt[2] (%)	50 %	0%	0.015

3.4 MEDIP ANALYSIS OF GENOME-WIDE PROMOTER DNA METHYLATION

The procedure used for the MeDIP analysis was adapted from previously published protocols, as described in previous studies (Provencal et al., 2012). Briefly, 2µg of DNA were sonicated, and methylated DNA was immunoprecipitated using 10µg of anti-5-methyl-cytosine (Eurogentec; Cologne; Germany). The monkey study involved DNA pools from (i) 6 MR and 5 SPR monkeys sampled twice (i.e. at postnatal days 14 and day 30) and from (ii) pools of DNA of 6 MR and 4 SPR monkeys sampled at age 2 years. For the rat study, samples of 4 Ctrl and 4 PNS rats (for each sex and brain region) sacrificed on PND62 have been used. The DNA-antibody complex was immunoprecipitated with 5 mg of protein G, and the methylated DNA was resuspended in 250 ul of digestion buffer (50 mM TRisHCl pH8; 10 mM EDTA; 0.5% SDS) and treated with 40 mg of proteinase K overnight at 55°C. The input and bound fractions were purified. Specificity for methylated DNA and the absence of unspecific binding were validated through PCR analysis of an unmethylated and a methylated control gene (respectively, *β-actin* and *H19*).

The input and bound fraction were purified and then amplified using the Whole Genome Amplification Kit (Sigma-Aldrich; Schnelldorf; Germany). The amplified input and bound fractions were labeled for microarray hybridization with Cy3-dUTP and Cy5-dUTP, respectively, using the CGH Enzymatic Labeling Kit (Agilent Technologies; Waldbronn; Germany) in accordance with the manufacturer´s instructions. For the rhesus macaque experiment, DNA samples were hybridized in triplicates.

3.4.1 MeDIP microarray design, hybridization, scanning and analysis

Custom designed tiling arrays were used (Agilent Technologies; Waldbronn; Germany). For the human studies, a 400K promoter tiling array was designed in 2009 using the eArray array design platform from Agilent. Probes were selected to tile all known gene promoters, i.e. intervals roughly 1200 bp upstream to 400 bp downstream of the transcription start sites of genes described in the Ensembl database (version 55) at 100 bp-spacing. For the analysis of genome-wide DNA methylation in *Macaca mulatta*, custom 400K promoter tiling array designs were used for the T cells studies (Agilent Technologies; Waldbronn; Germany). Microarray probe sequences were selected to tile all gene promoter regions defined as the genomic interval from -2000bp upstream to 400bp downstream of each transcription start site as defined for the Rhesus Macaque by the Ensembl database (version 64.10) (http://www.ensembl.org).

For the rat studies, a custom 400K promoter tiling microarray design was used. Probes were selected to tile all known gene promoters, i.e. intervals from -1000bp upstream to 200bp downstream around each transcription start site defined in the Ensembl database (version 62). Probes were placed approximately every 100bp. All genomic coordinates are given with respect to the rn4 (RGSC 3.4).

All the steps of the hybridization, washing scanning, and feature extraction procedures were performed in accordance with the Agilent Technologies protocol for chip-on-chip analysis. Probe intensities were extracted from scan images using Agilent´s Feature Extraction 9.5.3 Image Analysis Software. Extracted microarray intensities were processed and analyzed using the R software environment for statistical computing. Log-ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray. Microarrays were normalized to one another using quantile-normalization under the assumption that all samples had identical overall methylation levels. Methylation levels were estimated from normalized probe intensities by applying a Bayesian deconvolution algorithm. Promoter methylation levels were obtained by calculating the median estimated methylation level across each promoter. Promoters were defined as the region within 2000 to -400bp of the transcription start sites of each gene. Differential methylation between groups of samples was determined in several stages to ensure both statistical significance and biological relevance. In the first stage, linear models implemented in the 'limma' package of Bioconductor were used to combine the two dye labeling schemes from the dye swaps and to compute a modified t-statistic for each probe. An individual probe was classified as being differentially methylated if the *p*-value of its *t*-statistic was a maximum of 0.05 (uncorrected for multiple testing), and the associated difference of means between the groups was at least 0.5. Given that the DNA samples were sonicated prior to hybridization, the assumption was made that probes within 500bp should show approximate agreement. Therefore, the genome was into 1000bp regions and the significance of enrichment for high or low *t* statistics of probes within each region (containing at least 1 probe) was calculated. Significance was determined using the Wilcoxon rank-sum test comparing *t* statistics of the probes within the region against those of all the probes on the microarray and then adjusted to obtain false discovery rates (FDR) for each region. A probe was classified as being differentially methylated if it satisfied each of the following criteria:

1. the significance of its *t*-statistic was a maximum of 0.05 and the difference of means between the groups was at least 0.5

2. it belonged to a region whose FDR was a maximum of 0.2

A promoter was classified as being differentially methylated if it contained a differentially methylated probe.

Each row in a heat map shows the relative normalized intensities of a selected probe across all microarrays in the study. Colors are assigned according to a gradient from red to green such that high relative intensities appear as red and low relative intensities appear as green. Rows and columns are clustered using the Ward algorithm with correlation as the distance metric. The unsupervised heat map is based on the 500 most variable probes across all microarrays. The supervised heat map is based on the 500 probes most strongly associated with sample group assignment.

The DNA methylation data were deposited in GEO.

3.4.2 Validation

Gene-specific validation of the MeDIP data was performed applying quantitative-real time PCR (QPCR) to the samples used in the MeDIP microarray analysis. QPCR was performed in 1X Power SYBR Green Master Mix (life technologies; Darmstadt; Germany) for the human analysis, and in Light Cycler 480 SybrGreen I Master (Roche Diagnostics; Penzberg; Germany) for the rat and monkey analyses using 4 - 10ng of DNA and 1.5 - 4 µM gene specific primers which were designed using Primer 3 (http://frodo.wi.mit.edu/) software. Relative enrichment of triplicate reactions was determined after normalizing from the input fraction in each sample using the $2^{-\Delta\Delta Ct}$ method. All data are expressed as group means ± SEM. The Graphpad 5 software (La Jolla; CA; USA) was used to perform one-sample *t*-test statistical analyses.

3.4.3 Gene-based analysis

Morc1 genotypes were investigated using GWAS data of a previous study of MDD. The patients were recruited from consecutive admissions to the Department of Psychiatry of the University of Bonn, and the control subjects were drawn from three population-based epidemiological studies in Germany, as described elsewhere (Rietschel et al., 2010). All samples were individually genotyped using either Illumina HumanHap 550v3 or Illumina human 610 W quad BeadChips (San Diego; California; USA). After stringent quality control, the data of 593 patients with a DSM-IV diagnosis of MDD and 1307 control subjects were analyzed. In the present study, Entrez Gene (NCBI build 36.3: http://www.ncbi.nlm.nih.gov/gene/) was searched for single nucleotide

polymorphisms (SNPs) across the *Morc1* gene. To test for association between MDD and the whole set of *Morc1* gene variants in the dataset, the set-based test was performed (with default options and 106 permutations), as implemented in PLINK (v1.0.7).

4. RESULTS

4.1 COGNITION AND NEURONAL PLASTICITY

4.1.1 Animals exposed to prenatal stress displayed cognitive impairment when tested in the object recognition task

We first evaluated the effects of prenatal stress on cognitive functionality by subjecting adult animals to the object recognition test (ORT), since cognitive disabilities are one of the common symptoms that characterize different psychiatric disorders (Disner et al., 2011; Lapiz-Bluhm et al., 2008; Lesh et al., 2011; Lewis et al., 2012). This test was performed in both male and female adult rats whose mothers underwent immobilization stress during the last week of gestation (PNS) as compared to control animals (Ctrl).

The ORT is a test based on the spontaneous behavior of rodents that measures the memory of a previously presented, and thus now familiar object. It is widely used to assess cognitive functions, particularly visual working memory and recognition memory, in animal models of disorders of the Central Nervous System (Grayson et al., 2007; Lyon et al., 2012), taking advantage of the natural tendency of rodents to explore novelty. The choice to explore the new object reflects the use of these two types of memory.

As shown in Fig. 4, prenatal exposure to stress led to a significant cognitive deficit in both adult male and female rats (PND80). Ctrl and PNS animals, of both genders, spent the same time exploring, during the familiarization phase, the two identical objects (right and left), showing no preference for either of the two positions (Fig. 4a, b). During the test phase (Fig. 4c, d) Ctrl rats definitely differentiated the familiar and the novel object as they spent significantly more time exploring the latter compared to the first one (males: +300% New vs Fam, $p<0.01$, Student's *t*-test; females: +164% New vs Fam, $p<0.01$, Student's *t*-test). On the contrary, the ability to discriminate familiar and novel object was abolished by prenatal exposure to stress, as also shown by the significant reduction in the discrimination index, an impairment that seems to be more severe in female rats (males: $p<0.05$, Student's *t*-test, Fig. 4e; females: $p<0.01$, Student's *t*-test, Fig. 4f).

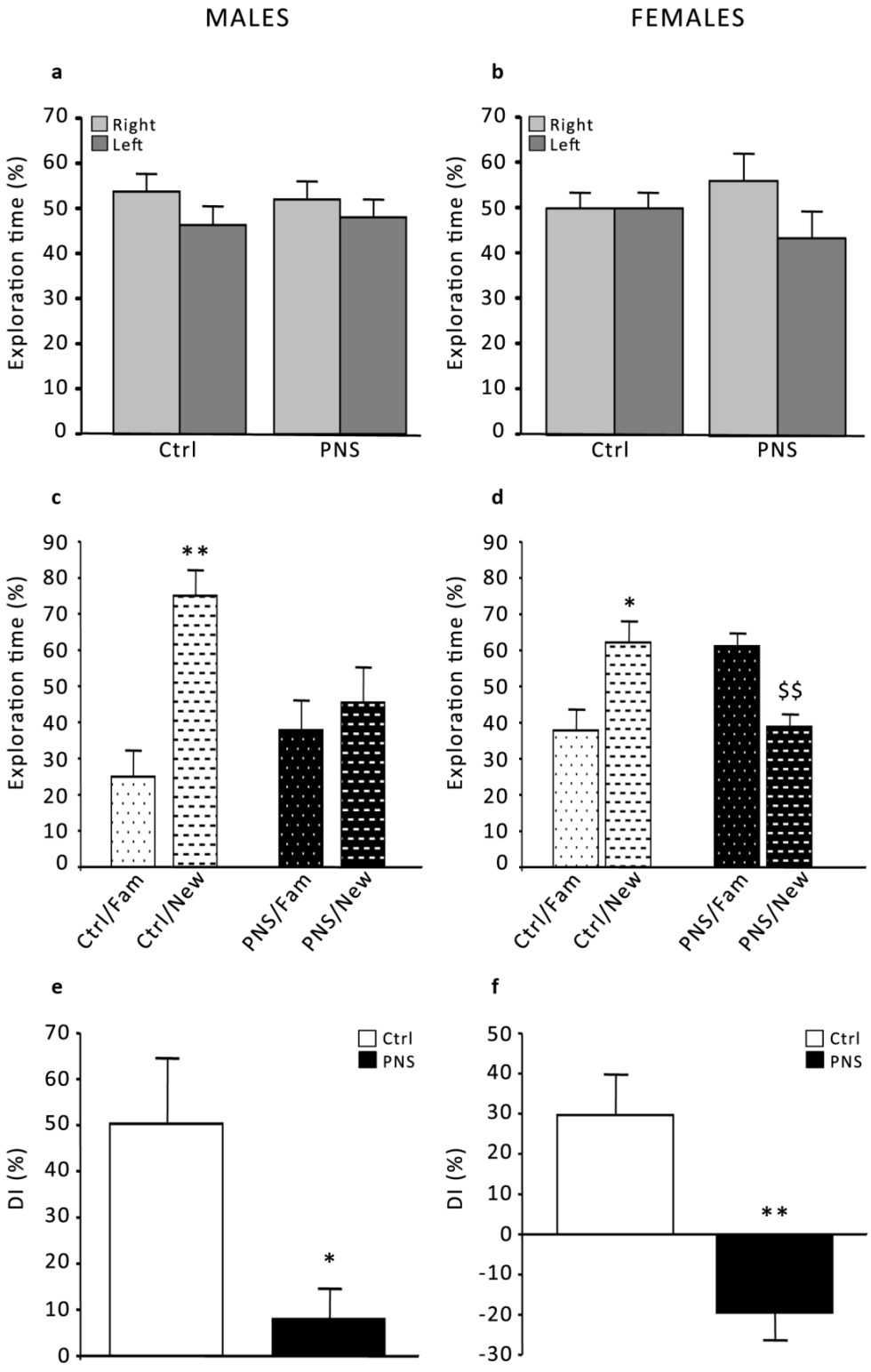

Figure 4 *Effect of prenatal stress (PNS) on recognition memory measured at adulthood (PND80) with the object recognition test.* (a, b) Percentage of exploration time of identical objects during the familiarization trial. (c, d) Percentage of exploration time of the familiar (Fam) vs. the novel (New) object during the test phase. (e, f) Discrimination index calculated as indicated in Methods. Data are expressed as mean ± SEM of at least 4 animals per group.

(c, d) $^{*}p<0.05$ and $^{**}p<0.01$ vs. Ctrl/Fam; $^{\$\$}p<0.01$ vs. PNS/Fam (Student's *t* test)

(e, f) $^{*}p<0.05$ vs. Ctrl (Student's *t* test)

4.1.2 Neuronal plasticity: anatomical and temporal characterization of the BDNF system in rats exposed to prenatal stress

Several studies have shown that impairment of cognition could be ascribed to alterations of neuronal plasticity. The neurotrophin Brain-Derived Neurotrophic Factor (BDNF) has emerged as an important player in such mechanisms as it regulates the formation of synaptic connections in an activity-dependent way, leading to enhanced long-term potentiation and to a facilitation of memory formation (Gomez-Pinilla and Vaynman, 2005; Lu et al., 2008; Savitz et al., 2006). We thus focused our attention on this neurotrophin, which has also emerged as an important mediator of long-term functional deterioration associated with mental illness (Calabrese et al., 2011; Castren and Rantamaki, 2010; Chourbaji et al., 2011; Cirulli et al., 2009; Duman and Monteggia, 2006).

We first analyzed the mRNA levels of total *Bdnf* and its transcripts containing the long 3'-UTR, which represent a neurotrophin pool that undergo dendritic targeting and may contribute to synaptic function (An et al., 2008). We performed the analysis in adult (PND62) male and female rats exposed to prenatal stress (PNS) focusing on the hippocampus and the prefrontal cortex, two brain regions that play a key role in psychopathology and in recognition memory. As shown in Fig. 5, total *Bdnf* mRNA levels were not altered by PNS at adulthood, with the exception of female hippocampus where gestational stress produced a significant decrease of the neurotrophin expression (-18% vs. Ctrl, $p<0.05$, Student's *t*-test) (Fig. 5a). In contrast, long 3'-UTR *Bdnf* expression was significantly reduced both in adult male and female PNS-animals within the prefrontal cortex (males: -28% vs. Ctrl, $p<0.05$, Student's *t*-test; females: -22% vs. Ctrl, $p<0.05$, Student's *t*-test) (Fig. 5d).

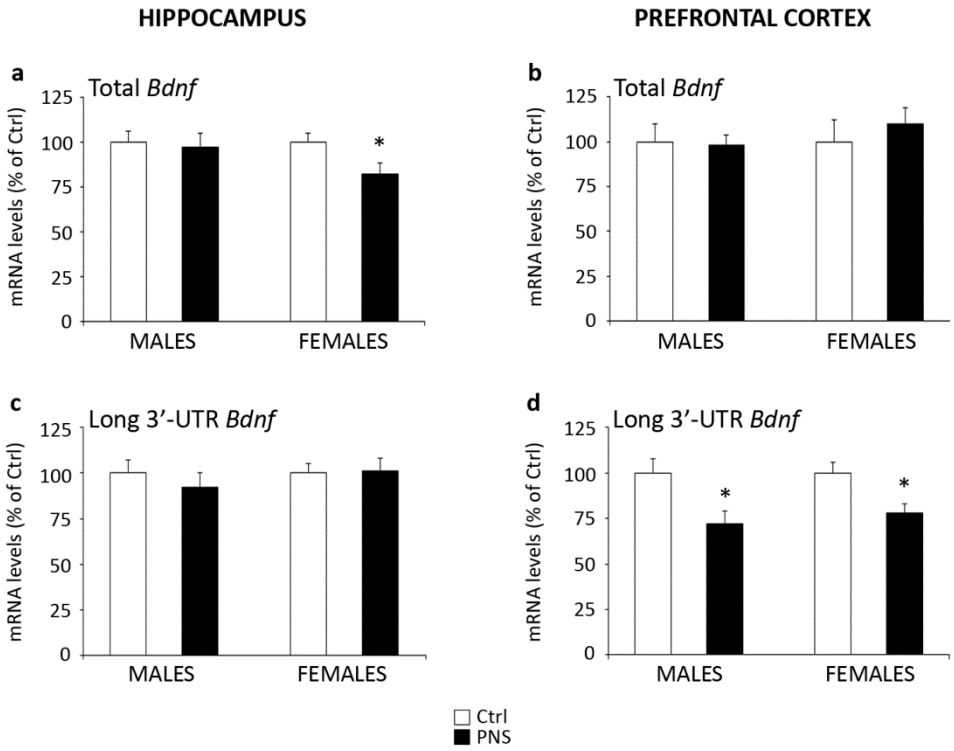

Figure 5 *Exposure to prenatal stress reduces Bdnf expression in adult rats.* The mRNA levels of total (a, b) and long 3'-UTR (c, d) *Bdnf* were analyzed in adult male and female rats exposed to prenatal stress (PNS), as compared to control animals (Ctrl). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at lease 6 animals per group.
* $p<0.05$ vs. Ctrl of the same sex (Student's t test).

In order to establish whether the neurotrophin expression could be affected by PNS earlier in development, we performed a time-course analysis from birth to adulthood: male and female pups were killed immediately after birth (PND1), at the end of the first week of life (PND7), at weaning (PND 21), during adolescence (PND40), as compared to young adulthood (PND62).

As shown in Fig. 6, total *Bdnf* as well as *Bdnf* with the long 3'-UTR have a comparable expression profile in male and female Ctrl rats within the hippocampus. It is possible to observe an increase of the total expression of the neurotrophin during development, which seems to be compromised by the exposure to PNS in a sex-specific manner (males: $F_{1,57}=0.008$, $p>0.05$, 2-way ANOVA; females: $F_{1,59}=10.699$, $p<0.01$, 2-way ANOVA) (Fig. 6a, b). In particular, female rats exposed to PNS showed a significant reduction of total *Bdnf* expression starting from PND40 (PND40: $p<0.01$ and PND62: $p<0.05$). Conversely, when we looked at the expression of *Bdnf* with the long 3'-UTR, although we did not observe any change at adult level (Fig. 6c), we found a significant PNS effect in males ($F_{1,57}=10.464$, $p<0.01$, 2-way ANOVA) and a significant PNS x AGE interaction in females ($F_{3,58}=3.218$, $p<0.05$, 2-way ANOVA). In fact, while male rats exposed to gestational stress

displayed an overall reduction of long 3'-UTR *Bdnf* levels throughout the postnatal development, which is however significant only at PND21 and PND40 (p<0.01 and p<0.05, respectively), PNS females showed a transient increase of its levels immediately after birth, to reach control levels after (PND7: p<0.05 and PND21: p<0.05) (Fig. 6c, d).

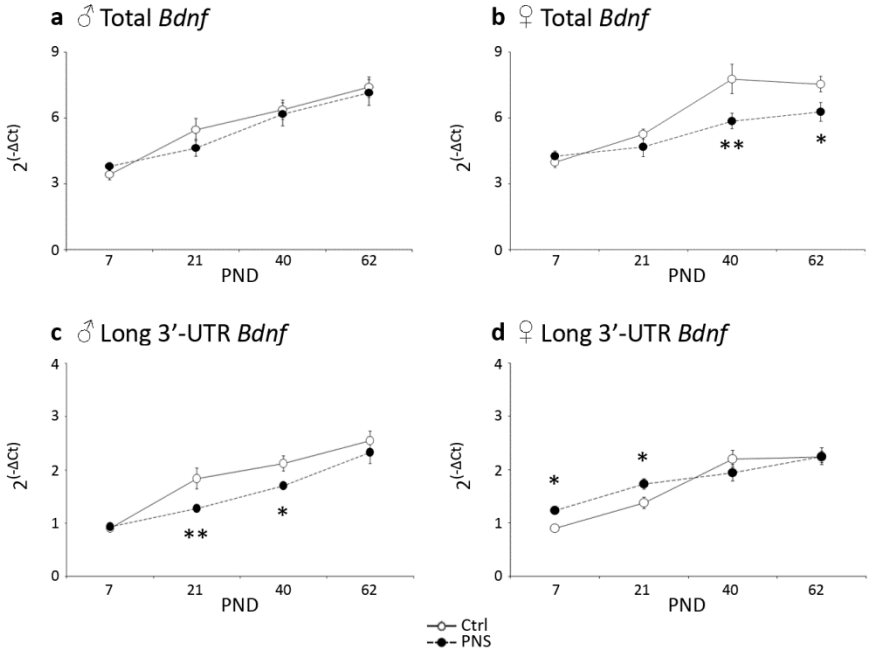

Figure 6 *Postnatal developmental changes of hippocampal Bdnf expression and modulation by gestational stress.* Postnatal developmental analysis of the mRNA levels for total (a, b) and long 3'-UTR (c, d) *Bdnf* were carried out in the hippocampus of male (a, c) and female (b, d) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 5 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p$<0.05 and $^{**}p$<0.01 vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

Moreover, in order to gain further insight into PNS-induced *Bdnf* changes during development, we analyzed the mRNA levels of three major brain splice variants of the neurotrophin, namely exon I, IV and VI. We found that adult male rats exposed to gestational stress displayed a significant reduction of all *Bdnf* exons analyzed, an effect that also became manifest at adulthood. Once again, in Ctrl animals, the expression of *Bdnf* isoforms increased with age (Fig. 7). However, in male rats we found a significant effect of PNS exposure (exon I: $F_{1,55}$=9.604, p<0.01, 2-way ANOVA; exon VI: $F_{1,58}$=5.673, p<0.05, 2-way ANOVA) and also a significant interaction PNS x AGE for exon VI ($F_{3,58}$=4.438, p<0.01, 2-way ANOVA) (Fig. 7a, e). Indeed, PNS males showed, at PND62, a significant reduction of exon I and VI expression (p<0.01 and p<0.01, respectively) (Fig. 7a, e). When we looked at female rats, we observed a different pattern of expression (Fig. 7b, d, f). We

found a significant interaction PNS x AGE for exon I, IV and VI ($F_{3,59}$=7.768, p<0.001, 2-way ANOVA; $F_{3,59}$=2.917, p<0.05, 2-way ANOVA; $F_{3,59}$=3.684, p<0.05, 2-way ANOVA, respectively), as the expression of all these transcripts is higher in PNS animals in the first period after birth (at least until PND21, but the effect is not statistically significant) and then significantly falls below Ctrl levels later. In fact, exposure to PNS produced a significant reduction of *Bdnf* exon I mRNA levels starting from PND40 (PND40: p<0.01 and PND62: p<0.01), while *Bdnf* exon IV and VI show a selective decrease at PND62 (exon IV: p<0.05 and exon VI: p<0.01) (Fig. 7b, d, f).

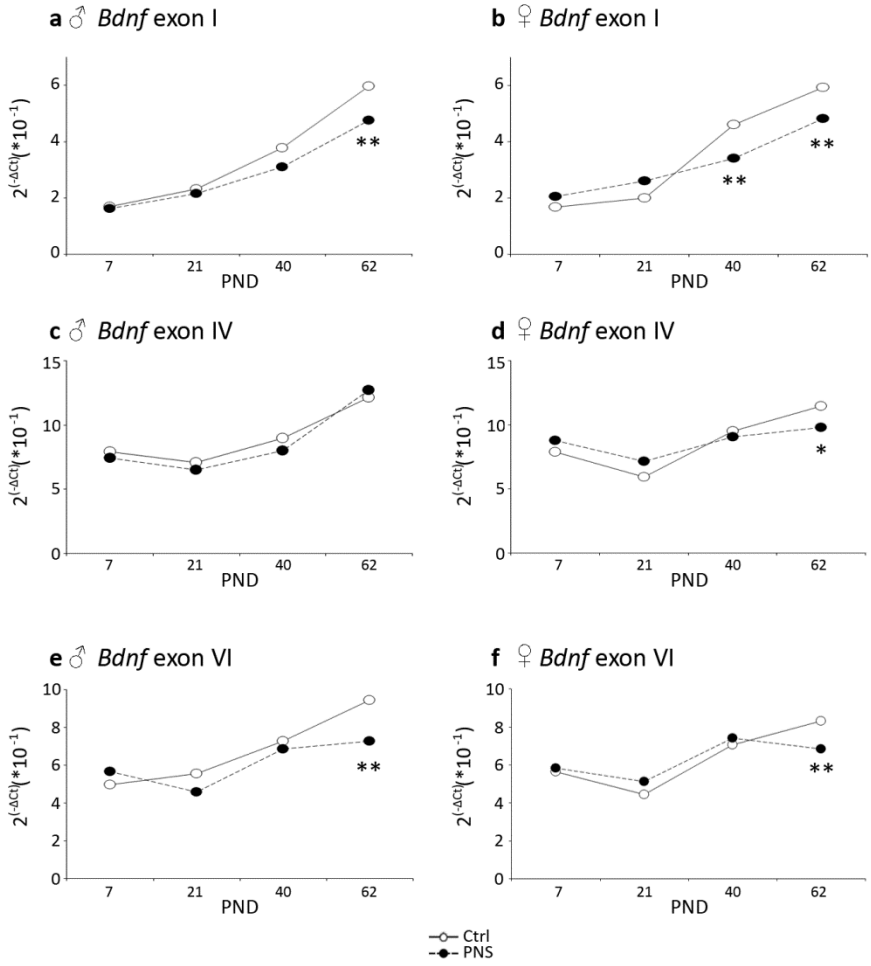

Figure 7 *Postnatal developmental changes of hippocampal Bdnf exons expression and modulation by gestational stress.* Postnatal developmental analysis of the mRNA levels for *Bdnf* exon I (a, b), *Bdnf* exon IV (c, d) and *Bdnf* exon VI (e, f) were carried out in the hippocampus of male (a, c, e) and female (b, d, f) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 5 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene). $^{*}p$<0.05 and $^{**}p$<0.01 vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

We next analyzed the developmental expression of *Bdnf* in the prefrontal cortex. We found that, in Ctrl animals, total *Bdnf* mRNA levels sharply increased from PND1 to PND21 and then leveled off to adult levels, with a similar profile in both sexes (Fig. 8a, b). The developmental pattern of *Bdnf* expression was substantially similar in PNS animals (Fig. 8a, b), suggesting that global *Bdnf* expression was not altered by gestational stress. The expression of long 3'-UTR *Bdnf* also showed a progressive increase during postnatal life, reaching a plateau between adolescence and adulthood, with no differences between males and females (Fig. 8c, d). Interestingly, this pattern was altered in PNS rats (Fig. 8c, d). In particular, when compared to Ctrl animals, PNS-male rats showed a transient increase during adolescence (PND40: $p<0.05$), followed by a decrease in adulthood (PND62: $p<0.001$) (Fig. 8c). Conversely, the mRNA levels of the long 3'-UTR *Bdnf* of PNS female rats increased from birth until weaning and leveled off thereafter, with a significant decrease, when compared to Ctrl animals, during adolescence (PND40: $p<0.01$) and adulthood (PND62: $p<0.01$) (Fig. 8d).

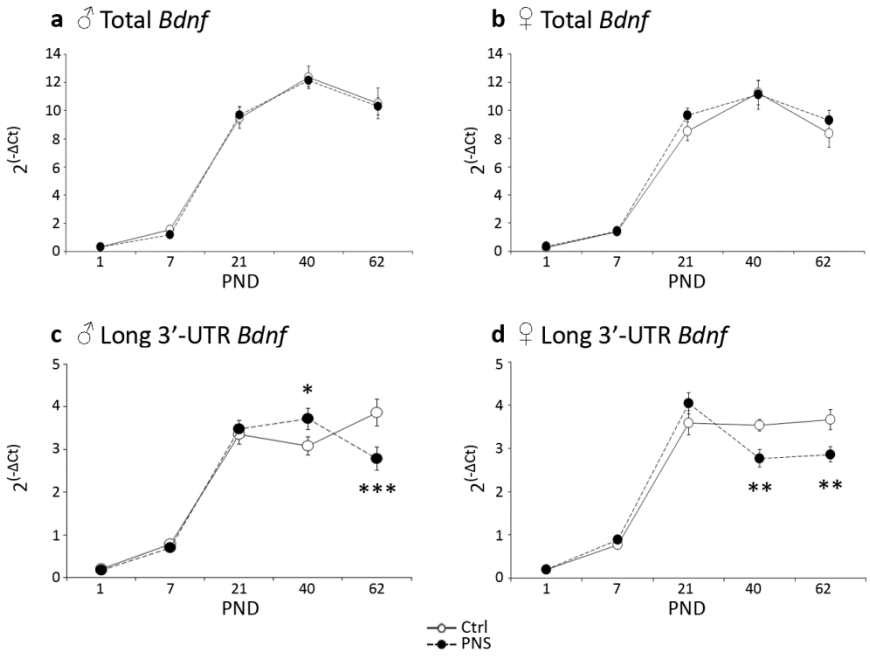

Figure 8 *Postnatal developmental changes of prefrontal cortex Bdnf expression and modulation by gestational stress.* Postnatal developmental analysis of the mRNA levels for total (a, b) and long 3'-UTR (c, d) *Bdnf* were carried out in the prefrontal cortex of male (a, c) and female (b, d) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 5 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

We next deepened the analysis investigating the effect of PNS on the expression of *Bdnf* exon I, IV and VI in the prefrontal cortex (Fig. 9). We found sex- and time-specific changes following gestational exposure to stress (PNS). In particular, in PNS male rats, the expression profile of the *Bdnf* isoforms was similar to the Ctrl counterpart until adolescence, while their mRNA levels were significantly reduced when compared to Ctrl animals at PND62 (exon I: $p<0.001$; exon IV: $p<0.001$; exon VI: $p<0.001$) (Fig. 9a, c, e). In contrast, PNS female rats showed a significant decrease only of *Bdnf* exon I mRNA levels at PND62 ($p<0.05$) (Fig. 9b), while the expression levels of exon IV and VI were not significantly affected by the prenatal manipulation (Fig. 9d, f).

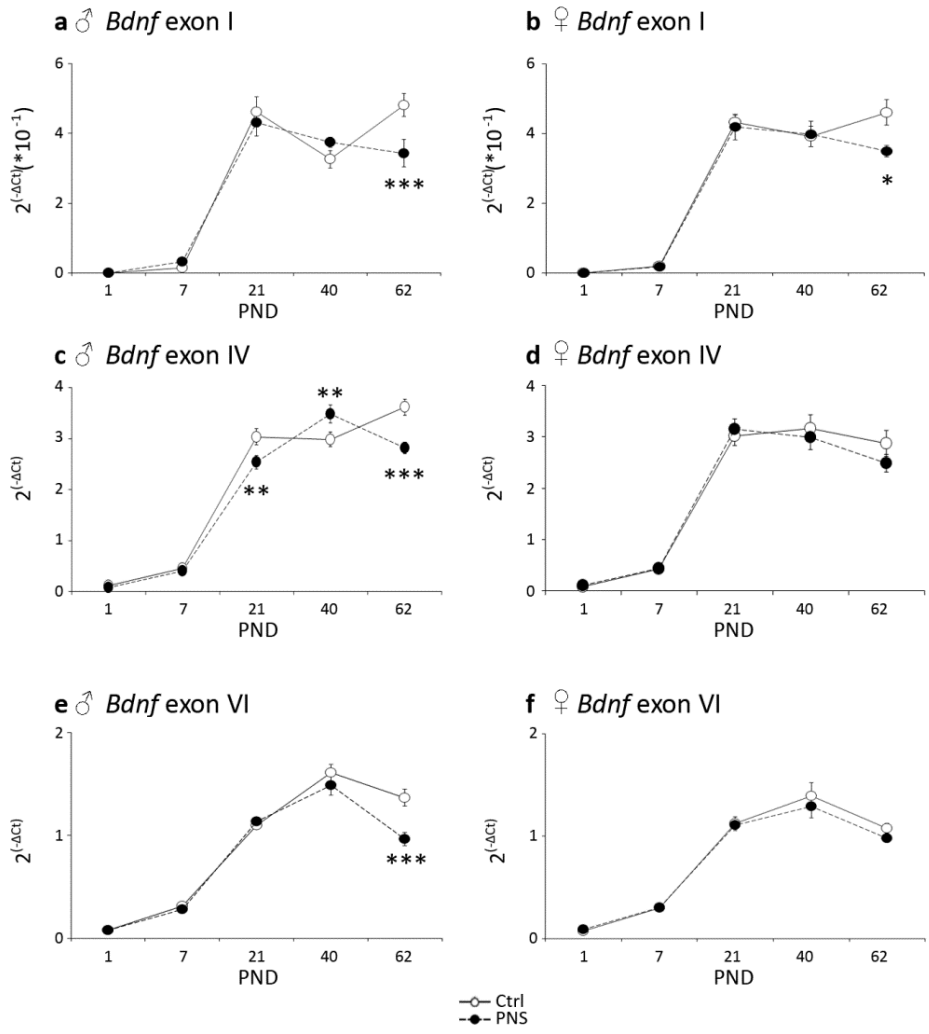

Figure 9 *Postnatal developmental changes of prefrontal cortex Bdnf exons expression and modulation by gestational stress.* Postnatal developmental analysis of the mRNA levels for *Bdnf* exon I (a, b), *Bdnf* exon IV (c, d) and *Bdnf* exon VI (e, f) were carried out in the prefrontal cortex of male (a, c, e) and female (b, d, f) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 5 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

4.1.3 Pharmacological intervention during adolescence prevents *Bdnf* alterations in male rats exposed to prenatal stress

Since the effect of PNS on *Bdnf* expression becomes manifest after adolescence, we reasoned that pharmacological intervention during this critical time window may be able to prevent the alterations set in motion by stress exposure during gestation. It has been demonstrated that prenatal stress produces persistent changes in the expression of different neurotransmitter receptors, including dopamine D_2 and serotonin $5HT_{2a}$ (Berger et al., 2002; Holloway et al., 2013). In order to confirm and extend this information, we investigated the expression of selected dopaminergic and serotonergic receptors in adolescent rats exposed to PNS. Indeed, as shown in Fig. 10, we found that prenatal stress exposure (PNS) had a significant effect on the expression of dopaminergic and serotonergic receptors measured during adolescence. Specifically, we found a significant up-regulation of the mRNA levels of dopamine D_2 (+51% $p<0.01$), as well as serotonin $5HT_{2a}$ (+46% $p<0.001$) and $5HT_7$ (+55% $p<0.05$) receptors, whereas the mRNA levels of D_1, $5HT_{1a}$, $5HT_{1b}$ and $5HT_{2b}$ receptors were not significantly affected by the prenatal manipulation. On these bases, we decided to repeat the prenatal stress procedure and to use as preventive intervention the novel multi-receptor antipsychotic drug lurasidone, which is characterized by potent $5HT_7$ receptor antagonism in addition to the blockade of dopamine D_2 and serotonin $5HT_{2A}$ receptors (Ishibashi et al., 2010; Tarazi and Riva, 2013).

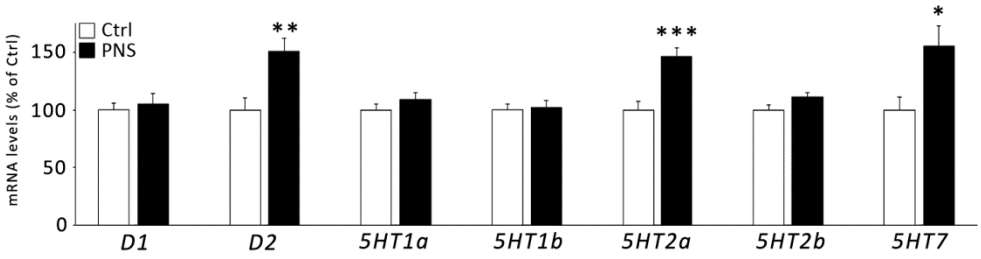

Figure 10 *Analysis of the mRNA levels of different dopaminergic and serotonergic receptors in adolescent male rats that were exposed to gestational stress (PNS), as compared to control animals (Ctrl).* The results, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 6 animals per group.
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl (Student's *t* test)

We thus injected the drug for two weeks, starting from PND35, and sacrificed the rats 24 hours or two weeks after the end of the treatment. In this experiment we only used male rats due to limitations in the number of pups from each litter.

In the hippocampus we found that prenatal stress exposure produced a transient significant increase of total *Bdnf* expression followed by a significant decrease at adult level (PND49: $F_{1,40}$=12.386, p<0.01, 2-way ANOVA; PND62: $F_{1,41}$=19.310, p<0.001, 2-way ANOVA) (Fig. 11a). In addition, we found a significant effect of lurasidone administration and also a significant PNS x LUR interaction at PND62 ($F_{1,41}$=57.309, p<0.001, 2-way ANOVA; $F_{1,41}$=5.288, p<0.05, 2-way ANOVA). In fact, while PNS exposure or lurasidone administration, per se, produced a decrease of total *Bdnf* levels at adulthood (-22% and -32% vs. Ctrl/Veh, respectively, p<0.001), the combination of these two variables led to a further decrease of its expression (-39% vs. Ctrl/Veh, p<0.01 vs. PNS/Veh) (Fig. 11a). Moreover, as shown in Fig. 11c, lurasidone treatment had a unfavorable effect also on the pool of *Bdnf* with the long 3'-UTR, 24 hours after the last administration as well as 2 weeks later ($F_{1,41}$=18.225, p<0.001, 2-way ANOVA; $F_{1,41}$=99.198, p<0.001, 2-way ANOVA). In fact, lurasidone administration determined a reduction of the neurotrophin expression in Ctrl as well as in PNS animals both at PND49 (Ctrl: -12% vs. Ctrl/Veh, p<0.05; PNS: -15% vs. PNS/Veh, p<0.01) and at PND62 (Ctrl: -30% vs. Ctrl/Veh, p<0.001; PNS: -29% vs. PNS/Veh, p<0.001) (Fig. 11c).

Conversely, as shown in Fig. 11b, total *Bdnf* mRNA levels were not significantly affected by PNS at adolescence (PND49: +23% vs. Ctrl/Veh, p>0.05) or early adulthood (PND62: +10% vs. Ctrl/Veh, p>0.05) in the prefrontal cortex. Nevertheless, lurasidone administration produced a significant increase of its mRNA levels 24h after the last administration (PND49: lurasidone effect p<0.01), an effect that persisted up to two weeks later (PND62: lurasidone effect p<0.001). In particular, at PND49, lurasidone given to PNS animals increased total *Bdnf* mRNA levels (+36%, p<0.01 vs. PNS/Veh) and this effect persisted up to PND62, where the effect was present also in Ctrl animals (+31% vs. Ctrl/Veh, p<0.001 and +32% vs. PNS/Veh, p<0.001).

The expression of long 3'-UTR *Bdnf* was not altered in PNS adolescent rats but, in line with results previously shown, it was significantly reduced at PND62 (-22% vs. Ctrl/Veh, p<0.01) (Fig. 11d). Interestingly, sub-chronic treatment with lurasidone did not alter long 3'-UTR *Bdnf* expression at PND49 in Ctrl rats (+14%, p>0.05 vs. Ctrl/Veh), while it produced a significant increase of long 3'-UTR *Bdnf* mRNA levels in PNS animals (PND49: +26% vs. PNS/Veh, p<0.01). More importantly, we found that lurasidone was able to prevent the significant reduction of long 3'-UTR *Bdnf* transcripts that we observed at PND62 in PNS animals (+53% vs. PNS/Veh, p<0.001), leading to an up-regulation of long 3'-UTR *Bdnf* mRNA levels (Fig. 11d).

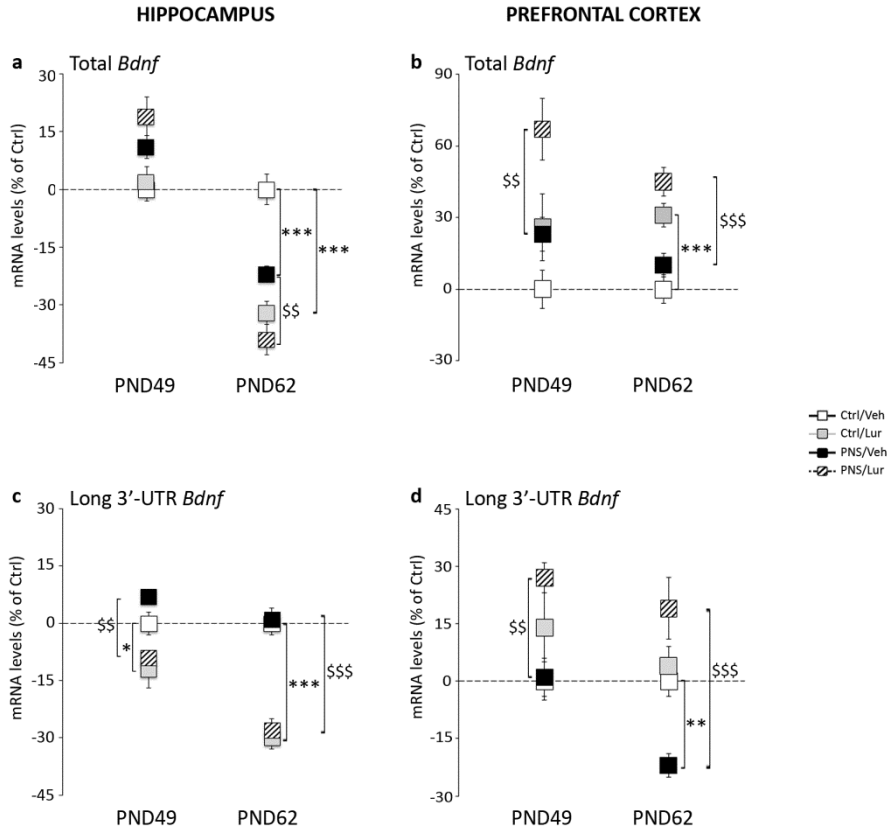

Figure 11 *Effect of sub-chronic lurasidone administration during late adolescence on Bdnf expression in animals exposed to gestational stress.* The effect of lurasidone treatment on total (a, b) and long 3'-UTR (c, d) *Bdnf* mRNA levels were analyzed in control or PNS male rats exposed to gestational stress, both in the hippocampus (a, c) and in the prefrontal cortex (b, d). The neurotrophin expression was investigated at PND49 (24 h after the last lurasidone administration) or at PND62 (14 days after the last lurasidone administration). The results, expressed as % change of Ctrl/Veh (animals treated with vehicle not exposed to prenatal stress, set at 0%), represent the mean ± SEM of at least 7 animals per group.

$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl/Veh of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison);

$^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. PNS/Veh of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

4.2 THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND STRESS-RESPONSIVENESS

4.2.1 Placental weight and corticosterone circulating levels in adult offspring

Human epidemiological studies consistently provide evidence for an association between environmental challenges during pregnancy, altered fetal growth and the development and occurrence of psychopathology later in life (Seckl, 1998). The placental unit partly represents the source of nutrients for the growing fetus, partly a protective barrier that shields from maternal glucocorticoids (Mark et al., 2009). Since one of the common features of perinatal exposure to stress is intrauterine growth retardation, we measured the placental weight as an index of the proper fetal development. As shown in Fig. 12, the placentas derived from dams that underwent gestational stress displayed a significant reduction of the global weight (-22%, $p<0.05$).

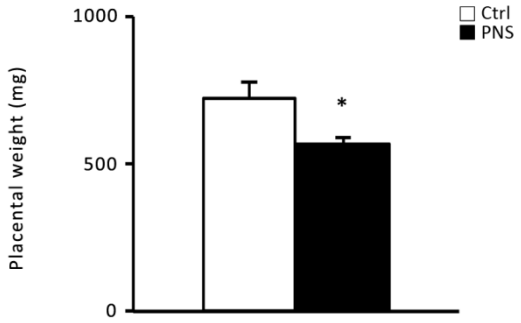

Figure 12 *Exposure to prenatal stress reduces the placental weight at birth.* The results, expressed in mg, represent the mean ± SEM of at least 5 dams per group.
* $p<0.05$ vs. Ctrl (Student's t test)

Glucocorticoids (GCs) play an important role in fetal tissues, yet excess levels of such hormones affect placental and fetal growth (Khulan and Drake, 2012; Wyrwoll et al., 2011). Also, the HPA system is designated to the body control of stress response and to the regulation of circadian circuits. The hypothalamus produces the corticotropin-releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH, in turn, regulates the release of glucocorticoids (corticosterone in rats, cortisol in humans) from the adrenal glands. Negative feedback occurs at the brain level through the binding of GCs to their receptors (glucocorticoid receptors, GR).

We thus quantified, using radioimmunoassay (RIA), the plasma corticosterone concentration of adult rats born to both Ctrl and PNS dams. As shown in Fig. 13, PNS rats displayed a significant

increase in corticosterone circulating levels when compared to the Ctrl counterpart (+222 ng/ml vs. Ctrl, p <0.05).

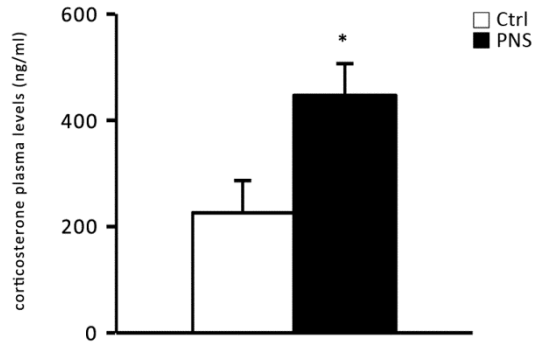

Figure 13 *Exposure to prenatal stress increases circulating corticosterone levels in adult rats.* The results, expressed in ng/ml, represent the mean ± SEM of at least 10 animals per group.
$^{*}p$<0.05 vs. Ctrl (Student's t test)

4.2.2 Quantification of *Nr3c1* mRNA levels in animals exposed to prenatal stress

We next evaluated, using qRT-PCR, the mRNA expression levels of the glucocorticoid receptor (gene name: *Nr3c1*) in the adult brain, both in the hippocampus and prefrontal cortex, of rats exposed to gestational stress as compared to controls.

We found that the exposure to prenatal restraint stress produced, in adult animals, both in male and female rats, a significant decrease of *Nr3c1* expression levels, in both brain region analyzed (male hippocampus: - 39% vs. Ctrl, p<0.001; female hippocampus: - 46% vs. Ctrl, p<0.001; male prefrontal cortex: - 51% vs. Ctrl, p<0.01; female prefrontal cortex: - 26% vs. Ctrl, p<0.05) (Fig. 14).

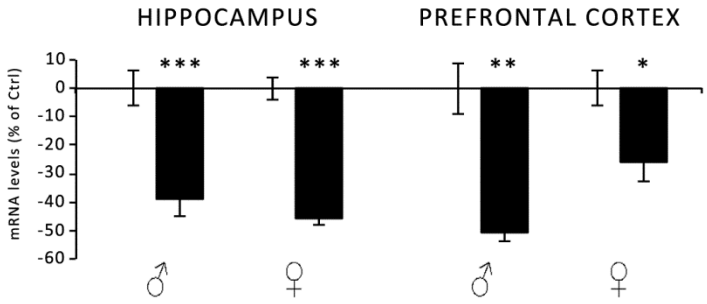

Figure 14 *Exposure to prenatal stress reduces Nr3c1 mRNA levels in adult rat brain.* The mRNA levels of the glucocorticoid receptor (*Nr3c1*) were analyzed in the hippocampus and prefrontal cortex of adult (PND62) male and female rats exposed to prenatal stress (PNS, fill bars), as compared to control animals (Ctrl). The data, expressed as a % change of Ctrl, represent the mean ± SEM of at least 7 animals per group.
$^{*}p$<0.05, $^{**}p$<0.01 and $^{***}p$<0.001 vs. Ctrl (Student's t test)

To better understand if the observed findings in the adult animals were a consequence of a dysregulation of *Nr3c1* expression during the postnatal development, we carried out a time-course analysis in both gender and both brain regions, focusing on the following time points: PND1, PND7, PND21, PND40 and PND62.

We found that in Ctrl animals, *Nr3c1* expression levels raised progressively until adulthood. On the contrary, prenatal exposure to stress affected this developmental pattern (Fig. 15) (PNS effect: $p<0.001$ in a, b, c and $p<0.05$ in d). In particular, within the hippocampus, PNS rats showed a significant decreased expression of *Nr3c1* starting from PND40 (PND40: PNS males -34% vs. Ctrl and PNS females -23% vs. Ctrl, $p<0.01$; PND62: PNS males -39% vs. Ctrl and PNS females -46% vs. Ctrl, $p<0.001$) (Fig. 15a, c). In the prefrontal cortex, while gestational stress seems not to affect *Nr3c1* mRNA levels in the first period of life, with the exception of a transient decrease at PND21 (PNS males -20% vs. Ctrl, $p=0.05$; PNS females -20% vs. Ctrl, $p<0.05$), the significant reduction of its expression become fully manifest at PND62 (PNS males -51% vs. Ctrl, $p<0.001$; PNS females -26% vs. Ctrl, $p<0.01$) (Fig. 15b, d).

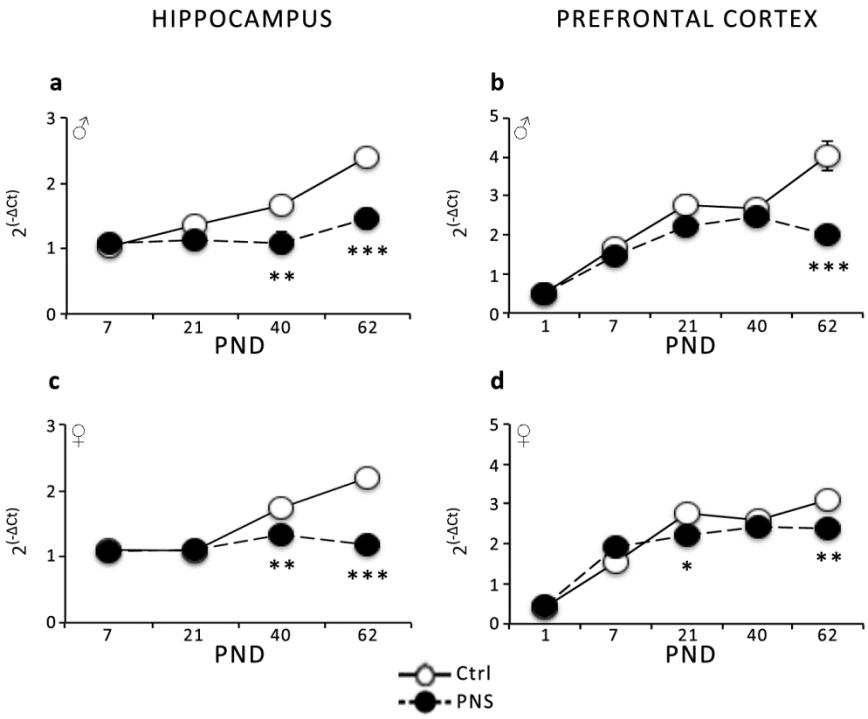

Figure 15 *Postnatal developmental changes of the glucocorticoid receptor (Nr3c1) expression and modulation by gestational stress.* Postnatal developmental analysis of the mRNA levels for *Nr3c1* were carried out in the hippocampus (a, c) and prefrontal cortex of male (b, d) of male (a, b) and female (c, d) rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 5 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

4.2.3 Correlation between *Nr3c1* expression levels in the brain and corticosterone plasma levels in animals exposed to prenatal stress

In order to assess the functionality of the HPA axis, we examined whether there is a correlation between corticosterone plasma levels and *Nr3c1* gene expression levels in the brain. For each animal, we took into account the value of circulating corticosterone (expressed in ng/ml) and the mean value of GR expression levels in the hippocampus and the prefrontal cortex (expressed as a percentage of the Ctrl group) at PND62. As shown in Fig. 16, the decrease of *Nr3c1* mRNA levels correlates with an increase in the circulating levels of corticosterone. Hence the two parameters showed a statistically significant inverse correlation (r = -0.550, n = 19, $p<0.05$, Pearson's correlation), suggesting that reduced expression of *Nr3c1* in animals exposed to prenatal stress leads to a persistent elevation of corticosterone levels, which in turn could impair the ability to cope with adverse or stressful events.

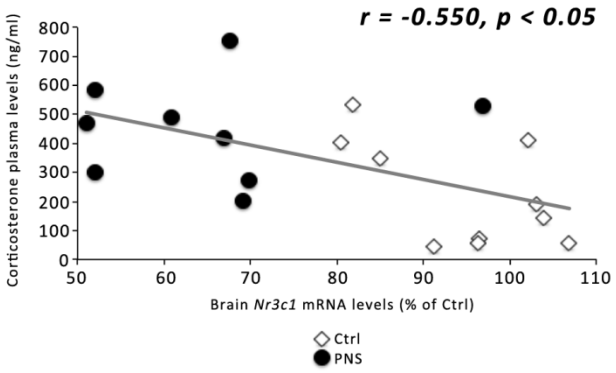

Figure 16 *Effects of prenatal exposure to stress on the HPA-axis functionality.* The figure shows the correlation analysis between plasma corticosterone levels and *Nr3c1* mRNA expression levels in the brain of adult rats (PND62) exposed to prenatal stress (PNS) as compared to control animals (Ctrl). Each spot represents the intersection between the value of peripheral corticosterone expressed in ng/ml and the level of mRNA expression (expressed as a mean between the two percentage values in the hippocampus and prefrontal cortex).
Pearson's correlation coefficient: r = 0.550, $p < 0.05$

4.2.4 Altered stress-responsiveness in adult rats exposed to prenatal stress

The results presented so far demonstrated that animals exposed to gestational stress are characterized by alterations of mechanisms involved in neuronal plasticity at adulthood and by an impairment of the HPA axis system.

As the expression of the neurotrophin *Bdnf* can also undergo rapid changes following acute manipulations, which may be considered an index of adaptive plasticity under challenging conditions (Fumagalli et al., 2012; Gourley et al., 2012; Molteni et al., 2009; Waterhouse and Xu, 2009), we investigated whether gestational stress may alter *Bdnf* responsiveness to an acute stress (AS) during adult life. In order to do so, we exposed Ctrl or PNS adult animals to the acute forced swim stress and sacrificed the animals 1 hour later.

As shown in Fig. 17, within the hippocampus the exposure to prenatal stress significantly reduced the expression of total *Bdnf* in female rats (PNS effect: $F_{1,30}$=10.159, p<0.01, 2-way ANOVA) (Fig. 17a), without affecting stress responsiveness within this brain region in male as well as in female rats. In fact, the expression of the neurotrophin is not modulated by the acute stress in Ctrl as well as in PNS animals of both genders. We only observed an interaction of the two variables (PNS and AS), although it did not reach statistic significance (interaction: $F_{1,29}$=4.173, p=0.052, 2-way ANOVA), in the modulation of the pool of *Bdnf* with the long 3'-UTR (Fig. 17d). Indeed, while in Ctrl females the acute stress exposure did not modulate the expression of the neurotrophin, in the PNS counterpart it produced a trend toward a decrease of its expression.

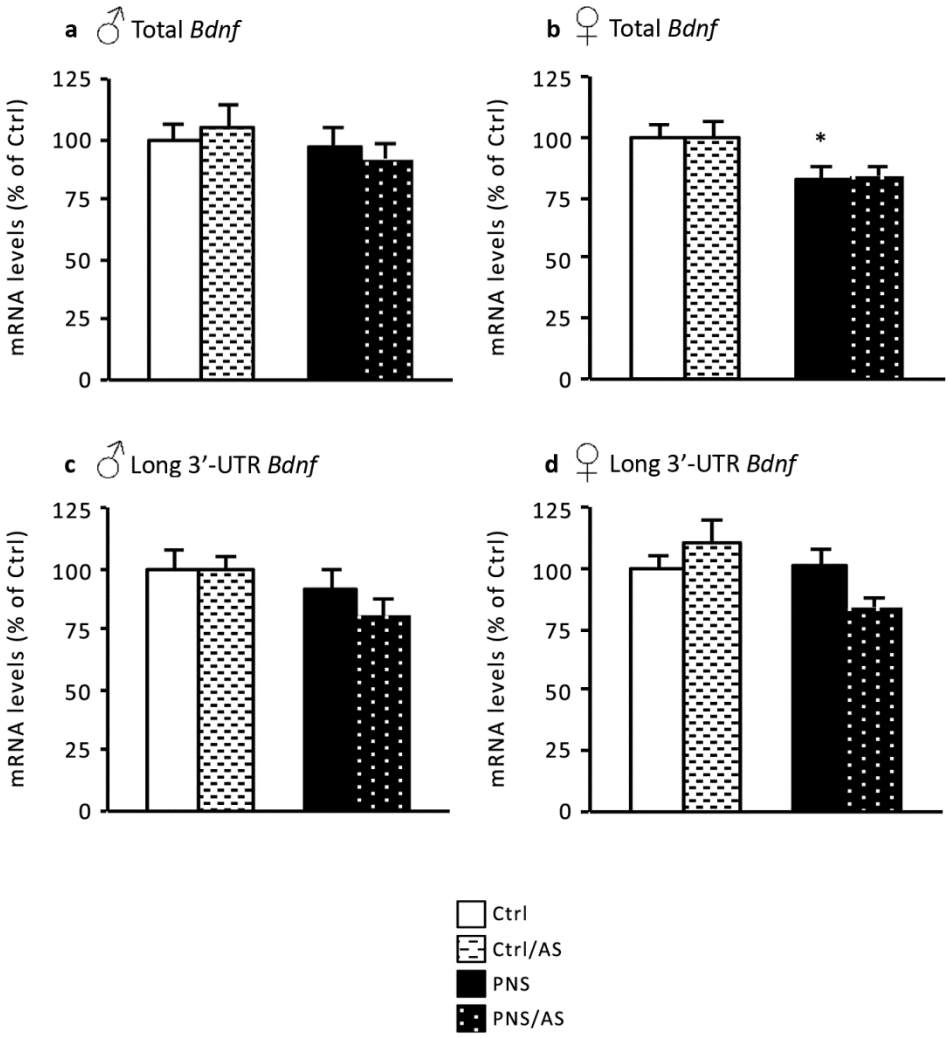

Figure 17 *Modulation of Bdnf mRNA levels following prenatal stress and forced swim stress exposure at adulthood.* The mRNA expression levels of total (a, b) and long 3'-UTR (c, d) *Bdnf* were analyzed in the hippocampus of control or PNS adult (PND62) male (a, c) and female (b, d) rats under resting conditions or following exposure to an acute forced swim stress (AS) at adulthood (PND62). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 7 animals per group.

$^{*}p<0.05$ vs. Ctrl (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

We further investigate, in this brain region, the expression of the main transcripts of *Bdnf*, namely exon I, exon IV and exon VI. As shown in Fig. 18, and in line with what we observed in Fig. 17b, *Bdnf* exon IV and VI were significantly affected by the exposure to gestational stress in female animals (PNS effect: $F_{1,29}=13.121$, $p<0.01$ and $F_{1,30}=12.442$, $p<0.01$, respectively, 2-way ANOVA), and we observed an effect on *Bdnf* exon VI also in the male hippocampus (PNS effect: $F_{1,30}=23.253$, $p<0.001$, 2-way ANOVA). Conversely, the acute exposure to stress once again had a modulatory effect on the expression of *Bdnf* only in female rats (AS: $F_{1,29}=6.713$, $p<0.05$, 2-way

ANOVA). In particular, as shown in Fig. 18d, the exposure to the acute stress produced an increase of *Bdnf* exon IV in Ctrl females (+19% vs. Ctrl/Sham, $p<0.05$), an effect that was not observed in PNS females.

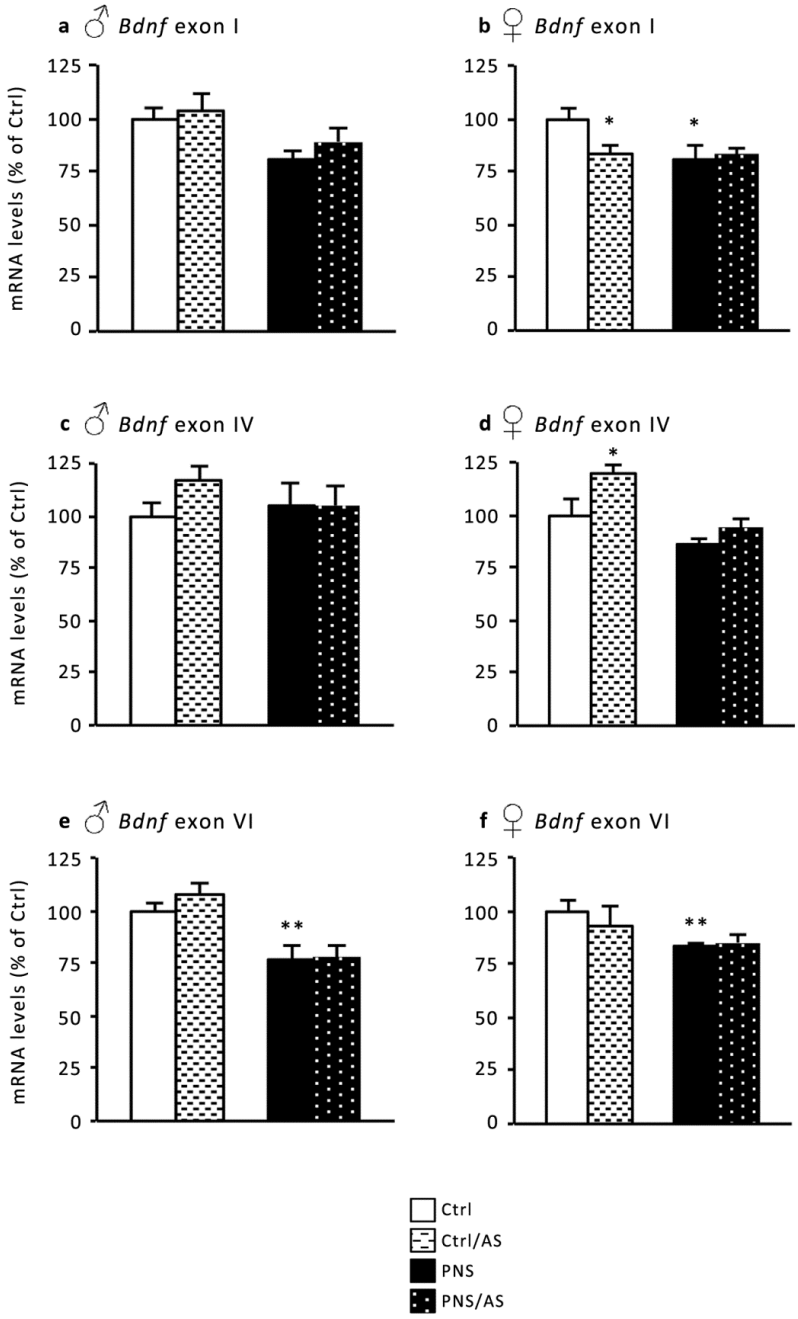

Figure 18 *Modulation of the mRNA expression for Bdnf exons following prenatal stress and forced swim stress exposure at adulthood.* The mRNA levels of exon I (a, b), exon IV (c, d) and exon VI (e, f) of *Bdnf* were analyzed in the hippocampus of control or PNS adult (PND62) male (a, c, e) and female (b, d, f) rats under resting conditions or following exposure to an acute forced swim stress (AS) at adulthood (PND62). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 7 animals per group.

$^{*}p<0.05$ and $^{**}p<0.01$ vs. Ctrl (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

In the prefrontal cortex we observed a different pattern of *Bdnf* modulation. As shown in Fig. 19a, we found a significant effect of AS, but no PNS X AS interaction on total *Bdnf* mRNA levels in male rats (AS effect: $F_{1,30}$=5.789, p<0.05, 2-way ANOVA), the latter reflecting that the expression was increased by the AS in both cohorts (Ctrl and PNS). Furthermore, as displayed in Fig. 19c, we found a significant effect of PNS ($F_{1,30}$=13.837, p<0.01, 2-way ANOVA) as well as of AS ($F_{1,30}$=19.793, p<0.001, 2-way ANOVA), but no PNS X AS interaction on long 3'-UTR *Bdnf* expression in male rats. Indeed, PNS exposure significantly reduced the expression of this pool of neurotrophin transcripts (-28% PNS/Sham vs. Ctrl/Sham, p<0.05), while the AS exposure produced a similar up-regulation in Ctrl and PNS animals (+40% Ctrl/AS vs. Ctrl/Sham, p<0.01 and +47% PNS/AS vs. PNS/Sham, p<0.01).

A different pattern of changes was found for female animals. Indeed, with respect to total *Bdnf* expression (Fig. 19b), we found a significant effect of AS and a significant PNS X AS interaction ($F_{1,30}$=15.285, p<0.01 and $F_{1,30}$=9.160, p<0.01 respectively, 2-way ANOVA). In fact, the AS increased total *Bdnf* mRNA levels in Ctrl animals (+69% vs. Ctrl/Sham, p<0.001), but not in PNS female rats (+9% vs. PNS/Sham, p>0.05). When considering long 3'-UTR *Bdnf* expression (Fig. 19d), we found a significant effect of PNS, AS as well as a significant PNS X AS interaction ($F_{1,26}$=30.742, p<0.001, $F_{1,26}$=4.848, p<0.05 and $F_{1,26}$=5.695, p<0.05, respectively, 2-way ANOVA). As shown before, PNS produced a significant reduction of the mRNA pool with long 3'-UTR *Bdnf* (-22% vs. Ctrl/Sham, p<0.05). Moreover, similar to what we observed for total *Bdnf* (Fig. 19b) AS increased its mRNA levels in Ctrl females (+32% vs. Ctrl/Sham, p<0.01), without affecting its expression in PNS females (-1% PNS/Sham, p>0.05).

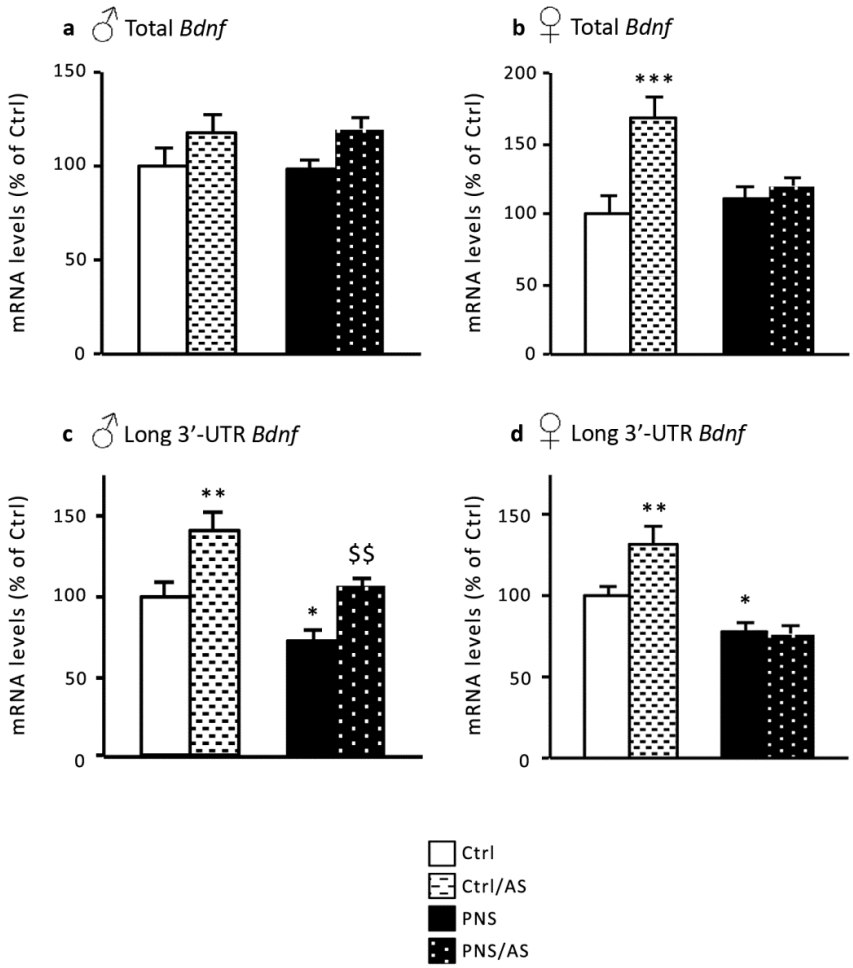

Figure 19 *Modulation of Bdnf mRNA levels following prenatal stress and forced swim stress exposure at adulthood.* The mRNA expression levels of total (a, b) and long 3'-UTR (c, d) *Bdnf* were analyzed in the prefrontal cortex of control or PNS adult (PND62) male (a, c) and female (b, d) rats under resting conditions or following exposure to an acute forced swim stress (AS) at adulthood (PND62). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 7 animals per group.

$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl (2-way ANOVA followed by Fisher's LSD post-hoc comparison);

$^{\$\$}p<0.01$ vs. PNS (2-way ANOVA followed by Fisher's LSD post-hoc comparison);

The expression of *Bdnf* transcripts in the prefrontal cortex was similarly affected by PNS and acute exposure to swim stress (Fig. 20). In agreement with the data reported above, PNS male animals showed decreased expression of all *Bdnf* exons analyzed (exon I: -29% vs. Ctrl/Sham, $p<0.05$; exon IV: -22% vs. Ctrl/Sham, $p<0.001$; exon VI: -29% vs. Ctrl/Sham, $p<0.01$) (Fig. 20a, c, e). Moreover, we observed a significant effect of AS on *Bdnf* exon IV ($F_{1,30}=8.101$, $p<0.01$, 2-way ANOVA) (Fig. 20c), but no PNS x AS interaction, suggesting that its expression was similarly modulated by the acute challenge in Ctrl and PNS male rats. With respect to female rats, we found a significant effect of PNS on *Bdnf* exon I and VI ($F_{1,28}=11.788$, $p<0.01$ and $F_{1,29}=6.528$, $p<0.05$, respectively, 2-way

ANOVA) (Fig. 20b, f), but no significant effect of AS, although single contrast analysis of exon VI shows that AS significantly increased its mRNA levels in Ctrl, but not in PNS rats (+28% vs. Ctrl/Sham, $p<0.05$ and +7% vs. PNS/Sham, $p>0.05$, respectively) (Fig. 20f).

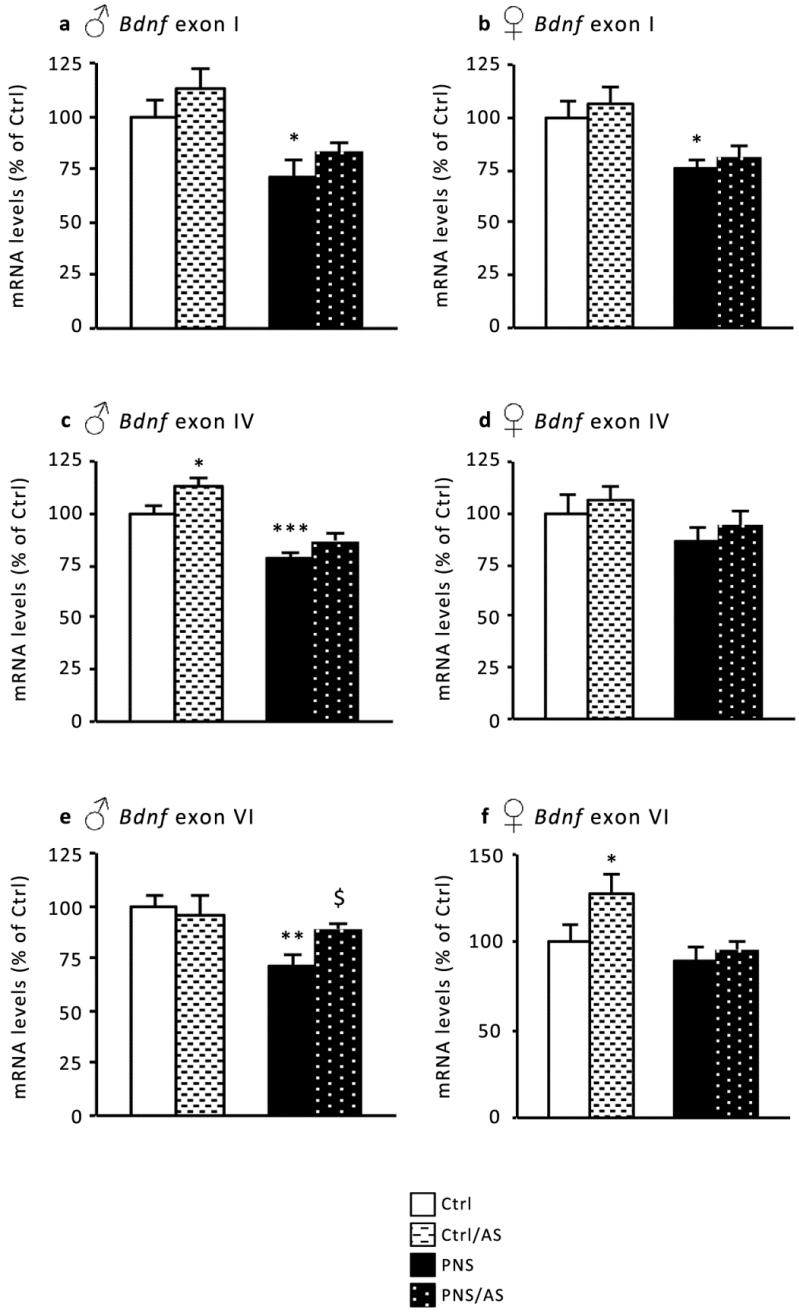

Figure 20 *Modulation the mRNA expression for Bdnf exons following prenatal stress and forced swim stress exposure at adulthood.* The mRNA expression levels of exon I (a, b), exon IV (c, d) and exon VI (e, f) of *Bdnf* were analyzed in the prefrontal cortex of control or PNS adult (PND62) male (a, c, e) and female (b, d, f) rats under resting conditions or following exposure to an acute forced swim stress (AS) at adulthood (PND62). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 7 animals per group.

$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl (2-way ANOVA followed by Fisher's LSD post-hoc comparison);

$^{\$}p<0.05$ vs. PNS (2-way ANOVA followed by Fisher's LSD post-hoc comparison);

We next determined whether the transcriptional changes observed following PNS were paralleled by modifications of BDNF protein levels in the prefrontal cortex. As shown in Fig. 21, in male as well as in female rats proBDNF levels were not affected by PNS or acute challenge (Fig. 21a, b). However, we found that mBDNF protein expression was significantly reduced in PNS male rats (-36% vs. Ctrl/Sham, $p<0.01$) (Fig. 21c), but not in female animals (-15% vs. Ctrl/Sham, $p>0.05$) (Fig. 21d). The acute challenge, *per se*, produced a reduction of mBDNF levels both in male and female Ctrl animals (respectively -29% vs. Ctrl/Sham, $p<0.05$ and -21% vs. Ctrl/Sham, $p<0.05$), but it did not exacerbate the already reduced mBDNF levels in PNS animals (Fig. 21c, d).

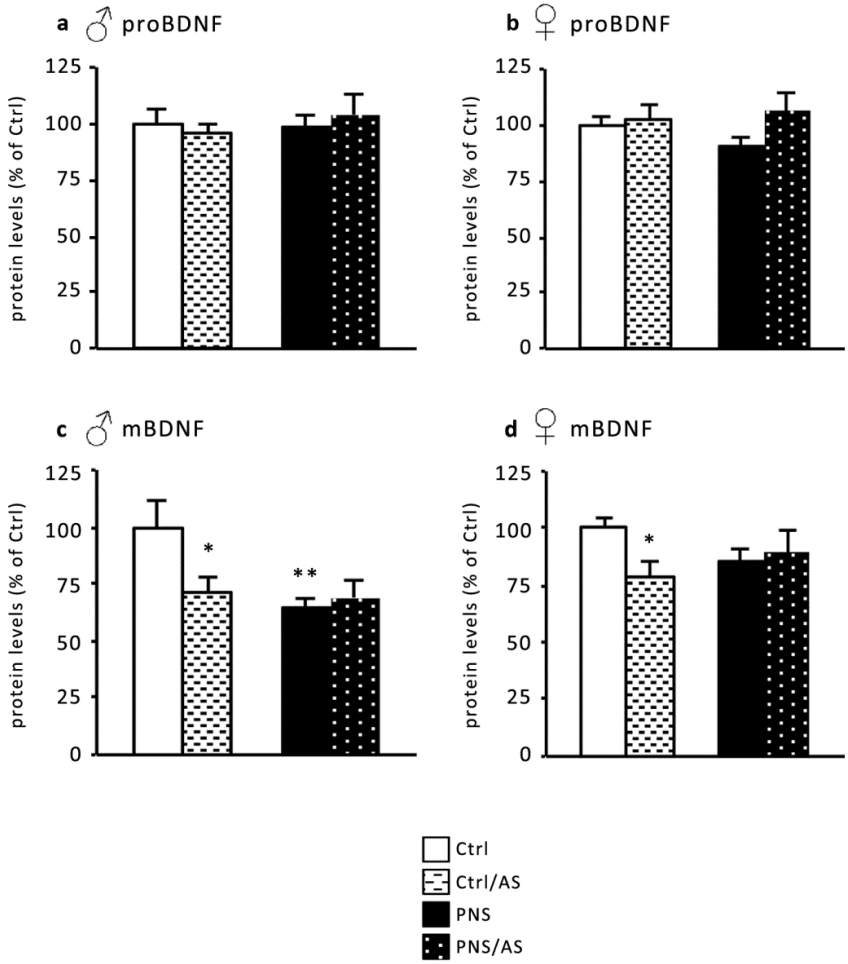

Figure 21 *Modulation of Bdnf protein levels following prenatal stress and forced swim stress exposure at adulthood.* The protein levels of proBDNF (a, b) and mBDNF (c, d) were analyzed in the prefrontal cortex of control or PNS adult (PND62) male (a, c) and female (b, d) rats under resting conditions or following exposure to an acute forced swim stress (AS) at adulthood (PND62). The data, expressed as a percentage of Ctrl (set at 100%), are the mean ± SEM of at least 5 animals per group.

$^{*}p<0.05$ and $^{**}p<0.01$ vs. Ctrl (2-way ANOVA followed by Fisher's LSD post-hoc comparison)

4.3 EPIGENETIC MECHANISMS

4.3.1 Exposure to prenatal stress alters expression levels of epigenetic regulators

Epigenetic modifications have been proposed to translate environmental cues into persistent cellular memories. In particular, it has been hypothesized that environmental stimuli early in life could influence adult behavior leaving a lasting mark in epigenetic signatures, which reshape the normal developmental trajectory of several tissues and systems leading to increase vulnerability to develop mental or physical disorders (Lv et al., 2013; Szyf, 2011).

As a first step, we investigated the expression of some epigenetic regulators that are involved in key mechanisms, including DNA methylation and histone acetylation, in order to establish their contribution to the enhanced vulnerability observed in our animal model.

As shown in Fig. 22, 23, 24 and 25, we analyzed, in the hippocampus and prefrontal cortex of both male and female rats, the developmental expression profiles of two enzymes that catalyze DNA methylation, *Dnmt3a* and *Dnmt1* (respectively a *de novo* and a maintenance methyltransferase), of Growth Arrest and DNA-Damage-Inducible beta (*Gadd45β*), which plays active role in DNA demethylation, and of three histone deacetylases (*Hdac1*, *Hdac2* and *Hdac5*) that, through their ability to regulate histone post-translational modifications, confer a further level of complexity.

As shown in Fig. 22, in the male hippocampus we observed a significant effect of prenatal stress exposure on *Dnmt1* ($F_{1,56}$=8.616, p<0.01, 2-way ANOVA), *Hdac1* ($F_{1,56}$=27.547, p<0.001, 2-way ANOVA), *Hdac2* ($F_{1,59}$=17.708, p<0.001, 2-way ANOVA) and *Hdac5* ($F_{1,54}$=18.319, p<0.001, 2-way ANOVA). In particular, male rats that suffered gestational stress displayed a significant reduction at adulthood of the mRNA levels of such epigenetic regulators (*Dnmt1*: p<0.01; *Hdac1*: p<0.001; *Hdac2*: p<0.01; *Hdac5*: p<0.01), an effect that is already evident at earlier developmental stages (*Dnmt1* at PND40: p<0.01; *Hdac1* at PND40: p<0.01; *Hdac2* at PND40: p<0.001 and at PND7: p<0.05; *Hdac5* at PND21: p<0.05).

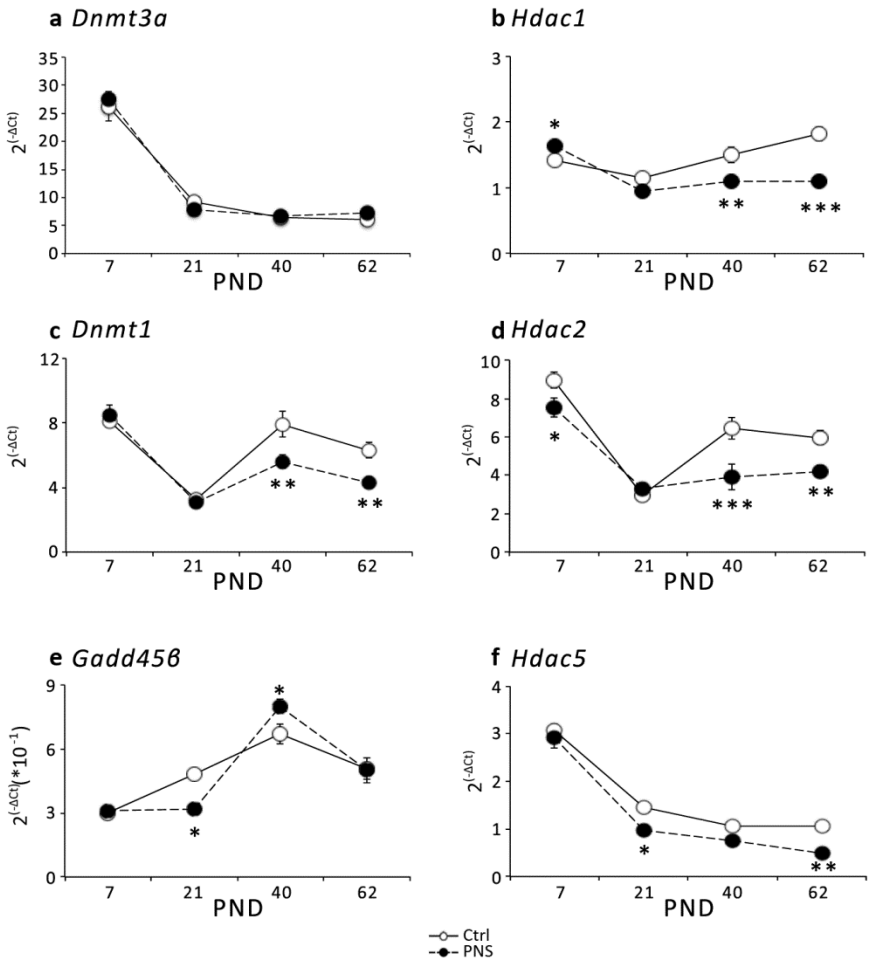

Figure 22 *Postnatal developmental changes in the expression of epigenetic regulators and modulation by gestational stress.* Postnatal developmental analyses of the mRNA levels for *Dnmt3a* (a), *Dnmt1*(c), *Gadd45β* (a), *Hdac1* (b), *Hdac2* (d) and *Hdac5* (f) were carried out in the hippocampus of male rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 6 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

On the contrary, the exposure to PNS determined a significant overall effect in the hippocampus of PNS female rats only for *Hdac5* mRNA expression ($F_{1,58}=4.268$, $p<0.05$, 2-way ANOVA) (Fig. 23). Nevertheless, adult PNS female rats showed a significant decrease of the expression levels of *Gadd45β* ($p<0.05$, Fig. 23e), *Hdac1* ($p<0.001$, Fig. 23b), *Hdac2* ($p<0.05$, Fig. 23d) and *Hdac5* ($p<0.01$, Fig. 23f).

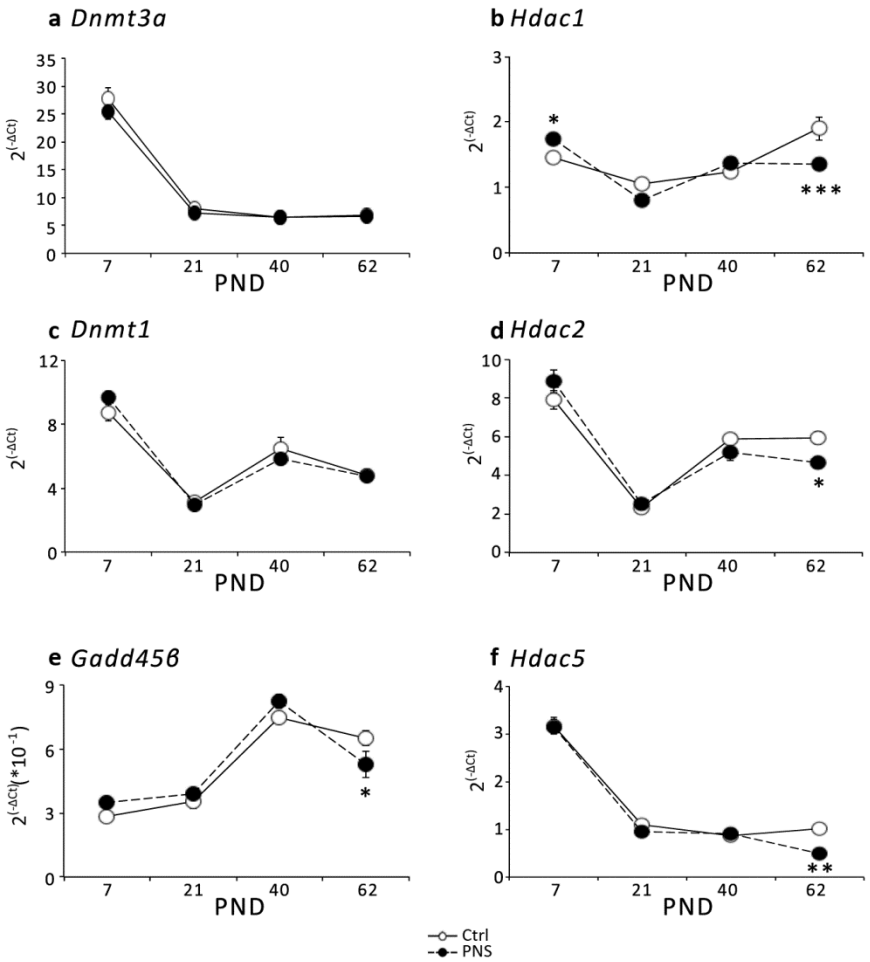

Figure 23 *Postnatal developmental changes in the expression of epigenetic regulators and modulation by gestational stress.* Postnatal developmental analyses of the mRNA levels for *Dnmt3a* (a), *Dnmt1*(c), *Gadd45β* (a), *Hdac1* (b), *Hdac2* (d) and *Hdac5* (f) were carried out in the hippocampus of female rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 6 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

When examining the prefrontal cortex of male rats (Fig. 24) we found that PNS affected the expression of all the epigenetic regulator under investigation (*Dnmt3a*: $F_{1,70}=9.429$, $p<0.01$, 2-way ANOVA; *Dnmt1*: $F_{1,70}=5.567$, $p<0.05$, 2-way ANOVA; *Hdac1*: $F_{1,68}=26.687$, $p<0.001$, 2-way ANOVA; *Hdac2*: $F_{1,64}=9.050$, $p<0.01$, 2-way ANOVA; *Hdac5*: $F_{1,67}=6.291$, $p<0.05$, 2-way ANOVA), with the exception of *Gadd45β*. In particular, *Dnmt3a* mRNA levels are transiently reduced in the PFC of PNS rats during early development, between PND 7 ($p<0.001$) and PND 21 ($p<0.05$) (Fig. 24a), whereas *Dnmt1* expression was significantly reduced only in adult PNS rats (PND62, $p<0.01$) (Fig. 24b). On the contrary, gestational stress affected *Hdac*'s expression across the whole postnatal period until adulthood, with an overall reduction of their mRNA levels. The changes in *Hdac1* were observed at all time points starting from PND7 (PND7: $p<0.01$; PND21: $p<0.05$; PND40: $p<0.05$;

PND62: p<0.001) (Fig. 24b), whereas the modifications of *Hdac2* and *Hdac5* were limited to PND7 and PND62 (*Hdac2:* PND7: p<0.05; PND62: p<0.05) (Fig. 24d) (*Hdac5:* PND7: p<0.01; PND62: p<0.001) (Fig. 24e).

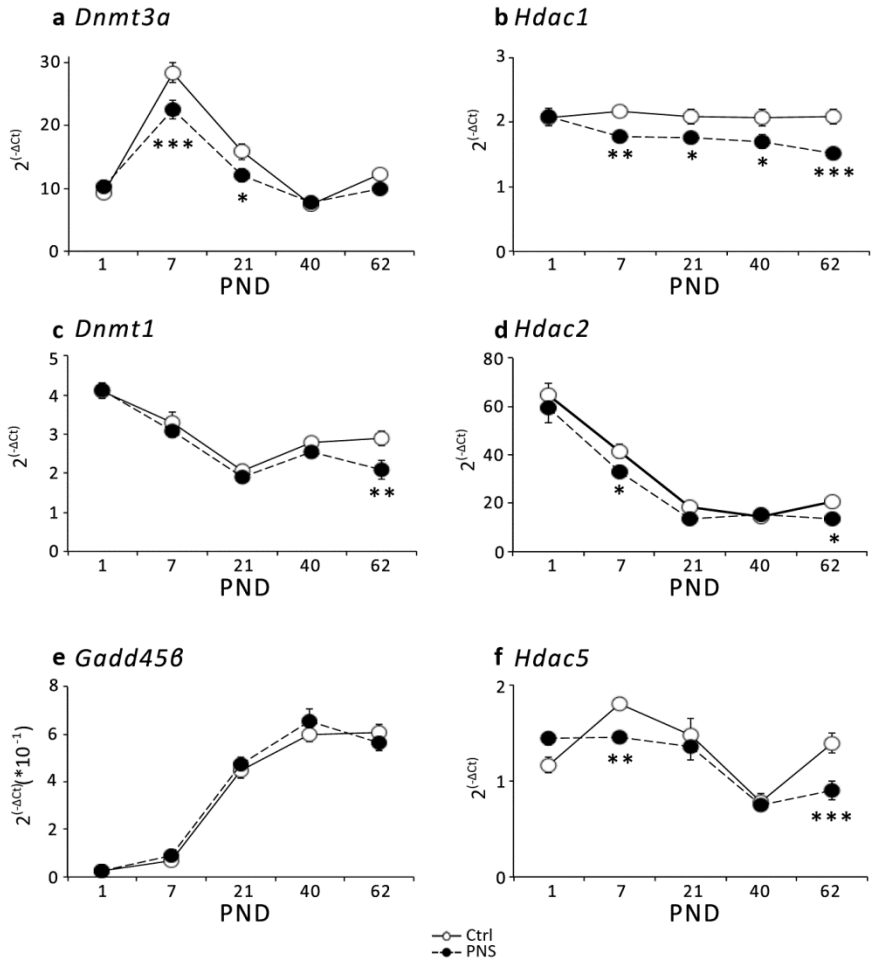

Figure 24 *Postnatal developmental changes in the expression of epigenetic regulators and modulation by gestational stress.* Postnatal developmental analyses of the mRNA levels for *Dnmt3a* (a), *Dnmt1*(c), *Gadd45β* (a), *Hdac1* (b), *Hdac2* (d) and *Hdac5* (f) were carried out in the prefrontal cortex of male rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 6 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene). $^{*}p$<0.05, $^{**}p$<0.01 and $^{***}p$<0.001 vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

In the prefrontal cortex of female rats the pattern of modulation is quite different when compared to males (Fig. 25). We found that PNS led to a significant modulation of *Dnmt3a* ($F_{1,73}$=4.287, p<0.05, 2-way ANOVA), *Dnmt1* ($F_{1,75}$=4.823, p<0.05, 2-way ANOVA), *Gadd45β* ($F_{1,74}$=14.422, p<0.001, 2-way ANOVA) and *Hdac5* ($F_{1,73}$=12.097, p<0.01, 2-way ANOVA). Similar to the changes observed in male rats, *Dnmt3a* and *Dnmt1* expression is transiently reduced by PNS at PND7 (*Dnmt3a*: p<0.001; *Dnmt1*: p<0.01). On the contrary, gestational stress led to a decreased expression of *Gadd45β* starting from adolescence (PND40: p<0.01; PND62: p<0.01), while the

mRNA levels encoding for *Hdac5* are reduced immediately after birth and at adulthood (p<0.05 and p<0.01, respectively).

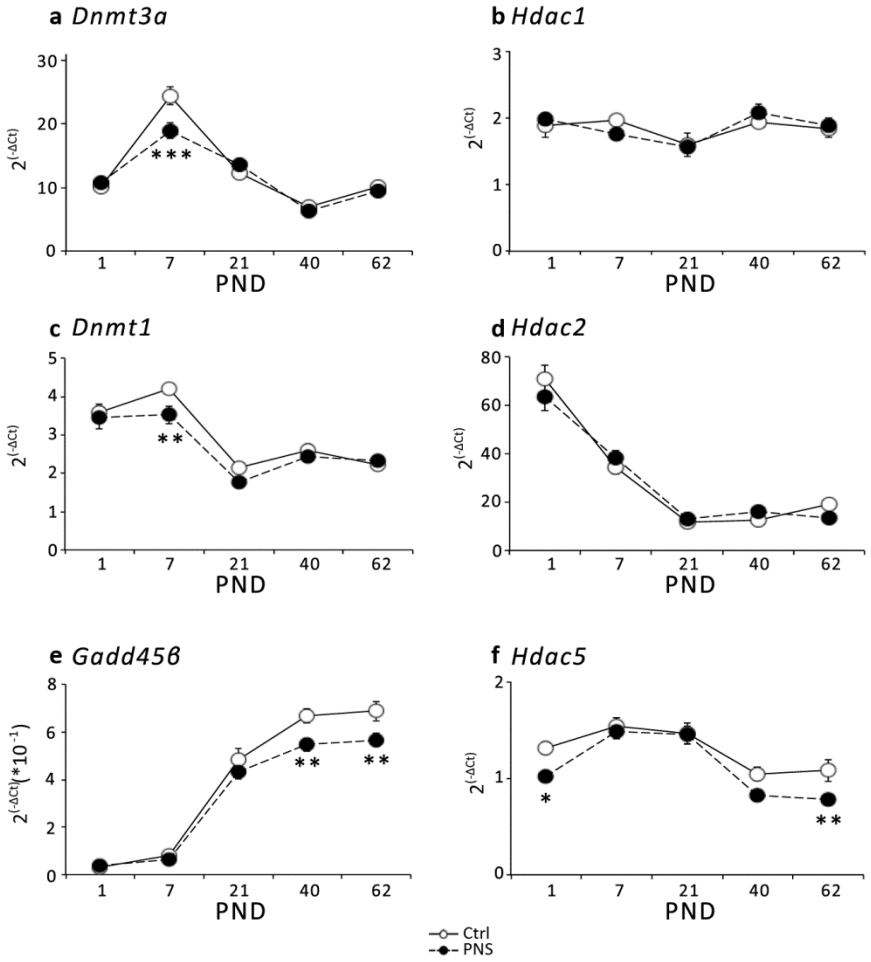

Figure 25 *Postnatal developmental changes in the expression of epigenetic regulators and modulation by gestational stress.* Postnatal developmental analyses of the mRNA levels for *Dnmt3a* (a), *Dnmt1*(c), *Gadd45β* (a), *Hdac1* (b), *Hdac2* (d) and *Hdac5* (f) were carried out in the prefrontal cortex of female rats exposed to gestational stress (PNS), as compared to control animals (Ctrl). The results represent the mean number of $2^{(-\Delta Ct)}$ ± SEM of at least 6 animals per group (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene).
$^{*}p$<0.05, $^{**}p$<0.01 and $^{***}p$<0.001 vs. Ctrl of the same postnatal age (2-way ANOVA followed by Fisher's LSD post-hoc comparison).

4.3.2 Methylome analysis in the hippocampus and prefrontal cortex of adult rats exposed to prenatal stress

We next analyzed genome-wide promoter methylation profiles of hippocampus and prefrontal cortex from eight adult (PND62) male and eight adult female rats. Methylation profiles were created using the method MeDIP with microarray hybridization (see "Materials and Methods").

Prenatal stress-associated promoter methylation in the hippocampus of adult male rats.

We found 21835 probes from 3668 distinct gene promoters whose normalized intensities were significantly associated with prenatal stress exposure (p<0.05; FDR<0.01). Of these promoters 2110 were more methylated in the Ctrl group, and 1363 were more methylated in the PNS group. For 195 gene promoters, a mixed methylation pattern was observed. A heat map of the clustering analysis of the probes that best differentiate between groups is shown in Fig. 26a. Table 4 provides a list of genes associated with the 25 most significant probes.

We subjected gene promoters containing probes associated with exposure to prenatal stress in the hippocampus of adult male rats to enrichment analysis using Ingenuity Pathways Analysis (IPA) software (version 8.0, http://www.ingenuity.com). Pathways associated with molecular transport (p=5.76E-18), neurological disease (p=1.51E-11), nervous system development and function (p=1.01E-10) were most prominently enriched.

Table 4 List of genes associated with the 25 top differentially methylated probes in the hippocampus observed in the comparison of control (Ctrl) and prenatal stress exposed (PNS) male rats at PND62.

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Lrif1	25	Ctrl	1.02E-09

Prenatal stress-associated promoter methylation in the hippocampus of adult female rats.

A total of 12296 probes mapping to 3955 distinct genes whose normalized intensities were significantly associated with the exposure to prenatal stress (p<0.05, FDR<0.05) have been found. Of these genes, 2111 were less methylated, and 1574 were more methylated after prenatal exposure to stress. For 270 gene promoters, a mixed methylation pattern was observed. Fig. 26b shows a supervised heat map based on the probes that differentiate most strongly between the Ctrl and the PNS group. Table 5 provides a list of genes associated with the 25 most significant probes.

Pathway analysis of the differentially methylated genes in adult female hippocampus identified pathway associated with transport of molecules (p=1.35E-18), neurological disease (p=1.70E-11), neurotransmission (p=2.42E-10), GABA-receptor signaling (p=2.57E-07) and Huntington's Disease Signaling (p=3.33E-07).

Table 5 List of genes associated with the 25 top differentially methylated probes in the hippocampus observed in the comparison of control (Ctrl) and prenatal stress exposed (PNS) female rats at PND62

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Ube2d3	20	Ctrl	9.51E-08
Fam103a1	5	Ctrl	1.23E-07

Prenatal stress-associated promoter methylation in the prefrontal cortex of adult male rats.

We found 3080 probes from 1130 distinct gene promoters whose normalized intensities were significantly associated with prenatal stress exposure (p<0.05; FDR<0.05). Of these promoters 395 were more methylated in the Ctrl group, and 741 were more methylated in the PNS group. For 22 gene promoters, a mixed methylation pattern was observed. A heat map of the clustering analysis of the probes that best differentiate between groups is shown in Fig. 26c. Table 6 provides a list of genes associated with the 25 most significant probes.

Pathway analysis of the differentially methylated genes in adult female hippocampus identified pathway associated with molecular transport (p=9.34E-08), neurotransmission (p=1.45E-05), action potential of cells (p=2.39E-05), response of neurons (p=1.29E-04) and schizophrenia (p=1.25E-03).

Table 6 List of genes associated with the 25 top differentially methylated probes in the prefrontal cortex observed in the comparison of control (Ctrl) and prenatal stress exposed (PNS) male rats at PND62

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Asb10	10	PNS	3.22E-07
Gnas	2	PNS	3.22E-07
Kcnk2	13	PNS	9.44E-06

Prenatal stress-associated promoter methylation in the prefrontal cortex of adult female rats.

A total of 8255 probes mapping to 2999 distinct genes whose normalized intensities were significantly associated with the exposure to prenatal stress (p<0.05, FDR<0.05) have been found. Of these genes, 1240 were less methylated, and 1614 were more methylated after prenatal

exposure to stress. For 145 gene promoters, a mixed methylation pattern was observed. Fig. 26d shows a supervised heat map based on the probes that differentiate most strongly between the Ctrl and the PNS group. Table 7 provides a list of genes associated with the 25 most significant probes.

Pathway analysis of the differentially methylated genes in adult female hippocampus identified pathway associated with proliferation of cells (*p*=9.72E-21), neuritogenesis (*p*=9.80E-15), neurotransmission (*p*=2.06E-12), gene expression (*p*=3.79E-12) and behavior (*p*=7.28E-11).

Table 7 List of genes associated with the 25 top differentially methylated probes in the prefrontal cortex observed in the comparison of control (Ctrl) and prenatal stress exposed (PNS) female rats at PND62

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Prom2	10	Ctrl	5.01E-06
Esm1	12	Ctrl	5.01E-06
Mrps27	1	Ctrl	5.01E-06
Esrra	2	Ctrl	5.01E-06

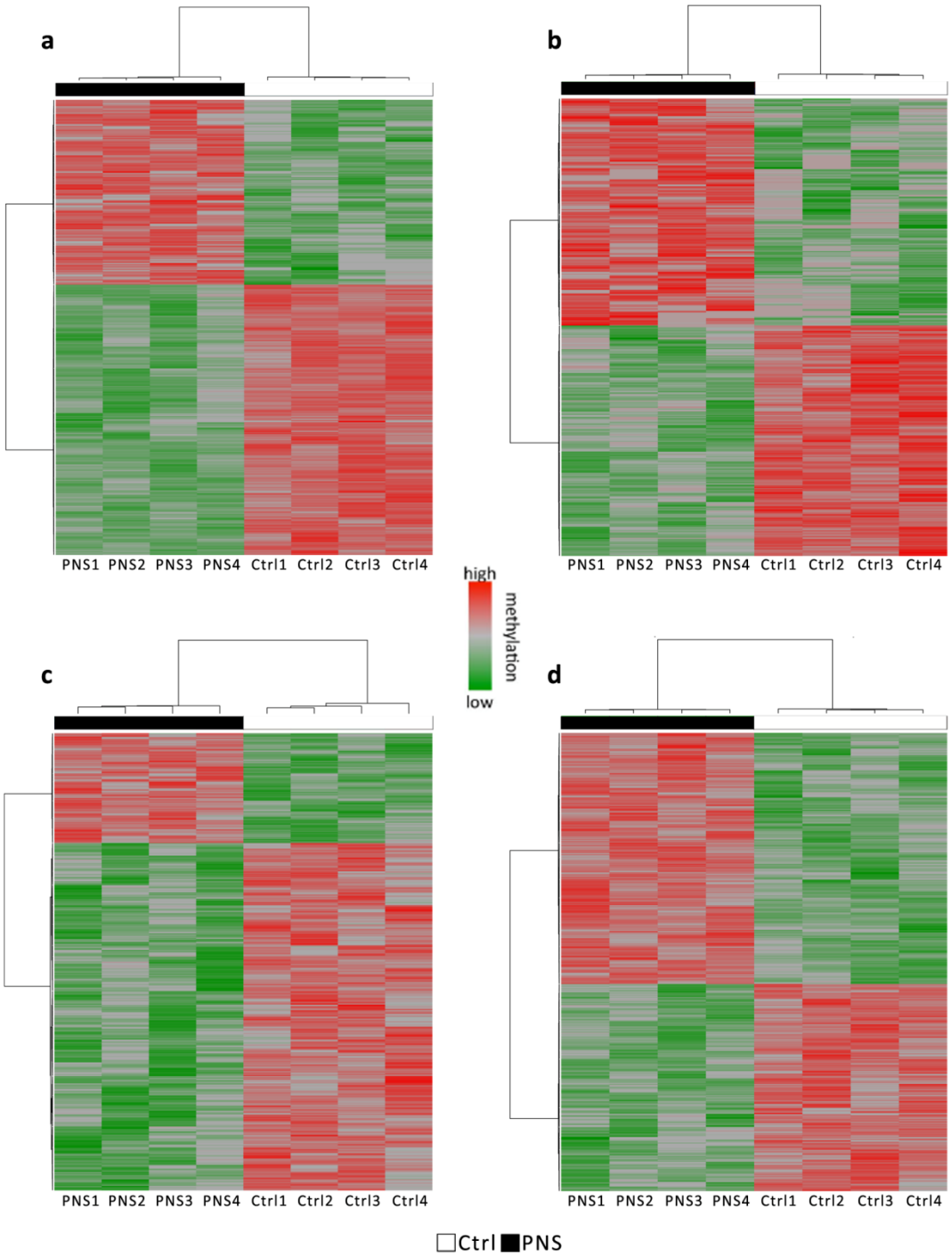

Figure 26 *Heat map depicts normalized intensities of microarray probes contained in promoters that differentiate most strongly between Ctrl and PNS groups in adult rats.* Rows correspond to promoters and columns to animals. Red indicates higher methylation in a row and green indicated lower methylation. (a) male hippocampus, (b) female hippocampus, (c) male prefrontal cortex, (d) female prefrontal cortex.

4.3.3 Overlap of the methylation analyses in the hippocampus and prefrontal cortex of male and female adult rats exposed to prenatal stress

We decided to cross all our analyses in order to identify genes that may be regulated by prenatal stress in different brain regions and genders, which may represent a signature of the early life event at adulthood.

Interestingly, an overlap of 893 differentially methylated genes was observed between the hippocampus and prefrontal cortex of adult male and female rats that were exposed to gestational stress (Fig. 27). Such an overlap is larger than expected by chance (p<1.00E-200, hypergeometric test).

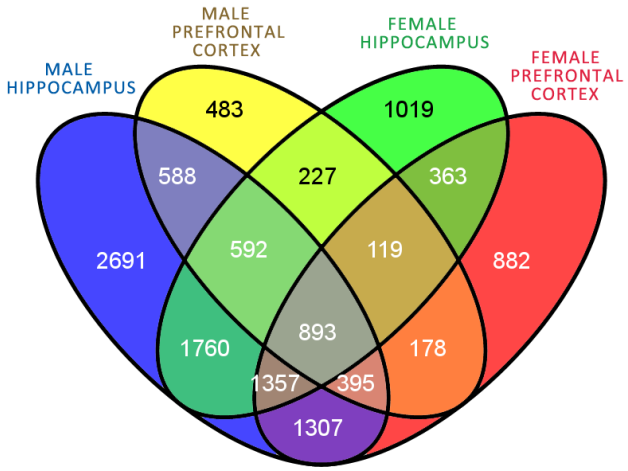

Figure 27 *Venn diagram of genes that are differentially methylated after exposure to prenatal stress in the hippocampus and prefrontal cortex of adult male and female rats.* The numbers within the diagram represent the number of genes characterized as having differential methylation profile between these groups, using a false discovery rate of 0.2

The IPA analysis showed enrichment in molecules involved in neurological disease (p:3.03E-21 - 2.48E-03, 239 molecules), molecular transport (p:4.30E-22 - 2.05E-03, 169 molecules) and nervous system development and function (p:7.43E-15 - 2.99E-03, 191 molecules). Genes involved in psychological disorders, gene expression, behavior and developmental disorders were also significantly enriched.

4.3.4 Convergent approach: comparison with two models of early-life exposure to stress in monkeys and humans

The genome-wide methylation analyses in the rat brain let us to identify important genes and pathways affected, which may be relevant for long-term effects of gestational stress in adult animals.

In order to prioritize the list of genes whose methylation signature could be used as a predictive and diagnostic marker, we took advantage of an European consortium, namely POSEIDON (*Pre-, peri- and postnatal stress in human and non-human offspring: a translational approach to study epigenetic impact on depression*) that offered us the possibility to overlap the list of genes identified in the hippocampus and prefrontal cortex of male and female adult rats, with the methylome results from a model in human of maternal adversities and from a model in monkeys reared under different conditions (for details, see "Materials and Methods"). We thus have been able to shortlist candidate genes that are commonly affected by early life stressors (ELS) in humans, rats and monkeys, which persist into adulthood and which are differentially methylated in both peripheral tissue and the brain.

For the human sample, the focus was on identifying the earliest postnatal DNA methylation response to prenatal ELS in the newborn using DNA of CD34+ hematopoietic stem cells derived from umbilical cord blood. We analyzed extreme ELS groups (10 newborns with very high levels of prenatal stress and 10 newborns with low levels of prenatal stress). For selection of the extreme groups, three different categories of prenatal stress (perceived maternal stress, symptoms of depression and anxiety of the mother, maternal socioeconomic and psychosocial stress) have been taken into account.

We found 6442 probes mapping to 3405 distinct gene promoters whose normalized intensities were significantly associated with the exposure to prenatal stress ($p<0.05$; FDR<0.2). Of these promoters, 1786 were less methylated, and 1750 were more methylated after the exposure to ELS. For 131 gene promoters, a mixed methylation pattern was observed. A supervised heat map based on the 500 probes most strongly associated with the sample group assignment is shown in Fig. 25a, while Table 8 provides a list of genes associated with the 25 most significant probes.

Table 8 List of genes associated with the 25 top differentially methylated probes in the ELS extreme group analysis of human CD34+ cells

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Krtap10-3	4	Low ELS	7.49E-09
Mir889	2	Low ELS	0.00062
Al591025.1	4	Low ELS	0.00062
Mir539	3	Low ELS	0.00062
Syne2	3	Low ELS	0.00062
B3gat2	5	High ELS	0.00077
Casp14	2	High ELS	0.001
Znf672	1	Low ELS	0.001
Pitpnm2	1	Low ELS	0.001

Persistent changes in gene methylation secondary to ELS exposure that can be identified in venous blood cells were of particular interest, as these genes are of potentially high value in follow-up studies in humans. Venous blood of human infants cannot be obtained for ethical reasons. Therefore, we analyzed the ELS signature of CD3+ T cells derived from venous blood of newborn (14 - 30 days old) and adolescent (or 2 years old) monkeys exposed to different rearing conditions. CD3+ T cells have been chosen as CD34+ stem cells, which are progenitors of CD3+ T cells, are not sufficiently abundant in venous blood. In brief, one group of animals has been raised by their biological mothers and served as the control group (MR), whereas the other group of animals was raised by an inanimate surrogate, which triggered ELS (SPR).

In the CD3+ T cells of the 14 - 30 day old monkeys, a total of 4924 distinct genes were significantly associated with the exposure to postnatal stress ($p<0.05$, FDR<0.2). Of these genes, 2803 were less methylated and 2424 were more methylated in SPR monkeys. For 303 gene promoters, a mixed methylation pattern was observed. Table 9 provides a list of genes associated with the 25 most significant probes.

Table 9 List of genes associated with the 25 top differentially methylated probes in CD3+ cells observed in the comparison of maternally reared (MR) and surrogate-peer reared (SPR) primates at postnatal day 14-30

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Loc705164	7	MR	2.44E-07
Calml5	9	MR	2.44E-07
Loc694416	9	MR	2.44E-07

In the CD3+ T cells of adolescent monkeys (2 years old), a total of 2547 distinct genes associated with the exposure to postnatal stress (p<0.05, FDR<0.2) have been identified. Of these genes, 1744 were less methylated, 873 were more methylated in SPR monkeys and 70 gene promoters displayed a mixed methylation pattern. Table 10 provides the list of genes associated with the 25 most significant probes.

Table 10 List of genes associated with the 25 top differentially methylated probes in CD3+ cells observed in the comparison of MR and SPR 2 year old primates

Gene name	Number of Probes	More methylated in	*p* value (FDR corrected)
Urod	6	MR	9.05E-06
Myom2	2	MR	1.08E-05
U7	1	MR	1.08E-05
mml-mir-1240	5	MR	1.13E-05
U6	2	MR	1.13E-05
Paxip1	1	MR	1.66E-05
Harbi1	1	MR	1.77E-05
Loc717746	2	MR	1.77E-05
Tctex1d4	4	MR	2.45E-05
Loc100428810	1	MR	3.01E-05

Heat maps of the clustering analysis of the probes that best differentiated most strongly between rearing groups are shown in Fig. 28b and c.

A total of 1180 genes were found differentially methylated in both 14 - 30 days and 2 years old monkeys (p<2.6E-10, hypergeometric test). These data support the hypothesis that ELS is associated with a pervasive signature in the methylome of CD3+ T cells which arises early after exposure to stress, and that a number of methylation changes persists later in life.

In order to establish which of the genes identified in peripheral specimens from humans and monkeys could be relevant for brain function, these data were crossed with genome-wide methylation analyses derived from the hippocampus and prefrontal cortex of adult rats. We identified 30 genes whose methylation status was associated with ELS in all tissues and all species analyzed. Interestingly, this list includes *Cacna1c*, one of the best-supported and replicated risk genes for affective disorders (Ferreira et al., 2008; Green et al., 2010). By restricting the analyses to promoter regions (-2000 to +500 from the transcription start site) of individual transcripts, we identified 7 gene promoters whose DNA methylation status is affected the same way (hypo- or

hypermethylation, respectively) in all species and tissues. *U6*, *Pde4dip*, *Adarb2*, and *Morc1* were hypomethylated in the ELS groups. *7SK*, *Prmt5*, and *Csrnp3* were hypermethylated in the ELS groups.

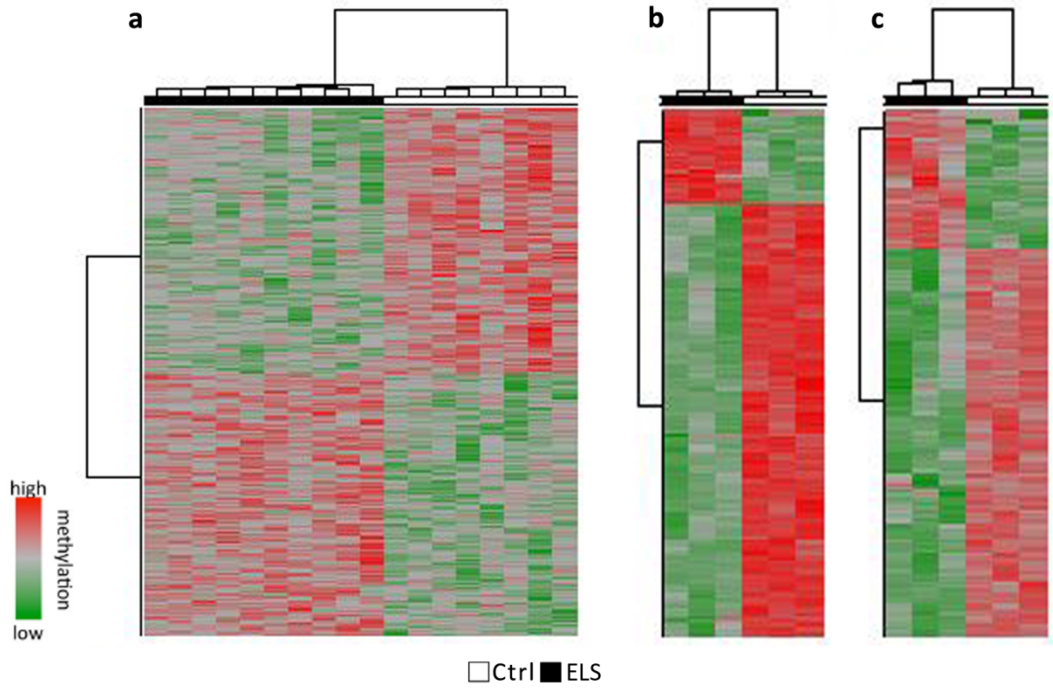

Figure 28 *Heat map depicts normalized intensities of microarray probes contained in promoters that differentiate most strongly between Ctrl and ELS groups.* Rows correspond to promoters and columns to animals. Red indicates higher methylation in a row and green indicated lower methylation in (a) human CD34+ cord blood, (b) 14-30 day old monkey CD3+ T cells and (c) 2 years old monkey CD3+ T cells.

4.3.5 Association with depression

Major depressive disorder (MDD) is one of the well-established ELS-associated phenotypes (Agid et al., 1999; McCrory et al., 2012; Widom et al., 2007). We therefore took advantage of a previous GWAS study on MDD (Rietschel et al., 2010) and performed a gene-based case-control analysis to test for an association between genetic variants in those overlapping genes and MDD. We excluded U6 and 7SK from our analysis, as multiple copies of those genes exist throughout the genome. We additionally had to exclude *Prmt5* and *Pde4dip* as no genetic variants in *Pde4dip* and only one genetic variant in *Prmt5* were represented in the quality controlled GWAS dataset. Of the remaining genes, *Morc1* and *Csrnp3* showed a nominally significant association with MDD (p=0.005 and 0.031, respectively). Only the association of *Morc1* with MDD withstood Bonferroni's correction for the number of genes tested (p=0.014), thus providing evidence that *Morc1* is involved in MDD, a stress-associated disorder. QPCR was used to validate *Morc1* methylation changes observed in human CD34+ cells, monkey CD3+ T cells and rat brain (Fig. 29).

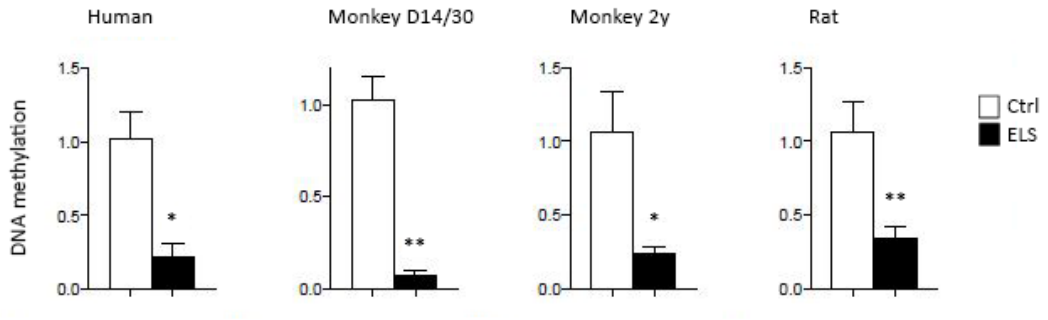

Figure 29 *QPCR analysis of DNA methylation differences in the Morc1 gene predicted by microarray analysis between ELS and Ctrl groups.* Relative bound fraction concentrations are shown. Error bars represent standard error of the mean (SEM).
$^{*}p<0.05$ and $^{**}p<0.01$ vs. Ctrl (Student's *t*-test)

5. DISCUSSION

During the perinatal period, the body may be exposed to complex environmental influences that act on essential maturational processes. Several studies have shown that prenatal (eg. exposure to synthetic glucocorticoids, nutritional deficiency, viral infections or stressful events during gestation) or postnatal (eg. psychological maltreatment, physical abuse, child neglect, caregiver's depressive symptoms) adversities have a long-lasting influence on multiple systems, including the hypothalamic–pituitary–adrenal (HPA) axis functions, the development of the limbic system, as well as the expression of several markers of neuronal plasticity, including neurotrophic factors (Brixey et al., 1993; Brunson et al., 2001; Nishi et al., 2013; Shoener et al., 2006). Perinatal stress, therefore, induces alterations that can not be unambiguously associated with a single disease, but that may predispose to different psychopathologies, suggesting that other factors (including genetic susceptibility) will interact to determine the final pathological phenotype.

We choose to investigate the model of prenatal stress in rats based on the fact that it has been previously amply demonstrated to affect the neuroendocrine system of the offspring at adulthood (Maccari et al., 1995). In fact, the last third of gestation in rats, which corresponds closely to the second trimester of human gestation (Bayer et al., 1993), is a plastic time window for the development of the HPA axis, and it seems to be the most vulnerable period during which environmental risk factors could predispose the offspring/individual to the development of schizophrenia in adulthood (Imamura et al., 1999; van Os and Selten, 1998). In particular, rats born from dams stressed during the last days of gestation display a decreased ability to cope with stress (Darnaudery and Maccari, 2008; Maccari and Morley-Fletcher, 2007), an exacerbated anxiety-like behavior (Vallee et al., 1997) and depressive-like disturbances (Morley-Fletcher et al., 2003). Conversely, offspring do not show abnormal behavior if animals are exposed to stress during the second week of pregnancy, just prior to the expression of the glucocorticoid receptors in the fetal brain (Koenig et al., 2005).

The data obtained in our work show that prenatal stress in rats impairs cognitive abilities, the HPA axis functionality and the expression of neurotrophic molecules, such as *Bdnf*, reproducing molecular and behavioral defects similar to those observed in patients suffering from psychiatric illnesses, such as schizophrenia and depression.

In particular, our results provide novel and detailed insights with respect to early and late consequences of prenatal stress exposure on neuronal plasticity, demonstrating that the

reduction of the neurotrophin *Bdnf* occurs with a specific temporal profile, shows some degree of gender specificity, is sensitive to behavioral challenges and may be modulated by pharmacological interventions during adolescence.

More interesting, in this study, we demonstrate that such vulnerability could be mediated by epigenetic regulation, that in turn affects the physiological outcome in a genome-wide and system-wide fashion. We found that the differential methylation of 30 genes was associated with early-life stress (ELS) immediately after birth in human CD34+ cells, and additionally in peripheral CD3+ T cells of newborn and adolescent monkeys, as well as in the brain of adult rats. A gene-based case-control analysis to test for an association between genetic variants and major depressive disorders (MDD) identified MORC1 as being epigenetically altered after exposure to ELS in peripheral tissue and the brain, and in addition associated with MDD on a genetic level in a different cohort. Our results provide evidence for a link between MORC1 and depression, thus proposing MORC1 as an excellent candidate gene that could be followed longitudinally in living humans.

Several studies have shown that cognitive deficits are a common feature of many psychiatric disorders such as MDD, schizophrenia and bipolar disorder (Iosifescu, 2012). In particular, the maternal exposure to stressful environments during pregnancy could predispose the offspring to develop an array of behavioral and cognitive disabilities (Bergman et al., 2007; Buitelaar et al., 2003; Entringer et al., 2009). Most of the preclinical models, regrettably, have focused their attention on the effects of prenatal exposure to stress on the offspring prior puberty (Wu et al., 2007; Yang et al., 2006), and it remains to be established whether the deficits persist until adulthood. Here, we show that prenatal stress causes long-term consequences on cognitive function, in line with the results of a recent study in which the authors observed an effect of variable prenatal stress exposure on recognition memory in adult animals, but not in adolescent animals (Markham et al., 2010). The test we used takes advantage of the natural tendency of rodents to interact more with novel objects compared to familiar ones, and the choice to explore one instead of the other reflects the use of working memory and recognition memory (Ennaceur, 2010). We chose to adopt ORT to test cognitive performance since it presents a lot of pros compared to other behavioral tests: first, it does not require food or water deprivation (as it is not based on food reward), it is devoid of stressful components and therefore does not generate anxiety or fear in animals and, last, does not require long periods of training.

Our data demonstrate that prenatal stress affects, in adult rats of both sexes, the index of discrimination between the new object and the familiar one. In other words, animals subjected to gestational stress seem, during the test phase, to not remember the object previously explored, spending about the same time exploring the two objects, thus demonstrating the existence of alterations in working and recognition memories. In particular, we observed that female rats exposed to prenatal stress display even less time spent in exploring the novel object. The control animals, on the other hand, recognize and remember the familiar object and tend to further explore the new object.

These effects may be caused by alterations in the hippocampus and cortical areas. Several lines of evidence show that the cytoarchitecture of the hippocampus of the rat is altered as a result of prenatal stress (Gould and Tanapat, 1999; Hayashi et al., 1998; Lemaire et al., 2000), indicating that these manipulations can determine morphological alterations of specific brain structures. Given that the hippocampus is essential for the integrity of the cognitive processes, the reduction of synapses in this area can be correlated with defects in memory and learning observed in rats exposed to prenatal stress (Hayashi et al., 1998). A reduction of hippocampal volume was observed in schizophrenic patients (Stefanis et al., 1999) and in depressed subjects (Hickie et al., 2005), further supporting the possibility that gestational stress could be etiologically relevant for the morphological changes that contribute the deterioration of psychiatric patients. Moreover, it has been shown that the perirhinal cortex plays a pivotal role in object recognition (Barker et al., 2007; Steckler et al., 1998) and, in cooperation with other cortical areas, such as the prefrontal cortex, they could modulate memory processes (Carboni et al., 2010; Fumagalli et al., 2009; Weinstock, 2001). The effects on cognition following exposure to gestational stress could may, in fact, be related to modulation of genes that are involved in synaptic plasticity in this brain area (Kinnunen et al., 2003), similar to what occurs in the schizophrenic brain (Beneyto and Meador-Woodruff, 2008; Broadbelt et al., 2002).

In addition to cognitive function, the prefrontal cortex plays a role in the negative feedback regulation of the HPA axis (Diorio et al., 1993; Vermetten and Bremner, 2002) and, together with the hippocampus, they are critically involved in the developmental programming of adult disease mediated by the glucocorticoids (Cottrell and Seckl, 2009). Undoubtedly, one of the most consistent alterations shared by various experimental paradigms that analyze the effects of short- and long-term stressful perinatal events is the alteration of the activity of the HPA axis (Henry et al., 1994; Maccari et al., 2003; Nyirenda and Seckl, 1998; Seckl, 2004). First, maternal stress has

been associated with intrauterine growth retardation (IUGR), and low birth weight is a hallmark of a suboptimal intrauterine environment. In this study, we found that placentas from PNS pups weighted significantly less when compared to the respective controls, suggesting that our PNS paradigm has an immediate effect on the fetus that is mediated by the placenta, the main source of fetal nutrition, which also acts as a barrier to protect the fetus from the excess of maternal glucocorticoids.

Our data show that rats born from mothers exposed to prolonged stress during the last week of gestation display an altered functionality of the HPA system at adulthood. In fact, the plasma levels of corticosterone are significantly increased in PNS adult rats compared to Ctrl. We also observed a statistically significant reduction of the glucocorticoid receptor mRNA expression in adult rats subjected to prenatal stress, both in the hippocampus and in the prefrontal cortex. Interestingly, this effect is evident starting from adolescence, with some latency with respect to the stress exposure. Of note, the plasma corticosterone levels and *Nr3c1* expression levels in the brain show an inverse correlation, suggesting that the increase in transplacental passage of maternal glucocorticoid during pregnancy lead to a reduced expression of glucocorticoid receptor signaling in the fetal brain that in turn increases basal corticosterone levels (Levitt et al., 1996). Hence, although glucocorticoids play an important role in brain function, the persistent exposure to high levels of these hormones following deregulation of the HPA axis may have toxic consequences for specific cell populations thus leading to long-lasting functional and structural alterations in specific brain regions (McEwen, 1999, 2000). These long-term effects on HPA axis show similarities with different psychiatric disorders, including major depression and schizophrenia (Arborelius et al., 1999; Corcoran et al., 2003; de Kloet et al., 2006; Holsboer, 1989; Tandon et al., 1991), suggesting that such alterations as a consequence of the exposure to early life adversities may represent an endophenotype for these pathologic conditions.

A possible consequence of the alteration of HPA axis functionality is represented by dysfunctions of synaptic plasticity and neurotrophic mechanisms. We therefore decided to deepen this aspect by analyzing the gene expression of the neurotrophin BDNF.

While BDNF has been consistently associated with psychiatric disorders (Autry and Monteggia, 2012; Boulle et al., 2012; Calabrese et al., 2009; Duman, 2002), the relationship between this neurotrophin and the pathologic condition depends on a number of variables, also due to the complexity of the BDNF system (Aid et al., 2007). We demonstrate that prenatal stress produces a significant down-regulation of the pool of *Bdnf* transcripts with long 3'-UTR in the prefrontal

cortex of male as well as of female rats. *Bdnf* transcripts can be processed at alternative polyadenylation sites giving rise to two different pools of mRNAs that harbor either a short or a long 3'-UTR (Baj et al., 2011; Fukuchi and Tsuda, 2010). Each *Bdnf* mRNA isoform encodes for the same BDNF protein, although the relative abundance of the short and long 3'-UTR *Bdnf* mRNAs differs in various brain regions (An et al., 2008). The 3'-UTR region provides a mechanism to specify subcellular localization of *Bdnf* transcripts and their regulation at translational level. Indeed, the long 3'-UTR is responsible for targeting *Bdnf* mRNA to dendrites, where activity-dependent translation may occur (An et al., 2008; Lau et al., 2010). Hence, the selective decrease of long 3'-UTR *Bdnf* mRNA levels after PNS is suggestive of an impairment of a sub-population of transcripts that undergo dendritic targeting and may contribute to local, activity-dependent, neurotrophin synthesis (Lau et al., 2010). It may be inferred that PNS leads to a reduced efficacy of cell-cell communication and synaptic function in the prefrontal cortex, which may contribute to cognitive and emotional deterioration associated with exposure to early life adversities (Michelsen et al., 2007; Murmu et al., 2006). We have previously shown that a reduction of 3'-UTR *Bdnf* mRNAs levels is also found in the prefrontal cortex of serotonin transporter knockout rats (Luoni et al., 2012), a genetic model of depression susceptibility, suggesting that, independent of the etiology, different models of depression may be characterized by profound alterations of synaptic mechanisms. The time course analysis of postnatal *Bdnf* changes in control and prenatally-stressed rats suggests that the reduced expression observed at adulthood is not directly linked to stress exposure, but it may represent an adaptive consequence of late stage maturation of the prefrontal cortex. Indeed, as shown here, and in agreement with previous data (Timmusk and Metsis, 1994), the expression of total *Bdnf* mRNA in control animals sharply increases between the second and third week of postnatal life to decline thereafter toward adult levels. Conversely, the mRNA levels of the long 3'-UTR show a more steady increase reaching maximal expression around adolescence, when the difference between control and PNS rats becomes fully manifest.

Conversely, within the hippocampus we observed a reduction of adult *Bdnf* expression in female rats that is mirrored by similar changes in all *Bdnf* transcripts analyzed. Moreover, even if total Bdnf mRNA levels were not significantly altered in the hippocampus of male rats, we did observe changes in the expression of some neurotrophin isoforms, in particular exon I and VI. While the reduction of the expression of *Bdnf* exon VI in the prefrontal cortex is in agreement with the reduction of the transcripts with the long 3'-UTR, we also have to mention that the promoter of this exon contains a glucocorticoid responsive element (GRE) (Hansson et al., 2006), suggesting

that the alteration of gene expression of this isoform, especially within the hippocampus, may be a consequence of the reduced glucocorticoid receptor expression found in adult PNS rats. Moreover, using a prenatal stress paradigm quite similar to the one we used in the present study, it has been demonstrated that PNS produces a transient alteration of synaptic plasticity in hippocampal CA1 region during adolescence, an effect that was due to impaired processing of proBDNF into mBDNF (Yeh et al., 2012). The abnormalities of pro and mBDNF in hippocampal CA1 were limited to early developmental stages, until adolescence, but disappeared at adulthood, a profile quite different from what we observed in the present study where maximal changes occurred in young adult rats. These observations suggest that changes in *Bdnf* expression and functioning following PNS may also imply abnormalities in the maturational program of selected brain regions (Andersen and Teicher, 2008; Kolb et al., 2012). One possibility is that the exposure to early adversity results in a reduced number of neurons expressing BDNF in selected brain structures. For example, patients suffering from psychiatric disorders, including major depression and schizophrenia, show a reduction in the number of neurons in the hippocampus and in the prefrontal cortex (Duman and Monteggia, 2006; Hashimoto et al., 2005; MacQueen et al., 2003a).

The reduced levels of *Bdnf* may also result from adaptive changes as a consequence of an altered function or release of neurotransmitters, such as dopamine and glutamate, which regulate the expression of *Bdnf* in some brain areas (Kuppers and Beyer, 2001; Zafra et al., 1991). A deficiency in the function of these neurotransmitters has also been described at cortical level in different psychiatric conditions and may contribute to specific disease symptoms, particularly cognitive deficits (Chudasama and Robbins, 2006).

It is known that exposure to stress early in life may prime the brain and enhance its susceptibility to a second hit, such as a further stressful experience at adulthood, which may lead to a precipitation of the psychiatric condition(Maccari et al., 2003). We found that the acute challenge upregulates *Bdnf* in control animals, specifically in the prefrontal cortex, while PNS rats did not show such response, suggesting that gestational stress may impair the proper activation of coping mechanisms at cortical level.

Actually, there is some degree of gender differences in the changes set in motion by PNS, which indicate females as more susceptible to the effects of this manipulation, as previously reported (Bowman et al., 2004). First, at behavioral level we observed a worse performance in PNS female rats, even if PNS male rats also showed a significant reduction of the discrimination index. Second, within the hippocampus, the reduction of *Bdnf* at PND62 occurs in PNS female rats only, and it is

mirrored by changes in the expression of exons I, IV and VI of *Bdnf*. On the contrary, in this brain region, PNS male rats showed a non-significant trend toward a decrease in the expression of the long 3'-UTR *Bdnf* levels, which is mirrored by a significant reduction of the exons I and VI. Conversely, within the prefrontal cortex, while the reduction of the long 3'-UTR mRNA levels of *Bdnf* was similar in males and females, the effect in females is already significant around adolescence and it is mainly sustained by changes in the expression of exon I, whereas also exon IV and VI contribute to the changes in male rats exposed to PNS. However, the most relevant sex difference in *Bdnf* modulation following PNS exposure is evident following an acute challenge at adulthood. Indeed, while the response to the acute forced swim stress is similar in control and PNS male rats in both brain region analyzed, total and long 3'-UTR BDNF mRNA levels were significantly up-regulated in control, but not so in PNS female rats within the prefrontal cortex.

We hypothesize that this acute transcriptional response may represent a coping mechanism set in motion to adapt to the adverse condition, also in line with a previous study in mice showing sex differences in *Bdnf* expression when altering environmental conditions (Chourbaji et al., 2012). As an example, it has been demonstrated that contextual fear learning is associated with a significant up-regulation of *Bdnf* expression, which may participate to the encoding of salient environmental cues (Lubin et al., 2008). Behavioral studies have shown that moderate acute stress facilitates classical conditioning and associative learning and may therefore improve memory (Joels et al., 2006; Shors et al., 1992; Tamashiro and Moran, 2010). Specifically, stress within the context of a learning situation leads to the release of corticosteroids, resulting in focused attention and improvements in memory (Joels et al., 2006). Considering that stress-induced glucocorticoid release may be sensitized in PNS animals (Harris and Seckl, 2011; Meaney, 2001), alternative mechanisms may contribute to stress-induced alteration of *Bdnf* transcription. We have previously shown that enhanced *Bdnf* transcription following acute swim stress is associated with a significant translocation of GR to the nucleus (Molteni et al., 2009). On these bases, the impaired up-regulation of *Bdnf* seen in PNS females exposed to the acute stress may be indicative of impaired GC response, which has been postulated in mood disorders (Anacker et al., 2011; Oberlander et al., 2008).

Our results clearly demonstrate that alterations in *Bdnf* expression in PNS rats have a delayed onset, suggesting that they may represent a long-term adaptive consequence of early manipulation. This is in agreement with the observation that traumatic events early in life may not directly lead to an overt pathologic phenotype, but will eventually set the stage for enhanced

vulnerability later in life. This temporal profile suggests that adolescence may indeed represent a critical time during development that may, on one hand, precipitate a pathologic phenotype but may, on the other hand, offer windows of opportunity for therapeutic intervention.

We specifically addressed this issue and demonstrated the ability of chronic treatment with the antipsychotic lurasidone during adolescence to prevent the alterations of *Bdnf* expression produced by PNS. Indeed, the second generation antipsychotic drug (APD) lurasidone was able, selectively in the prefrontal cortex, to prevent the PNS-induced down-regulation of long 3'-UTR *Bdnf* levels that occurred from late adolescence into young adulthood. Moreover, in line with our previous studies, chronic lurasidone was able to up-regulate *Bdnf* in normal animals (Fumagalli et al., 2012). These results are in good agreement with our previous data in serotonin transporter knockout rats, showing that chronic lurasidone can normalize the defects of neurotrophin expression in the prefrontal cortex of these animals (Luoni et al., 2012). It is worth noticing that lurasidone treatment has a selective impact on the pool of *Bdnf* transcripts with the long 3'-UTR, which are enriched in the dendrites and mediate activity-dependent translation at synapses (An et al., 2008; Lau et al., 2010). Interestingly, antidepressant drugs can facilitate BDNF function at synaptic level. For example, Autry and colleagues have shown that ketamine exerts rapid antidepressant activity by increasing BDNF translation at synaptic level (Autry et al., 2011). Moreover, we have previously demonstrated that chronic antidepressant or antipsychotic drug treatment can facilitate the transcription of *Bdnf* exons that undergo dendritic targeting (Calabrese et al., 2013; Calabrese et al., 2010; Luoni et al., 2012), suggesting that local (synaptic) control of BDNF production may represent an important aspect for pharmacological intervention in mental illness.

Previous studies have tried to address the issue of developmental exposure to psychotropic drugs in order to counteract the outcome of gestational stress. However, in these studies antidepressants, such as fluoxetine, were given to breastfed pups, which is to say during a different time window when compared to our work (Nagano et al., 2012; Rayen et al., 2011). While developmental fluoxetine was able to counteract functional deficits associated with PNS exposure (Nagano et al., 2012; Rayen et al., 2011), it must be kept in mind that deleterious effects of such early treatment have also been reported in normal animals (Ansorge et al., 2008), suggesting that several variables may come into play to determine the final outcome. Furthermore, it has been demonstrated that pharmacological interventions with antidepressants as well as with the glutamate mGlu2/3 receptor agonist LY379268 at adulthood can be effective in

normalizing long-term molecular and functional defects that originate from PNS exposure (Matrisciano et al., 2012; Morley-Fletcher et al., 2004). Our results suggest a novel opportunity for a preventive intervention with the innovative antipsychotic lurasidone during adolescence, which represents a critical window for vulnerability to psychiatric disorders. This may indeed open important venues with respect to therapeutic approaches aimed at preventing the manifestation of full-blown phenotypes in vulnerable individuals.

It has been shown that persistent changes in gene expression of neurotrophic factors, such as long-term consequence of prenatal stress, may also be associated with epigenetic mechanisms (Weaver et al., 2004). Several studies in animal models have demonstrated that environmental risk factors (such as chronic stress in major depression) alter the levels of *Bdnf* expression via epigenetic mechanisms, and pharmacological treatment (for example with antidepressants) is able to restore the levels of *Bdnf* by changes in the epigenome (Tsankova et al., 2006).

It is worth mentioning that also the glucocorticoid receptor system is regulated by epigenetic modifications in response to external stimuli. In fact, first Mairesse and co-workers have shown that the expression and activity of 11β-hydroxysteroid dehydrogenase type 2 (*11beta-HSD2*), an enzyme that in the placental compartment converts glucocorticoids (cortisol/corticosterone) into inactive metabolites (cortisone/11beta-deidrocorticosterone), is significantly reduced in the placenta of mothers exposed to restraint stress during the last week of gestation (Mairesse et al., 2007). In addition, Jensen Peña and co-workers, in agreement with the previous study, showed that, in this animal model, the reduction of *11beta-HSD2* expression is associated with an hypermethylation at its promoter (Jensen Pena et al., 2012). Moreover, in rats, exon 1_7 of the *Nr3c1* gene has higher methylation in pups receiving low maternal care compared to controls (Weaver et al., 2004).

However, it is unlikely that the plethora of systemic consequences brought on by early adaptation to an adverse environment can be the result of epigenetic alterations of these two systems alone. Indeed, we demonstrated that gestational stress lead to an overall dysregulation of epigenetic regulators involved in DNA methylation and post-transcriptional modifications of histones, in line with a study from Blaze and Roth, that uses a postnatal paradigm of early exposure to stress, namely caregiver maltreatment (Blaze and Roth, 2013). Again, we found a certain degree of gender-specificity, as the expression of *Dnmt1* is specifically affected in PNS males, while *Gadd45β* only in PNS female rats. We then investigated, using an unbiased approach, the methylome profile associated with exposure to prenatal stress in rats. Interestingly, an overlap of 893 differentially

methylated genes was observed between the hippocampus and prefrontal cortex of adult male and female rats that were exposed to gestational stress. Ingenuity Pathway Analysis (IPA) showed significant enrichment in molecules involved in neurological disease, molecular transport, nervous system development and function as well as psychiatric disorders. Interestingly, the list includes several genes previously associated with schizophrenia and other psychiatric conditions, such as calcium and potassium voltage operated channels (Lodge and Li, 2008) as well as GABA and glutamate receptor subunits (Coyle et al., 2012; Gaspar et al., 2012; Gonzalez-Burgos and Lewis, 2012; Sanacora et al., 2011).

Such an overlap is significantly larger than expected by chance and, in order to shortlist candidate genes that are affected by early life exposure to stress, we employed a cross-species and cross-tissue approach in humans, monkeys and rats. As postulated before (Szyf, 2012), our data provide evidence for genome-wide and system-wide changes in DNA methylation in response to ELS. This information might be useful in predicting lifelong behavioral and physical phenotypes, especially as we could identify such changes very early in life.

Although previous studies reported associations between ELS and DNA methylation in adult brain tissue or peripheral blood cells, critical questions remained unanswered. Firstly, whether ELS-associated DNA methylation changes are due to early life experiences or represent a consequence of the psychiatric phenotypes resulting from ELS. To address this question, we examined DNA changes immediately after exposure to ELS. Our data show that, in monkeys and humans, DNA methylation differences emerged soon after ELS exposure. Although it is well understood that there are many different exposures that the fetus encounters in utero, our data differentiates between the total impact of the in utero experience and later experiences. This supports the hypothesis that DNA methylation changes follow ELS and precede the appearance of the clinical phenotypes later in life.

In humans, unraveling DNA methylation caused by ELS from DNA methylation caused by other preexisting confounding factors, such as genetic variation and other environmental factors, remains impossible. We thus applied a convergent, translational approach comparing human data with data derived from animal models, since they can be randomized to high and low ELS and their environment can be controlled.

Since a wide variety of prenatal stressors in humans may result in epigenetic alterations and long-lasting effects on the health and behavior of the child, in the present study we applied a broad definition of prenatal adversities in humans. Indeed, in the monkey study we used *Macaca*

mulatta analyses and we focused on the effects of very early postnatal stress on the epigenome. This experimental condition was specific, standardized and adequately controlled. However, the comparison of a postnatal stress paradigm in the monkey to a gestational stress paradigm in humans could be considered a limitation of our study. Nonetheless, the maternal separation model applied in the non-human primates leads to neurobiological, physiological and behavioral consequences similar to those identified in humans after exposure to early adversity (Conti et al., 2012; Suomi, 1997). Our study provides additional evidence that ELS causes evolutionary conserved differential methylation of responsive genes as has been postulated previously (Suderman et al., 2012).

We reasoned that there exist robust and fundamental changes in DNA methylation, which will be conserved not just amongst species but also amongst tissues. The overlapping changes identified in both CD34+ and CD3+ cells are consistent with the hypothesis that changes in methylation in response to ELS appear early in progenitor cells which are then passed on to their different daughter lineages.

ELS is a condition associated with several health and psychiatric conditions including depression in later life (Agid et al., 1999; McCrory et al., 2012; McLaughlin et al., 2010; Widom et al., 2007). Taking advantage of an available GWAS in major depressive disorders (MDD) we were able to demonstrate that genetic variants in one of those persistently methylated genes, *Morc1*, are significantly associated with major depression. This supports the hypothesis that *Morc1* may be involved in at least one known consequence of ELS, the risk for depression in later life.

The role of MORC1 is largely unknown. In mammals MORC1 is mainly expressed in male germ cells. This expression commences in early embryonic development and is important for the completion of prophase of meiosis I during spermatogenesis (Inoue et al., 1999; Watson et al., 1998). Loss of function mutation of this gene in mice leads to male infertility. For other members of the gene family, expression is not restricted to the testis, which suggests a more general biological function for the MORC gene family. A recent study has suggested that MORC1 itself plays a more general biological role, as it encodes for an evolutionary conserved nuclear protein, which may influence gene silencing and chromatin structure, possibly through the detection of epigenetic marks (Iyer et al., 2008; Moissiard et al., 2012; Perry and Zhao, 2003). The identification of *Morc1* as a gene whose promoter is differentially methylated after exposure to different forms of ELS in different organisms, different tissues, and different time points also suggests that this gene plays a more general regulatory role in response to stress.

In conclusion, the paradigm of prenatal stress in rats that we adopted can be useful for the identification of molecular candidates whose alteration may lead to altered brain maturation and impaired cellular plasticity, affecting adult behavior and increasing the vulnerability to psychiatric disorders. Moreover, our systematic, genome-wide, cross-tissues and cross-species approach for the identification of genes that respond similarly to ELS in brain and blood cells is critical for studying behavioral epigenetics in humans and for potential diagnostics and therapeutic interventions. Indeed, we found *Morc1* as differentially methylated in all three investigated organisms, in peripheral tissues as well as in the brain and at different time points in the life span, and an association was demonstrated between *Morc1* and major depression, which is a disorder strongly linked to ELS. We therefore propose *Morc1* as a new candidate gene for stress-related disorders whose DNA methylation status reflects ELS, is amenable to longitudinal follow up in peripheral cells and may predict the emergence of ELS-related disorders such as depression in later life. This has important research, diagnostic and therapeutic implications.

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