



## Review

# Disruptions of the mother–infant relationship and stress-related behaviours: Altered corticosterone secretion does not explain everything

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## ABSTRACT

The hypothalamic–pituitary–adrenal (HPA) axis is the main neuroendocrine system of response to stress, and an imbalance of this system's activity is believed to be at the core of numerous psychiatric pathologies. During the neonatal period, the glucocorticoid response to stress is maintained at low levels by specific maternal behaviours, which is essential for proper brain development. Effective evaluation of the impact of increased secretion of corticosterone during an essentially anabolic developmental period on adulthood behaviour involved separation of the neonate from its mother for periods ranging from 3 to 24 h. It has been shown that disinhibition of the stress response is achieved by such procedures. The pioneering studies by Seymour Levine set the stage for a prolific and promising field of study that may help neuroscientists unveil the neurobiological underpinnings of stress-related disorders. Based on a series of studies, we propose that maternal separation and maternal deprivation change stress-related behaviours, but that corticosterone seem to be only partially involved in these changes in adulthood. It appears that extra-hypothalamic corticotrophin-releasing factor and neurotransmitter systems may be the primary mediators of these behavioural outcomes.

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## 1. Introduction

A growing body of data points to a fundamental interaction between genetic factors and early environmental experiences as determinants of later vulnerability to psychopathology and many stress-related disorders (Caspi et al., 2003; Plotsky et al., 1998). One of the most robust risk factors for the development of anxiety

and depressive disorders in adulthood is a history of severe childhood stress. The risk of developing depression and anxiety is higher in children exposed to family violence (especially when there is police involvement or same-sex parent victimization), insecure attachment or inconsistent rearing attitudes, than in children who had no such experiences (Penza et al., 2003). Epidemiological data also show that parental neglect and early maltreatment are risk factors for later psychopathologies (Gutman and Nemeroff, 2003). For instance, there is a greater likelihood of developing major depression with a history of emotional abuse during childhood, and of anxiety disorders and post-traumatic stress disorder (PTSD) in the context of a history of emotional

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abuse in addition to physical or sexual abuse (Gibb et al., 2007; Springer et al., 2007).

Pre-clinical research may help to shed light on the neurobiological consequences of early life adversity, although modelling of human conditions is extremely difficult and sometimes unfeasible. As discussed in Loman and Gunnar (2009) review, children are rarely submitted to a single type of adversity, as is the case in animal studies. Therefore, pre-clinical studies may be more helpful in establishing cause and effect relationships than clinical studies.

The development of the rat brain includes a spurt of development of specialized astrocytes and pyramidal cells before birth, whereas peak brain growth, myelination and the emergence of granule cells of the dentate gyrus and cells of the olfactory bulb and cerebellum takes place from postnatal days (PNDs) 7–14 (Morgane et al., 2002). This period of intense cellular change occurs during a time when the activity of the hypothalamic–pituitary–adrenal (HPA) axis, the most important neuroendocrine system involved in the stress response, exhibits a peculiar maturational profile known as the stress hypo-responsive period (SHRP). The SHRP extends from PNDs 4 to 14 and consists of a period of low activity of the adrenocortical system and refractory responsiveness to stressors that would normally induce a robust stress response in adult animals (Rosenfeld et al., 1992a). This period is considered a highly adaptive phase in development because the maintenance of low and stable levels of corticosterone (CORT) is required for normal growth and development of the central nervous system (CNS) during ontogeny (Ballard et al., 1979; Doupe et al., 1985; Meyer and Fairman, 1985; Meyer and Joy, 1985; Sawano et al., 1969). Although very well-characterized in rats, the SHRP is less well-understood in children, although the establishment of salivary cortisol assays has allowed for a breakthrough in the study of this system in very young humans and has revealed the existence of such a period in children raised by responsible and caring parents (Gunnar and Donzella, 2002). Although some studies have sought to determine the long-term consequences of disturbances in the activity of the HPA axis during this period in humans, such investigation is easier in animals, especially due to the much faster rate of maturation in rodents.

The present paper reviews studies of the short- and long-term effects of two of the most used environmental adversities during development, long maternal separation and maternal deprivation, in rodents. The focus of this review will be on the activity of the HPA axis and on behavioural and physiological processes that may be modulated by this system. In addition, it presents the results of original studies in which the main objectives were to determine whether high circulating levels of corticosterone during the SHRP affected emotional behaviours and whether they correlate with neuroendocrine responses in adulthood.

### 1.1. The stress hypo-responsive period in the neonate and the influence of maternal behaviours

The SHRP has been proposed to result from factors intrinsic to the pup, such as enhanced negative feedback at the brain and pituitary levels (Walker et al., 1990, 1991) and/or from immaturity of pathways leading to the neonatal hypothalamus (De Kloet et al., 1988). There is currently sufficient evidence, however, to show that negative feedback in the neonate is deficient. More specifically, when a stress response is triggered, CORT and ACTH levels remain elevated for at least 2 h after presentation of the stimulus (Suchecki et al., 1995, 1993b; van Oers et al., 1997).

Factors extrinsic to the pup (e.g., maternal presence) are major determinants of the SHRP. The mother represents a major source of interaction and regulation of physiological and psychological processes. One way to determine how maternal care regulates distinct processes is separation of the dam from her offspring and

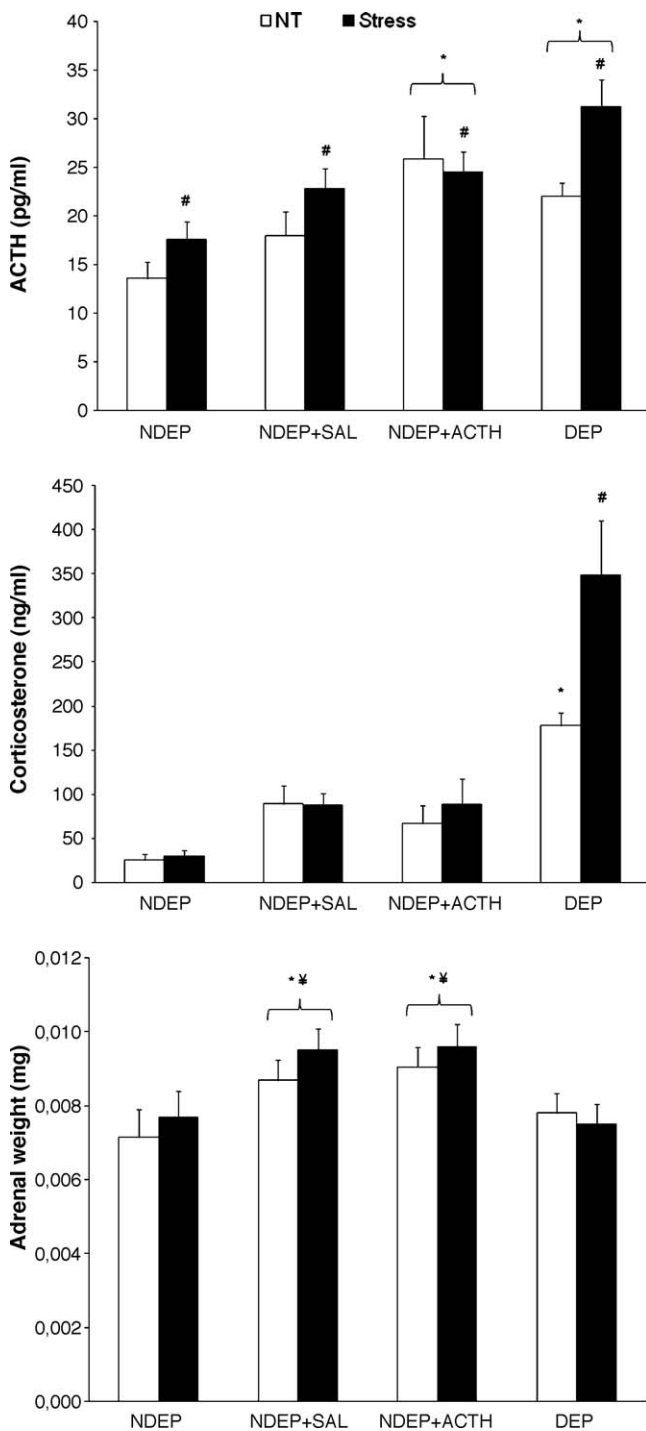
subsequent evaluation of changes in the course of development of any given system (Hofer, 1987). By using this separation approach, Miron Hofer elaborated his theory of hidden regulators within the mother–infant interaction in which some criteria must be fulfilled. The first criterion states that changes in physiological processes in the neonate should develop slowly over the course of many hours of separation, such that they would be distinguishable from the immediate reaction to separation from the mother, which would reflect a non-specific response to the loss of maternal care. The second criterion states that the replacement of specific maternal behaviours during the separation period should prevent or reverse the effects of maternal removal (Hofer, 1983). According to these criteria, the developing HPA axis is under maternal regulation in a tonic inhibitory fashion; however, the available data indicate that the main *locus* of the SHRP is the adrenal gland. For instance, it has been shown that the neonatal rat is quite capable of responding to stress with increased expression of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) at PNDs 6 and 12 (Dent et al., 2000b). Vasopressin (AVP) mRNA is also increased in the PVN of 12-day-old pups, as is *c-fos* expression, in an age-dependent manner (PND 6 < PND 12 < PND 18) (Dent et al., 2000a). Thus, even before weaning, the central component of the HPA axis is responsive to stress. The adrenal gland, on the other hand, is refractory to both direct and indirect stimulation. This physiological feature is dependent on maternal presence. In normally reared pups, administration of ACTH, which overcomes the neural processing of stress stimuli and the activation of brain mechanisms of the stress response, results in a U-shaped curve with adult-like CORT output at PNDs 1 and 2, followed by a progressive decline until it reaches a nadir between PNDs 8 and 12, only to start increasing again after PND 14 (Witek-Janusek, 1988).

The mechanisms involved in the onset and offset of adrenal insensitivity during the SHRP are not completely understood. Evidence suggests that metabolic signals may be a crucial factor for the SHRP. Feeding results in the maintenance of high plasma levels of leptin. This hormone blocks ACTH-induced steroidogenesis in cultures of adrenal cells from 10-day-old pups, strongly suggesting that it may be a mediator of the SHRP (Salzmann et al., 2004). In mice, however, leptin does not appear to play an important role. Instead, GHrelin and glucose seem to be stronger inhibitors of ACTH and corticosterone responses to 12-h maternal deprivation (Schmidt et al., 2006).

These regulatory factors do not explain why maternally deprived pups, which secrete more CORT in response to stress, do so in a similar age-dependent manner as non-deprived pups, i.e., in a U-shaped curve. One possibility could be that this negligible adrenal responsiveness results from insufficient stimulation by ACTH because the ACTH response is stimulus- and age-dependent during this period (Suchecki et al., 1993a, 1995; Walker et al., 1991). If this were indeed the case, exogenous administration of ACTH should sensitize the adrenal gland, rendering it responsive to a mild stressor. We tested this hypothesis by administering ACTH to non-deprived neonates (NDEP + ACTH) and comparing CORT levels between NDEP and deprived (DEP) pups (see legend of Fig. 1 for details on experimental procedures and results).

The results demonstrated that the adrenal gland responded to its trophic factor and to the stress of injection, given the augmented adrenal gland. Importantly, this effect did not translate into increased basal or stress-induced CORT levels, as seen in DEP pups. Thus, some aspect(s) of the mother–infant relationship functions as a shield for the adrenal glands. Overall, these findings suggest that the low CORT levels found in NDEP pups are not due to insufficient stimulation of the adrenal glands by low levels of ACTH.

Because the SHRP is such a sensitive period for CNS development, disruptions of the mother–infant relationship during this



**Fig. 1.** ACTH (pg/ml), CORT ( $\mu$ g/ml) and adrenal weight (mg) were measured from 12-day-old (NDEP) non-deprived, from NDEP pups injected twice with saline (0.9% saline injection, 0.1 ml/10 g body weight, NDEP + SAL) or ACTH (Synacthen<sup>®</sup> Depot, Novartis Pharma, Brussels, Belgium; at a dose of 0.1 ml/10 g body weight, NDEP + ACTH), and maternally deprived (DEP) pups. The ACTH solution was prepared immediately before being used by diluting 20  $\mu$ l of ACTH in 1980  $\mu$ l of 0.9% saline. Both saline and ACTH were administered twice in 11-day-old pups (at 10:00 a.m. and at 4:00 p.m.), and after the injections, pups were immediately placed back with their mothers until the time of testing. NDEP and DEP non-treated litters were used as comparison groups to provide reference values for ACTH and CORT basal and stress response. The breeding procedure was carried out according to Suchecki and Tufik (1997). The day of birth was designated PND 0. On PND 1, litters were culled to four males and four females. When necessary, cross-fostering was performed between litters born on the same day. The maternal deprivation procedure consisted of removing the pups from their mothers and placing them in another cage with sawdust from their home-cage on top of a heating pad set at 30–33 °C. Mothers and pups were kept in different rooms during the deprivation period,

period could be useful for the study of consequences of maternal negligence, parental loss and disturbed upbringing on processes mediated by the brain. Nonetheless, the multitude of experimental conditions has hindered the attainment of definite conclusions and has contributed to discrepancies in the results reported in the literature (Lehmann and Feldon, 2000). For the purpose of clarity, we will refer to *Long Maternal Separation* (LMS) as cases of repeated removal of either pups or mother from the nest for periods ranging from 3 to 8 h per day. The maternal separation paradigm typically involves multiple sessions, but there are some cases of a single separation event and to *Maternal deprivation* solely to a single 24-h period.

## 2. Models of disruption of the mother–infant relationship

### 2.1. Long maternal separation

#### 2.1.1. Short-term effects

Few studies have evaluated the immediate effects of either single or repeated maternal separations on the neuroendocrine and behavioural stress responses of neonatal rats. Among these studies, the separation procedures were variable both in regard to the length of separation and how pups were maintained during this period (e.g., isolated from siblings, in a warm or cold environment, etc.) The LMS procedure described in a large number of studies involves removal of the pups from the cage for an allotted period of time. Before returning the pups to the cage, the mother is taken away, and the pups are placed back in the cage. The mother is then placed back in the cage as well. Nonetheless, variations among these similar procedures could be the source of the inconsistency in the results.

Historically, the earliest studies focused on the paradigm of infantile stimulation due to the unexpected positive effects of early adversity on the stress response and maturational processes. The surprising finding reported in Seymour “Gig” Levine’s seminal work (Levine, 1957; Levine and Alpert, 1959; Levine et al., 1957, 1958) that electric foot shock (Levine, 1957) and other noxious stimuli during infancy [cited in (Zarrow et al., 1968)] rendered the animals less reactive to psychological stress during adulthood represented the starting point of a most prolific field of research. Because pups had to be away from their mothers during the stress session, a control group that was only separated for a brief period of time (usually 15 min) was used. This procedure, which is customarily termed early handling (EH), resulted in similar effects to those observed in the stressed pups, leading the authors to conclude that these effects were produced by the brief separation

and the procedure was carried out during the morning between 9:00 a.m. and 11:00 a.m. In each litter, two male and two female pups were sampled immediately after the end of maternal deprivation or at the corresponding time for NDEP groups (non-treated–NT), while the remaining pups received a saline injection and were sampled 30 min later (Stress). ACTH, CORT and adrenal weight data were analysed by two-way ANOVA (factors: Group and Stress). Analysis of the data showed a main effect of group for ACTH plasma levels [ $F(3,67) = 5.9$ ;  $p < 0.002$ ], with both DEP and NDEP + ACTH groups exhibiting higher ACTH levels than NDEP pups ( $p < 0.004$ ). A treatment effect was also observed [ $F(1,67) = 4.63$ ;  $p < 0.04$ ], in which stressed pups showed higher ACTH levels than non-stressed pups. For CORT plasma levels, main effects of group and treatment and an interaction between these two factors were observed [ $F(3,74) = 3.72$ ;  $p < 0.02$ ]. *Post hoc* analysis of this interaction showed that basal levels of DEP rats were higher than those of NDEP and NDEP + SAL rats, which did not differ from each other ( $p < 0.05$ ). Likewise, DEP rats exhibited the highest CORT stress response to the saline injection ( $p < 0.0002$ ), while none of the NDEP groups showed a stress response ( $p < 0.0005$ ). Finally, for adrenal weight, ANOVA revealed a main effect of group; therefore, the data were collapsed across treatments [ $F(3,79) = 5.1$ ;  $p < 0.003$ ], with NDEP + SAL and NDEP + ACTH pups showing heavier adrenal glands than NDEP and DEP pups ( $p < 0.01$ ). Values are expressed as mean  $\pm$  SEM of 20–24 pups/group. (\*) Different from NDEP group; (#) different from all other groups and respective treatments; (¥) different from DEP group ( $p < 0.05$ ).

of the pups from their mothers. EH was shown to increase CORT secretion in 2-day-old rat pups and to result in augmented binding of  $^{14}\text{C}$ -CORT in the hypothalamus (Denenberg et al., 1967). Moreover, it has been shown that mice tested at different ages during the first two weeks of life secrete more CORT in response to 15 min of exposure to clean bedding at PNDs 12 and 14 (D'Amato et al., 1992). At the level of the CNS, EH results in increased CRH mRNA expression in the central nucleus of the amygdala (CeA) and in the anterodorsal bed nucleus of the *stria terminalis*, but reduced expression in the PVN. In contrast, glucocorticoid receptor (GR) mRNA expression is reduced in the CeA and unchanged in the hippocampus and PVN, which explains the reduction of CRH expression in the PVN (Avishai-Eliner et al., 2001; Fenoglio et al., 2004).

In mice that are in the midst of the SHRP (PND 9), changes in the activity of the various components of the HPA axis take place with distinct temporal patterns. The first element to be affected by removal of the mother is CRH, for which mRNA expression in the PVN shows a slight drop after one hour of separation, followed by an increase by 2 h and a substantial reduction that remains until 24 h. ACTH levels, in turn, increase following 2 h of separation, with a further increase at 8 and 12 h; however, by 16 h, levels are only slightly elevated above those of non-separated pups. Finally, CORT levels increase steadily from 4 to 24 h of manipulation (Schmidt et al., 2004). These results indicate that consistent changes in the activity of the HPA axis in 9-day-old mice are undetectable earlier than 2 h into a single episode of maternal separation. Similar findings were described for 10-day-old homozygote and heterozygote Brattleboro rats (AVP-deficient rats, a model of diabetes insipidus) separated from the mother for 1, 4, 12 or 24 h. Both genotypes exhibit increased CORT levels after 4 h of separation; however, surprisingly, homozygous pups display even higher CORT levels than heterozygous pups, despite no change in ACTH levels. This finding suggests that ACTH levels are regulated almost exclusively by AVP and not by CRH (Zelena et al., 2008).

Consistent results have been observed with a period of separation ranging from 4 to 8 h, when a gradual increase in basal, stress-induced and ACTH-induced CORT secretion becomes apparent in PND 3, 7 and 11 pups (Levine et al., 1991). Interestingly, a single or multiple (three times) 8-h separation schedule results in similar CORT responses to novelty, saline or hypertonic saline. This finding indicates that 8 h of maternal separation does not lead to sensitization of the adrenocortical response to distinct stressors. There also seems to be no cumulative effect of multiple 8-h separations, suggesting that returning to the mother resets the system (Rosenfeld et al., 1992b).

### 2.1.2. Long-term effects

In the literature, there are major differences in the frequency, duration and age of onset of LMS. Reports of distinct outcomes, therefore, should come as no surprise and it is almost impossible to reconcile the discrepancies that have appeared in the literature. Subtle differences in procedure and in animal husbandry could account for some of the apparent successes or failures of some laboratories to observe the long-term effects of LMS on stress reactivity (Levine, 2005). Moreover, EH is commonly used as a comparison group for LMS, given that these two early manipulations can result in opposite effects, especially with regard to the activity of the HPA axis, but not for some other behaviours. This finding indicates that the activity of the HPA axis may not necessarily impact disruptions of the mother–infant relationship on later stress-related behaviours.

Many of the outcomes of repeated maternal separation have been attributed to changes in maternal care upon reunion of the pups with their mothers; however, this issue is controversial.

Initially, it was thought that manipulated pups vocalized more, thus stimulating maternal behaviour (Bell et al., 1974, 1971). This increase in maternal behaviour was shown later to be induced by pup ultrasonic vocalization, as became evident in a study in which handled pups that did not display ultrasonic callings received little maternal care, whereas those exposed to cold for brief periods vocalized intensely upon returning to the nest, leading the mothers to assume the lactation posture (Bell et al., 1974). A recent study reported opposite findings, such that pups isolated from the nest for 3–6 h emitted less ultrasonic vocalization, but increased, nonetheless, bouts and time of licking and grooming by the mothers (Zimmerberg et al., 2003). In addition, pups subjected to brief handling associated with ear punch or maintained out of the nest were tended by parents more often than pups that had been handled or not manipulated (Barnett and Burn, 1967). Demanding pups maintained isolated and away from the mother for 12 h and, consequently, in greater need of care, also receive large amounts of maternal care (Pereira and Ferreira, 2006). Lastly, a comparison of pup care dispensed by the mothers of early-handled or LMS rats showed that, although LMS pups received more maternal care, they exhibited more fearful behaviour and higher ACTH and CORT responses to restraint stress than early-handled animals, thus demonstrating an apparent dissociation between early care and later anxiety-like behaviour (Macri et al., 2004).

Long maternal separation in the rat has been used as an animal model of vulnerability to the development of depressive- and anxiety-like disorders because adult animals exhibit similar features to those observed in depressive and anxious patients. Among these features, there is a reduction in CORT negative feedback efficiency (Ladd et al., 2004) and increased neophobia (Caldji et al., 2000). There are, however, contradictory results with Wistar rats. In these rat strain, LMS produces lower levels of anxiety-like behaviour (Ploj et al., 2002; Roman et al., 2006). Because depression and anxiety present high co-morbidity with other behavioural changes that can be triggered by stressful events in adolescence and adulthood, we describe how LMS may influence the manifestation of some of these co-morbidities in the following sections.

*2.1.2.1. Effects of stress-induced sleep.* Sleep alterations are closely associated with stress-related disorders and several psychopathologies. For instance, depressed patients complain of insomnia, with difficulty falling asleep, early wake-up and several awakenings throughout the night. Polysomnographic recordings confirm these alterations and show changes specifically in rapid eye movement (REM) sleep, such as reduced latency for the first REM sleep episode, increased time spent in REM sleep and decreased delta sleep (part of what is called NREM sleep) (Popa et al., 2008; Riemann et al., 2001).

Because adult rats submitted to repeated LMS exhibit depressive- and anxiety-like behaviours, which are closely associated with sleep disturbances in humans, we designed a series of experiments to test the possibility that early stress in the form of 3-h daily maternal separation would produce a vulnerable phenotype of stress-induced insomnia. The rationale for the use of the LMS model was based on the fact that anxious individuals are at greater risk to develop chronic insomnia after a stressful situation (Larsson et al., 2008; Morin et al., 2003; Waters et al., 1993). We initiated this series of studies by challenging adult Wistar rats submitted to EH with 1 h long restraint stress and assessed their sleep patterns before and after the challenge. We reasoned that early-handled rats would display more sleep rebound (an augmentation in sleep time usually observed after exposure to a stressful situation in the rat) than control rats, given that this manipulation has been shown to decrease hormonal stress response and that the hormones of the HPA axis are

primarily involved in waking (Bradbury et al., 1998; Chang and Opp, 2002; Dugovic et al., 1999; Lancel et al., 2002; Marinesco et al., 1999; Opp and Imeri, 2001; Vazquez-Palacios and Velazquez-Moctezuma, 2000). To our surprise, early-handled rats showed the same sleep rebound as control non-manipulated rats, i.e., increased time spent in NREM and REM sleep (Tiba et al., 2003). We then proceeded to test 3-h maternally separated rats because increased anxiety- and depressive-like behaviours would presumably exhibit changes consistent with these pathologies and possibly less sleep rebound. Surprisingly, we found that male rats displayed more REM sleep in the light phase (the resting phase of the rats) than control and early-handled rats (Tiba et al., 2004), whereas female rats exhibited a significant increment of REM sleep in the dark phase (the active phase of rats) after cold stress (Tiba et al., 2008). In both males and females, the manipulated groups (early-handled and LMS) exhibited similar secretion of CORT in response to the challenge (cold stress) compared to non-manipulated rats. Together, the hormonal results do not support the behavioural outcomes of these manipulations, suggesting that other mechanisms may underline sleep alterations found in LMS animals. The most provocative result was the increase in pup retrieval by 3-h-separated mothers upon returning the pups to the nest, indicating that, at least in this aspect, maternal behaviour was increased by LMS (Tiba et al., 2004).

In conclusion, for both male and female rats submitted to LMS, REM sleep time was increased, which parallels the change in depressed individuals [for review, see [Riemann, 2007](#)]. None of the other sleep change characteristics of depression (such as shortened latency to REM sleep or sleep fragmentation), however, were found in this animal model.

**2.1.2.2. Effects on memory.** Few studies have focused on the consequences of LMS on learning and memory in adulthood, although all of the alterations induced by this manipulation seem to be related to the hippocampus, a structure that is known to be involved in some mnemonic processes. Additionally, there are important cognitive disturbances in stress-related psychiatric disorders, such as deficits in working memory, selective attention and long-term memory in patients with major depression ([Hasler et al., 2004](#)), and declarative long-term memory and attention impairments in patients with PTSD ([Nelson and Carver, 1998](#)). It has been proposed that HPA axis hyperactivity interferes with cognitive function because glucocorticoids are known to play a role in learning and memory, and it is given that elevated levels of this hormone induce memory impairment ([McGaugh and Roozendaal, 2002](#)). Data on the cognitive effects of LMS, however, are controversial. In some studies, including one from our laboratory, LMS rats exhibit slight cognitive impairment in memory tasks, such as the Morris water maze ([Aisa et al., 2009a,b](#); [Huot et al., 2002](#)), contextual fear conditioning ([Guijarro et al., 2007](#)) and the novel object recognition test ([Aisa et al., 2007](#); [Marcos et al., 2008](#)). In other studies, this manipulation either improves performance in the Morris water maze and active avoidance task ([Pryce et al., 2003](#); [Schable et al., 2007](#); [Weiss et al., 2001b](#)) or leads to no observed change in behaviour ([Diehl et al., 2007](#); [Madruga et al., 2006](#)). One possible explanation for these discrepancies involves behaviours that are non-specific to learning and memory, but constitute important factors for performance of the tasks, including increased locomotor activity and impulsive behaviour and decreased oriented behaviour displayed by LMS rats ([Colorado et al., 2006](#)). Increased locomotion could be beneficial in active avoidance (because the animal must escape to the safe compartment in order to avoid foot shock) and counteracts freezing behaviour (used as a retention index for fear conditioning), while impulsivity and decreased oriented behaviour could interfere with context perception, impairing both water maze and fear con-

ditioning tasks (in which the animal must perceive the environment to construct a spatial map in order to find the hidden platform in the water maze task or must notice that the context was previously paired with a foot shock in the fear conditioning task). Therefore, performance could benefit from these behavioural changes in some tasks and be impaired in others. Other contributing factors include gender, the method of learning and memory evaluation in different tasks, and the use of different control groups for comparison.

Recent studies indicate that impairment of performance in the Morris water maze in LMS rats stems from higher susceptibility of cholinergic neurons in the basal forebrain to neurotoxic lesions, in addition to a reduced density of GR in the hippocampus (but not in the frontal cortex) and lower expression of hippocampal nerve growth factor ([Aisa et al., 2009b](#)). Moreover, LMS leads to reduced expression of BDNF and cell proliferation in the dentate gyrus ([Aisa et al., 2009a](#)). Overall, these data indicate that LMS alters the cholinergic system and markers of neuronal plasticity in key regions involved in spatial memory.

Regarding gender differences, for instance, in the study of [Kosten et al.](#), only females submitted to maternal separation exhibited impaired performance in contextual fear conditioning, while males performed as well as control animals. The authors' conclusion of impaired performance was based on differences in ultrasonic vocalizations instead of freezing behaviour, which showed no change ([Kosten et al., 2007](#)). In agreement with these results, a recent study performed in female rats submitted to either EH or LMS (6 h/day) reports that LMS reduces innate fear and freezing responses in the training session for tone-fear conditioning, with a concomitant reduction in the autonomic response to training or re-exposure to the conditioned stimulus ([Stevenson et al., 2009](#)).

Improved active avoidance in males and enhanced spatial learning and memory in LMS females has also been reported ([Pryce and Feldon, 2003](#)). The authors discuss the importance of using an adequate comparison group because non-manipulated animals perform poorly in memory tasks, leading to the conclusion that early-life manipulated rats display better learning and memory. In fact, when compared to rats that had been handled according to the animal facility routine (AFR) – in which the home-cage is cleaned two or three times per week – LMS males are not different with regard to their performance in the two-way avoidance task ([Pryce and Feldon, 2003](#)). Taken together, these findings indicate that confounding factors, such as anxiety, impulsive behaviour, motor components, and gender differences, should be taken into account, especially when evaluating emotional memory, in order to exclude behavioural consequences of early-life manipulations that might influence learning and memory.

**2.1.2.3. Effects on drug abuse.** The effects of LMS on drug abuse have been described in previous reviews ([Meaney et al., 2002](#); [Moffett et al., 2007](#); [Roman and Nylander, 2005](#)). Most studies in this field use one of the following paradigms to assess drug addiction [for more details, see ([Sanchis-Segura and Spanagel, 2006](#))]: (1) self-administration is widely used because it mimics human consumption behaviour and reveals the motivation for drug intake; and (2) behavioural sensitization, which refers to the increase in behavioural responses induced by repeated administration of the same dose of drug.

In general, LMS adult males display higher drug intake. Most studies use AFR as the control group because [Jaworski et al. \(2005\)](#) showed that non-manipulated animals consumed more alcohol than any other group (LMS, early-handled and AFR). Compared to non-manipulated controls, LMS rats acquire cocaine self-administration at the lowest dose tested ([Moffett et al., 2006](#)), exhibit a preference for morphine in the place preference test ([Vazquez](#)

et al., 2005), and consume more alcohol (Huot et al., 2001; Ploj et al., 2003; Roman and Nylander, 2005). Increased alcohol intake in LMS animals is reversed by chronic treatment with the antidepressant paroxetine (Huot et al., 2001). It has recently been shown that LMS male mice also consume more alcohol. Using the three-bottle choice procedure in which the mouse can choose between vehicle (saccharin) and two alcohol solutions (6 or 10% alcohol in saccharin), LMS mice prefer the 10% alcohol solution (Cruz et al., 2008). These results are in agreement with previous studies in which LMS rats consumed more of the high alcohol concentration solution compared with control animals, but showed no differences when low concentrations were offered (Ploj et al., 2003). There appears to be a gender-dependent effect of LMS in paradigms of drug intake because female LMS rats and mice do not differ from control animals (Advani et al., 2007; Gustafsson et al., 2005; Roman et al., 2004). Interestingly, there are rat strains that have been selectively bred for their preference to voluntarily consume alcohol, a substance that usually has an aversive taste for rats. In these strains, LMS increases drug intake in males, but decreases the behaviour in females, indicating an interaction between genetics and gender in determination of the effects of early life events (Roman et al., 2005).

The effects of LMS on the locomotor response to drugs of abuse are also gender-dependent. Male LMS animals, but not females, show higher amphetamine-induced locomotion (Pryce et al., 2001) and higher behavioural sensitization to morphine compared to control animals (Kalinichev et al., 2002), whereas female mice, but not males, exhibit faster development of behavioural sensitization to alcohol (Kawakami et al., 2007). Some studies have reported a lack of LMS effects on cocaine-induced locomotion (Marin and Planeta, 2004) and on behavioural sensitization to amphetamine (Weiss et al., 2001a), and one study reported lower behavioural sensitization in female LMS rats compared to AFR rats (Li et al., 2003). Again, these discrepancies may be attributable to the use of different control groups, different animal strains (Long-Evans and Wistar rats) and different species (mice or rats).

**2.1.2.4. Effects on the immune response.** Child maltreatment is related to an increased risk for the development of inflammatory disorders. Adults who were maltreated during infancy display increased levels of clinically relevant high sensitivity C-reactive protein, fibrinogen and white blood cells (Danese et al., 2007). Besides maturation of the nervous and endocrine systems, the neonatal period is also important for development of the immune system. The ontogenesis of these three systems appears to be interdependent (Jankovic et al., 1981), inasmuch as neonatal manipulations in rodents are related to differential immune responses and susceptibility to auto-immune diseases in adulthood. In a descriptive study, Groer et al. (2002) reported a reduction in thymus weight in BALB/c pups submitted to LMS, but observed no change in the mitogenic responses of cultured spleen cells.

LMS is a useful procedure for the study of inflammatory diseases. For instance, inflammatory bowel disease, which chronically affects the gastrointestinal tract, is characterized by numerous relapses throughout life and is closely associated with chronic stress and mood disorders, typified by an abnormal pattern of Th1 and Th2 cytokines (Blumberg and Strober, 2001; Mawdsley and Rampton, 2006). The balanced production of pro- (Th1 pattern) and anti-inflammatory (Th2 pattern) cytokines is essential, and its loss leads to numerous neoplastic, infectious, allergic, inflammatory and auto-immune diseases [reviewed in (Elenkov and Chrousos, 2002)]. In rats, exposure to foot shock in adulthood elicits a sensitizing effect to dextran sulfate sodium (DSS)-induced colitis in LMS animals, with more erosions and greater severity than in non-manipulated animals (Milde et al.,

2004). LMS mice show greater vulnerability to DSS-induced colitis, as they present higher histological damage and elevated secretion of IFN- $\gamma$  and TNF from mesenteric lymph node cells. Consistent with the relationship between heightened emotional behaviour and irritable bowel syndrome in humans, these animals also display increased anxiety-like behaviour in the elevated plus maze, and, after a paradigm of chronic psychosocial stress, they exhibit lower CORT levels, more pronounced adrenal hypertrophy and higher CRH mRNA expression in the PVN. These findings indicate enhanced emotionality and propensity to stress disorders (Veena et al., 2008).

The literature points to a major interplay between the immune system and altered behaviour induced by LMS. Inflammatory bowel disease corroborates this idea because it has been associated with depression (Addolorato et al., 1997; Kurina et al., 2001). In agreement with the induction of depressive-like behaviour reported in some LMS studies (Huot et al., 2001; Lee et al., 2007; Ruedi-Bettschen et al., 2006; Varghese et al., 2006), LMS C57BL/6 mice display more severe colitis than non-manipulated mice, with increased production of pro-inflammatory cytokines IL-6, IL-1 $\beta$  and TNF- $\alpha$ , which is successfully treated with the antidepressants desmethylimipramine and desipramine (Ghia et al., 2008; Varghese et al., 2006).

There seems to be an interaction between inflammatory bowel disease and irritable bowel syndrome, a disease that is characterized by abdominal pain caused by hypersensitivity of the colon to mechanical stimuli. Inflammatory and immune processes, although not yet well-understood, may be involved in the muscle dysfunction and gut hypersensitivity observed in irritable bowel syndrome (Quigley, 2005). LMS seems to be an interesting model of irritable bowel syndrome because many features of the human syndrome are mimicked [for review see (Barreau et al., 2007)]. The effects of LMS on the rat pup's gut include stimulated ion secretion, enhanced macromolecular permeability, increased adherence and bacteria penetration, and enhanced colonic mast cell density (Barreau et al., 2004a, 2008; Gareau et al., 2006). These alterations persist through the animals' lives inasmuch as adults also show an increased density of colonic mast cells (Barreau et al., 2004a, 2008). Gut paracellular permeability is increased by LMS (Barreau et al., 2004a, 2007), and enhanced stress-related intestinal permeability is associated with pro-inflammatory cytokines, such as IFN- $\gamma$  (Ferrier et al., 2003). Under these conditions, LMS Wistar rats show over-expression of pro-inflammatory (IL-1 $\beta$ , IL-2 and IFN- $\gamma$ ) and anti-inflammatory (IL-4 and IL-10) cytokine mRNA in the colon, spleen and liver (Barreau et al., 2004b). Treatment with some species of lactobacillus reverses LMS-induced augmented gut permeability and reduces the elevated CORT levels seen in these animals, suggesting that impaired gut colonisation in the neonatal period may be responsible for HPA axis and gastrointestinal tract disturbances, or vice versa (Eutamene et al., 2007; Gareau et al., 2007).

Some studies indicate that LMS influences susceptibility to infection insofar as adult rats submitted to LMS are more vulnerable to primary parasitic infection and exhibit exacerbated parasite-induced intestinal infections (Barreau et al., 2006). In addition, C57BL/6 female LMS mice show a more prominent rise in lung pro-inflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-12 and IFN- $\gamma$ ) mRNA expression after infection with influenza, and reduced host resistance is seen in both males and females during the early phases of infection, with higher viral titres (Avitsur et al., 2006).

With regard to auto-immune diseases, there seems to be some controversy over the impact of LMS and involvement of the HPA axis. For instance, in experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis with a Th1 cytokine pattern, the results are inconsistent. On one hand, Laban et al.

(1995) have shown decreased susceptibility to EAE, whereas Stephan et al. (2002) have reported early onset and worsening of EAE with a concomitant decrease in CORT levels in female Lewis rats submitted to daily 2-h maternal separation compared to non-manipulated control rats. Because physiological levels of glucocorticoids induce a shift from a Th1 to Th2 cytokine secretion pattern, the altered CORT secretion is possibly related to worsening of the disease. In line with these results, when periodic touching is added to the 2-h neonatal handling, further aggravation of EAE is observed in Lewis female rats (Manni et al., 1998). Recent findings indicate that early-handled male C57BL/6 mice may be more vulnerable to EAE because of a lower surge of testosterone secretion after immunization, given that this hormone displays a protective effect against auto-immune disease (Columba-Cabezas et al., 2009). As happens in models of inflammatory bowel disease, antidepressants can counteract the damage imposed by LMS in EAE; e.g., imipramine reverses the symptom aggravation of EAE and increases IL-4 levels, suggesting that the protective effect of the antidepressant may be partially due to a shift in the Th1 to Th2 cytokine pattern (Stephan et al., 2002).

On the other hand, for the adjuvant-induced arthritis model of rheumatoid arthritis, LMS showed no effect on either the severity of the disease or CORT secretion in Lewis (more susceptible) or Fischer (less susceptible) rats (Lariviere et al., 2006). These data partially corroborate results reported by our group, which showed no effect of LMS on the course of lupus in females of a mouse strain that develops spontaneous systemic lupus erythematosus, although a decrease in CORT levels was observed in these animals (Catallani et al., 2008). Interestingly, in non-manipulated mice, there was a clear association between basal secretion of CORT throughout life and disease-related parameters, whereas this association was lost in LMS mice (Catallani et al., 2008). Although activity of the HPA axis contributes to activity of the immune system, conferring protection against auto-immune diseases, there are clearly other mechanisms involved in this process, with secretion of leptin being a possible candidate (Columba-Cabezas et al., 2009).

## 2.2. Maternal deprivation

### 2.2.1. Short-term effects

Maternal deprivation results in increased basal, ACTH- and stress-induced CORT secretion immediately following maternal deprivation procedure (Cirulli et al., 1994; Levine et al., 1991; Rosenfeld et al., 1991; Stanton et al., 1988). With regard to the effects on ACTH, small and sometimes imperceptible changes in basal levels are observed in deprived compared to non-deprived (NDEP) neonates (Suchecki et al., 1993a, 1995), whereas the response to mild stressful stimuli is augmented after 24 h of maternal deprivation (Suchecki et al., 1993a). This observation suggests some control via the CORT negative feedback loop. Importantly, this control mechanism does not seem to be as effective as has been suggested by some authors (Walker et al., 1990, 1991), as ACTH will remain elevated for prolonged periods of time, even when CORT levels are elevated in response to different stressors (Suchecki et al., 1995).

Early studies by Gig Levine's group showed that specific maternal behaviours regulate specific aspects of the stress response in pre-weanling rats. Thus, feeding is important for regulation of the adrenal response because periodic infusion of milk to DEP pups lowers the CORT response to stress, albeit not to the levels seen in NDEP pups (Rosenfeld et al., 1993; Stanton and Levine, 1990; Suchecki et al., 1993b). Regulation of the ACTH response, in turn, is accomplished by anogenital stroking, inasmuch as DEP pups submitted to this simple manipulation during the deprivation period show a response to mild stress as

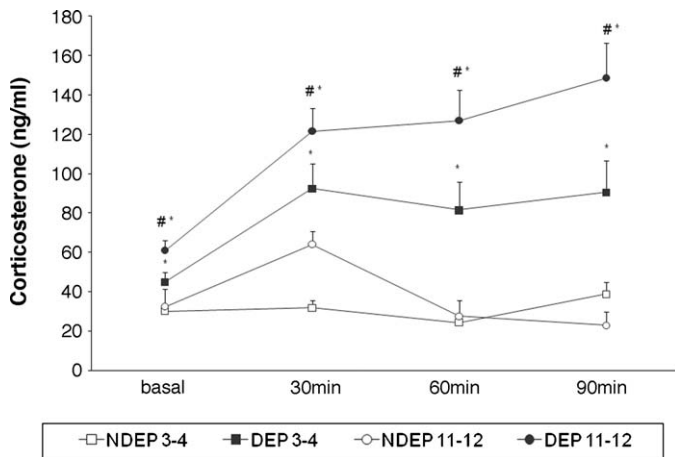
low as NDEP pups at both PND 9 and 12 (Suchecki et al., 1993b). Not only are peripheral hormones regulated by these two types of maternal behaviours, but the reduction in GR mRNA in the hippocampus is also reversed by feeding and stroking together, while the reduction in mineralocorticoid receptor (MR) mRNA in the hippocampus and CRH mRNA in the PVN is reversed by stroking alone (van Oers et al., 1998c). Based on these findings, it appears that the CNS components of the HPA axis are regulated by anogenital stroking, whereas the adrenal gland is regulated by feeding.

Besides the extrinsic regulation, maturational aspects also play a role in adrenal responsiveness because, once the pup is rendered responsive to stress after being subjected to maternal deprivation, the adrenal gland becomes responsive to exogenous ACTH with a similar U-shaped curve to that exhibited by normally reared pups (Rosenfeld et al., 1991). Moreover, it has been shown that 12-day-old NDEP pups respond to a saline injection with increased expression of c-fos and nerve growth factor-induced clone B (NGFI-B), two markers of neuronal activation, in the PVN, cingulate and piriform cortex (Smith et al., 1997). This response, however, is greatly augmented by maternal deprivation, indicating that the brain is responsive to stress, irrespective of the animal's previous experience, whereas the adrenal response to mild stressors is only observed after maternal deprivation (Kuhn et al., 1990; Smith et al., 1997).

Given the immediate impact of maternal deprivation on the activity of the entire HPA axis and the influence of excessive CORT secretion on brain development, we hypothesized that this manipulation could affect the behaviour of adult animals. In order to perform long-term studies, we first needed to replicate the results obtained with the hybrid offspring of Long-Evans and Sprague–Dawley rats because we use Wistar rats in our laboratory. Furthermore, because maternal deprivation performed in different phases of the SHRP (e.g., in the transition to (PNDs 3–4) or in the midst (PNDs 11–12) of this period) leads to distinct outcomes in pubertal rats (van Oers et al., 1997), we submitted 3- and 11-day-old Wistar rat pups to maternal deprivation and assessed their CORT stress response to an injection of hypertonic saline (2%) for up to 90 min after the stressor. As can be seen in Fig. 2, NDEP pups did not respond to the stressor, regardless of age. Maternal deprivation produced an increase in CORT basal levels at both ages and a robust CORT stress response, which was even more robust in PND 12 pups. The persistent elevation of the CORT response in DEP pups confirms a previous finding. More specifically, we had previously shown that, in DEP pups, an exaggerated CORT stress response was not capable of suppressing the ACTH response for at least 90 min (Suchecki et al., 1995, 1993b). Evaluation of CORT negative feedback signalling demonstrates that the reactive mode, i.e., that mediated by the low affinity GR, is not functional in PND 9 and PND 12 DEP pups (van Oers et al., 1998b). This result is explained by the reduction in GR mRNA in the hippocampal CA1 region and in the PVN (van Oers et al., 1998c). The present results assured us that the effects of maternal deprivation on the immediate response to stress were independent of rat strain and that we could pursue our main goal, e.g., to study the long-term effects of maternal deprivation on anxiety-like behaviour, which is discussed later in this paper.

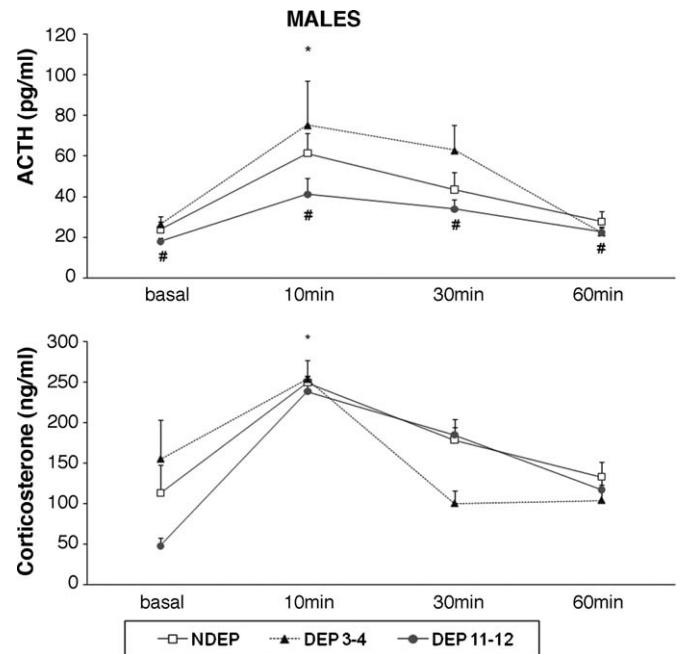
### 2.2.2. Middle- and long-term effects of maternal deprivation

2.2.2.1. *Effects on HPA axis functioning.* Most studies have focused on the immediate effects of manipulation. Few studies have assessed the middle- and long-term effects of maternal deprivation. These studies have focused on the activity of the HPA axis and stress-related behaviours. Approximately one decade ago, two studies were published that explored the middle-term effects of



**Fig. 2.** The aim of this experiment was to assess the effect of maternal deprivation on PND 3 or PND 11–12 on basal and hypertonic saline injection-induced corticosterone release at 30, 60 and 90 min after the stressor (2%; 1 ml/10 g). The breeding procedure was carried out according to Suchecki and Tufik (1997). The procedures after birth are described in Fig. 1 caption. Thirty-six litters were distributed in four different groups: non-deprived, tested on PND 4 (NDEP 3–4; 12 litters); maternally deprived for 24 h on PND 3 (DEP 3–4; 12 litters); non-deprived, tested on PND 12 (NDEP 11–12; 6 litters); and maternally deprived for 24 h on PND 11 (DEP 11–12,  $N = 6$  litters). Because the blood volume obtained from PND 4 pups was not sufficient to perform the radioimmunoassays, two pups of the same sex from the same litter were used to compose one blood sample in the NDEP 3–4 and DEP 3–4 groups. Corticosterone levels were measured in duplicate by a double antibody radioimmunoassay method specific for rats and mice, using a commercial kit (ICN Biomedicals, Costa Mesa, CA, USA) with a modification of the original protocol, as described by Thrivikraman et al. (1997). Three-way ANOVA with group (NDEP and DEP), age (PND 3–4 and PND 11–12) and time (basal, 30, 60 and 90 min) as main factors showed an interaction between the factors group  $\times$  time [ $F(3,154) = 6.8434$ ;  $p < 0.01$ ] and age  $\times$  group [ $F(1,154) = 8.3058$ ;  $p < 0.01$ ]. A Newman–Keuls test showed that basal levels were different among the groups. The hypertonic saline injection increased the CORT levels in both DEP groups, but the response of DEP 11–12 pups was higher than that of DEP 3–4 neonates ( $p < 0.05$ ). Values are expressed as mean  $\pm$  SEM. (\*) Different from respective NDEP; (#) different from DEP 3–4 ( $p < 0.05$ ).

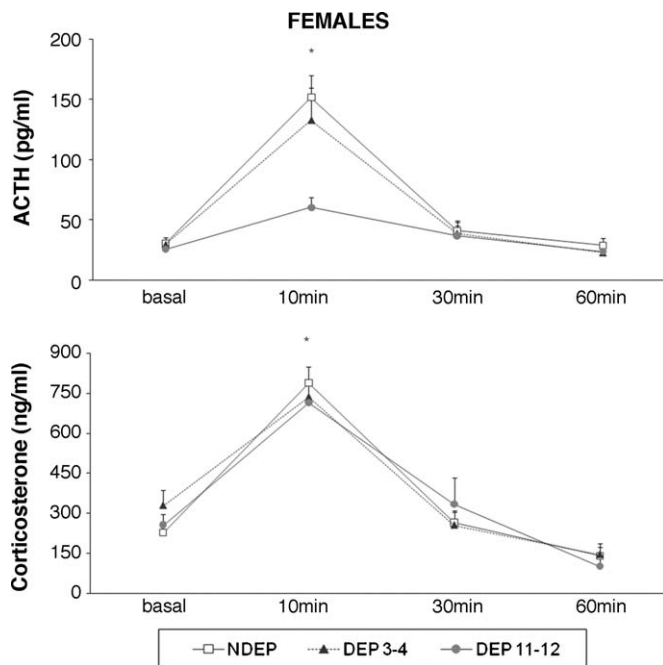
maternal deprivation. The first (Smith et al., 1997) reported that, contrary to the immediate disinhibiting effect of maternal deprivation on the ACTH stress response, PND 20 pups that had been maternally deprived on PND 11 showed lower ACTH secretion than NDEP pups. CORT basal levels were higher, but the return to unstressed values was faster, and c-fos and CRH mRNA expression levels in the PVN were lower than in NDEP pups (Smith et al., 1997). Using the same paradigm at PND 11, the second paper (Suchecki and Tufik, 1997) showed that, contrary to the high CORT stress response seen in 12-day-old DEP pups, the pups were no longer different from their NDEP counterparts at 16 and 22 days of age. Surprisingly, by 30 days of age, they displayed lower basal and smaller CORT responses to a mild stressor than NDEP animals (Suchecki and Tufik, 1997). Thus, both papers showed that maternal deprivation at PND 11 resulted in hyporesponsive juvenile rats. The profiles of HPA axis activation at PND 20, when pups were deprived on PND 11, were the opposite of those for pups deprived on PND 3. Thus, instead of the smaller ACTH response shown by pups deprived at PND 11, those deprived at PND 3 showed greater CRH mRNA expression and ACTH stress response (van Oers et al., 1998a). Maternal deprivation at PND 11 results in reduced GR mRNA expression in the PVN and in all hippocampal fields of PND 20 pups, while feeding and stroking reverse these effects (van Oers et al., 1999). The finding that maternal deprivation at different phases of development could lead to different outcomes was exciting, especially in view of human studies indicating distinct impacts of early trauma depending on the age when it occurred. Thus, before adolescence,



**Fig. 3.** ACTH (pg/ml) and CORT (ng/ml) responses to hypertonic saline (9%; 1 ml/kg) in male adult rats submitted to maternal deprivation on PND 3–4 or PND 11–12. Three groups of animals were tested: NDEP ( $N = 51$ ), DEP 3–4 ( $N = 47$ ), and DEP 11–12 ( $N = 44$ ). The breeding and maternal deprivation procedures were carried out as described in Fig. 1. Blood samples were obtained by decapitation at four different time-points: basal, 10, 30 and 60 min after hypertonic saline injection. ACTH levels were determined as described in Fig. 1, and CORT levels, as described in Fig. 2. Data were analysed by two-way ANOVA with group (NDEP, DEP 3–4, DEP 11–12) and time-point (basal, 10, 30 and 60 min) as main factors. With regard to ACTH secretion, there were main effects of time [ $F(3,130) = 13.7486$ ;  $p < 0.01$ ] and group [ $F(2,130) = 4.4445$ ;  $p < 0.05$ ]. A Newman–Keuls test showed that ACTH levels were lower in DEP 11–12 than in DEP 3–4 rats ( $p < 0.05$ ). The analysis of CORT data showed a main effect of time-point [ $F(3,128) = 41.4195$ ;  $p < 0.01$ ], with peak secretion at 10 min. The group factor was only marginally different [ $F(2,128) = 2.6250$ ;  $p = 0.07$ ]. *Post hoc* analysis revealed that CORT levels in DEP 11–12 tended to be smaller than those of DEP 3–4. Values are presented as mean  $\pm$  SEM. (\*) Different from the other time-points; (#) different from DEP 3–4 ( $p < 0.05$ ).

there is a similar chance of developing depression and PTSD, but after age 13, the risk for PTSD is higher (Maercker et al., 2004). These results, although intriguing, represent the starting point for a series of studies attempting to dissect the long-term neurochemical, neuroendocrine and behavioural impacts of high CORT levels during development.

As discussed above, increased levels of CORT induced by maternal deprivation could impact CNS development, especially in areas associated with emotional behaviour, such as the limbic system. Recently, we finished a study looking at some aspects of neuroendocrine and behavioural long-term effects of maternal deprivation at PND 3 or PND 11 in male and female adult rats. We tested the ACTH and CORT responses to an injection of hypertonic saline (9%) and replicated Van Oers' finding of higher ACTH secretion in males deprived at PND 3 and lower levels in animals deprived on PND 11 (Fig. 3). Our results are also in agreement with those obtained by Rots and co-workers (Rots et al., 1996), who reported increased ACTH and CORT secretion in adult males deprived on PND 3, but no other major differences from NDEP animals. Female rats did not show a remarkable difference in their ACTH response, although DEP 11 adult females did not show a peak in ACTH release in response to the stressor (although the difference was not statistically significant), in contrast to the other groups (Fig. 4). Because cyclic changes in the sexual steroid hormones inherent to the estrous cycle influence the stress response (Viau



**Fig. 4.** ACTH (pg/ml) and CORT (ng/ml) responses to hypertonic saline (9%; 1 ml/kg) in female adult rats submitted to maternal deprivation during infancy. Three groups were tested: NDEP ( $N = 48$ ), DEP 3–4 ( $N = 42$ ) and DEP 11–12 ( $N = 47$ ). The estrous cycle was determined for 14 consecutive days before the day of the experiment. Animals were tested only during diestrous, when the presence of leukocytes confirmed that the animals were in this phase. Experimental procedure, blood sampling, hormone assays and statistical analysis were identical to those described in Figs. 1 and 3. The analysis showed only a main effect of time-point for both ACTH and CORT secretion [ $F(3,125) = 1.21727, p < 0.01$ ; and  $F(3,123) = 59.4142, p < 0.01$ , respectively]. A Newman–Keuls test showed that the peaks in ACTH and CORT release occurred 10 min after the stress and returned to basal levels at 30 min post-stress. Values are presented as mean  $\pm$  SEM. (\*) Different from the other time-points ( $p < 0.01$ ).

and Meaney, 1991), we tested all females in diestrous. Therefore, we cannot rule out that maternal deprivation could alter the stress response of females in phases of the estrous cycle other than diestrous.

The ability to terminate the stress response when the stressful stimulus is no longer present is as important as adequate

activation of the HPA system in threatening situations. Thus, proper activity and sensitivity of the negative feedback system is fundamental for the stress response. The reported changes in ACTH levels in DEP male rats could result from changes in the sensitivity of this system at the brain and/or pituitary levels. To test whether maternal deprivation alters the function of the negative feedback system, we performed a dexamethasone suppression test (DST) using three doses of DEX. The results presented in Table 1 show that maternal deprivation did not affect this process. All groups exhibited complete suppression of CORT secretion from the lowest to the highest dose of DEX, indicating that the negative feedback system functioned well in these animals, despite a report of reduced GR mRNA expression in the PVN and hippocampus (van Oers et al., 1997). These findings indicate that maternal deprivation-induced changes in mRNA expression may not be permanent or, alternatively, that reduced mRNA levels may not necessarily translate into reduced protein levels or a functional response of receptor occupancy, as evidenced in the DST.

**2.2.2.2. Effects on emotional behaviour.** Stressful situations are well-known facilitators of the onset of psychopathologies; therefore, it is not surprising that a substantial portion of the scientific literature describes dysfunction of physiological mechanisms of the stress response to be at the core of some psychopathologies. Other aspects also play a role, such as gender. Numerous studies have shown that women are more likely to have depression and anxiety disorders than men (Kessler et al., 1994; MacMillan et al., 2001; Shea et al., 2005). Perhaps one of the most consistent epidemiological findings in psychiatric research is the two-fold higher chance of developing depression among women as compared to men (Weissman and Klerman, 1977; Wickramaratne et al., 1989). The impact of stressful life events is also greater in women than in men (Sherrill et al., 1997), as women seem to be more susceptible to the negative effects of adverse events and trauma during childhood (Breslau and Anthony, 2007). At the physiological level, studies disagree as to whether females display a greater magnitude of activation of the HPA axis in response to stress. Female rats and mice appear to secrete more ACTH and CORT in response to a variety of stressors (Iwasaki-Sekino et al., 2009; Kawakami et al., 2007; Sloten et al., 2006; Welberg et al., 2006). In human beings, however, the opposite scenario seems to occur: men secrete more free cortisol than women in response to

**Table 1**  
CORT release (ng/ml) in response to the dexamethasone suppression test (DST) in maternally deprived animals.

		Basal	6:00 p.m.	8:00 p.m.	8:00 a.m.	AUC of CORT release (ng/ml)
Control	Vehicle	52.2 $\pm$ 24.8	252.1 $\pm$ 39.1	155.4 $\pm$ 16.8	47.9 $\pm$ 9.2	2844.4 $\pm$ 78.2
	7.5 $\mu$ g/kg	38.9 $\pm$ 16.1	36.6 $\pm$ 7.5*	45.8 $\pm$ 17.2*	25.1 $\pm$ 8.1	809.9 $\pm$ 228.8*
	15 $\mu$ g/kg	73.1 $\pm$ 16.2	12.1 $\pm$ 2.6*	25.3 $\pm$ 9.8*	17.3 $\pm$ 4.9	633.3 $\pm$ 105.2*
	30 $\mu$ g/kg	61.9 $\pm$ 18.9	21.4 $\pm$ 8.7*	17.2 $\pm$ 6.2*	12.9 $\pm$ 3.1	551.9 $\pm$ 124.7*
DEP 3–4	Vehicle	33.5 $\pm$ 08.9	153.5 $\pm$ 38.8	177.5 $\pm$ 41.9	57.9 $\pm$ 11.2	2490.9 $\pm$ 503.5
	7.5 $\mu$ g/kg	31.4 $\pm$ 12.4	12.5 $\pm$ 3.3*	24.0 $\pm$ 5.1*	22.6 $\pm$ 13.2	491.6 $\pm$ 130.4*
	15 $\mu$ g/kg	40.7 $\pm$ 11.6	10.6 $\pm$ 1.7*	27.3 $\pm$ 9.2*	20.7 $\pm$ 6.9	530.8 $\pm$ 118.2*
	30 $\mu$ g/kg	37.2 $\pm$ 13.0	15.8 $\pm$ 6.3*	17.9 $\pm$ 6.5*	19.7 $\pm$ 7.9	470.7 $\pm$ 128.6*
DEP 11–12	Vehicle	46.8 $\pm$ 8.3	162.0 $\pm$ 29.0	220.8 $\pm$ 42.0	38.1 $\pm$ 11.7	2771.5 $\pm$ 346.8
	7.5 $\mu$ g/kg	42.6 $\pm$ 12.3	12.8 $\pm$ 4.0*	14.8 $\pm$ 5.9*	28.2 $\pm$ 8.4	507.8 $\pm$ 80.3*
	15 $\mu$ g/kg	41.3 $\pm$ 16.5	15.2 $\pm$ 5.3*	22.0 $\pm$ 8.3*	28.1 $\pm$ 9.1	563.0 $\pm$ 133.8*
	30 $\mu$ g/kg	25.7 $\pm$ 7.8	19.6 $\pm$ 9.0*	22.5 $\pm$ 9.8*	14.1 $\pm$ 7.5	443.3 $\pm$ 168.2*

The DST was performed in order to assess the efficiency of the negative feedback mechanism in maternally deprived male rats. Whole litters were submitted to maternal deprivation either on PND 3 (DEP 3–4;  $N = 32$ ) or on PND 11 (DEP 11–12;  $N = 32$ ), or were non-deprived (NDEP;  $N = 32$ ). The animals from each of the groups were distributed in four treatments: vehicle, 7.5, 15 and 30  $\mu$ g/kg of DEX ( $N = 8$ /treatment). The first blood sample was taken by a tail incision at 10:00 a.m. just before the subcutaneous administration of dexamethasone (DEX, diluted in alcohol/corn oil). Other 2 samples were collected at 6:00 p.m. and 8:00 p.m. of the same day and the last sample was taken at 8:00 a.m. of the following day. The results were analyzed by a three-way ANOVA for repeated measures. The main factors were: group (NDEP, DEP 3–4 and DEP 11–12), DEX treatment (vehicle, DEX 7.5, DEX 15 and DEX 30) and time (basal, 6:00 a.m., 8:00 p.m. and 8:00 a.m.). Analysis showed a main effect of treatment [ $F(3,84) = 36.729; p < 0.01$ ] and the Newman–Keuls test revealed that the groups treated with DEX, regardless of dose, presented significantly lower CORT levels and area under the curve (AUC) than the group treated vehicle. Values are expressed as mean  $\pm$  SEM.

\* Different from the respective vehicle-treated sub-group.

**Table 2**  
Free exploration paradigm test in maternally deprived rats.

		% Time spent in the unfamiliar compartment	Number of transitions	AUC of CORT release (ng/ml)
Males	NDEP	27.2 ± 5.3	45.2 ± 9.8	4518.5 ± 213.8
	DEP 3–4	34.0 ± 2.4	51.5 ± 5.7	4600.8 ± 469.5
	DEP 11–12	28.9 ± 3.6	42.3 ± 7.6	4752.5 ± 93.6
Females	NDEP	37.6 ± 10.4	61.9 ± 11.6	4426.8 ± 363.5
	DEP 3–4	47.8 ± 9.2	64.5 ± 5.0	4738.3 ± 237.4
	DEP 11–12	46.8 ± 6.6	56.6 ± 6.0	4100.2 ± 4475.0

Two groups of animals were maternally deprived either on PND 3 (DEP 3–4) or on PND 11 (DEP 11–12), and a third group was kept undisturbed (NDEP). The apparatus used in this test consisted in a wooden box (60 cm × 60 cm × 40 cm) divided in two compartments of equal size (60 cm × 60 cm × 20 cm) by a guillotine door. One of the compartments was designated familiar and the other one as unfamiliar. The first was supplied with sawdust, food and water, while the second was empty. Both compartments were subdivided into three sections (60 cm × 20 cm × 20 cm), with passage ways in the walls through which the animals could explore the apparatus. The animals were placed in the familiar compartment and, after 24 h of habituation, the test was performed. The test was carried out during the dark period, beginning at 7:30 p.m., when the guillotine door was open and the animals were allowed to explore the unfamiliar compartment for 15 min. The behaviour was recorded under infrared light, and analysed using a computerized system (Anymaze, Stoelting Co., Wood Dale, IL, USA). The main analysed parameters were the percentage of time spent in the unfamiliar compartment and total number of transitions in both familiar and unfamiliar compartments. Female rats were tested during diestrous, which was confirmed by the presence of leukocytes in the vaginal smear. Blood samples were collected from the tail in baseline conditions (around 7:30 p.m.) and 20 min after the test. After being handled for 5 days, the animal was held with a clean piece of cloth and a small incision was made at the end of the tail with sharp scissors. Baseline samples were taken on the day preceding the behavioural test in males and in the previous diestrous phase in the females. One-way ANOVA did not reveal differences in any of the behavioural parameters analysed in males or females. The area under the curve (AUC) of CORT release was not influenced by maternal deprivation. Values are expressed as mean ± SEM. Number of animals is given on the caption of Fig. 5.

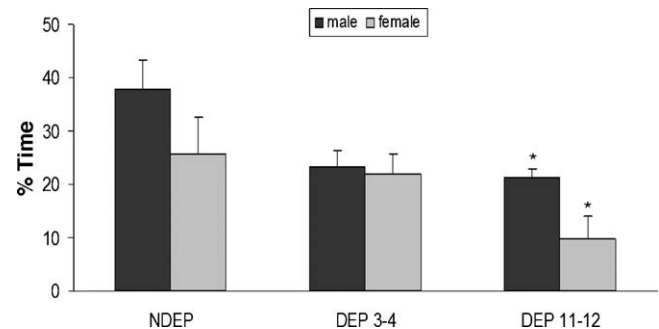
psychological stressors, whereas women secrete more total cortisol, but have a higher level of corticosterone binding globulin (CBG) (Kudielka et al., 2004; Kumsta et al., 2007; Uhart et al., 2006). This finding suggests that higher CBG levels may play a protective role, inasmuch as a greater proportion of cortisol remains bound to the protein, and, therefore, unavailable to act at target organs and that the effects of free cortisol may be similar between men and women.

We used maternal deprivation as a form of early adversity to explore the impact of increased endogenous activity of the HPA axis on the behavioural outcomes associated with emotional behaviours. We used the free exploration paradigm (Hughes, 1968), which proposes to assess “trait” anxiety-like behaviour, which does not change after repeated exposures (Calatayud et al., 2004; Griebel et al., 1993) (Table 2). In a second experiment, the same animals whose “trait” anxiety was tested in the free exploration paradigm were tested in the light/dark box test, a model of “state” anxiety-type behaviour (Fig. 5), which is sensitive to drugs used to treat generalized anxiety such as benzodiazepines (Chaouloff et al., 1997). Because the free exploration paradigm and light/dark box test assess different features of anxiety-like behaviour, the use of both tests provides a better characterisation of the consequences of maternal deprivation on emotionality. In addition, the measurement of CORT basal levels and stress response to these tests adds more information to the general emotional status of these animals.

Our results show that maternal deprivation on PND 11 resulted in increased “state,” but not “trait,” anxiety-like behaviour in adult male or female rats. The distinct behavioural profile exhibited by the PND 11 group was not accompanied by an adrenocortical differential response in that all rats exhibited a similar CORT response to the test. Ideally, we should have also assessed the ACTH response, which better reflects changes in CRH/AVP activity, but to do so would have required an enormous amount of animals because the volume of blood necessary would have required that animals be decapitated. Instead, we chose to obtain basal and test-induced CORT levels measured in the same animal, for which a small blood volume can be drawn from the tail vein (Table 3).

In a previous study in which we tested emotional behaviour in adolescent rats submitted to maternal deprivation on PND 11, we observed reduced anxiety-like behaviour in the open field in male and female DEP rats, and in response to the apparatus, DEP males secreted just as much ACTH and CORT as NDEP animals. On the

other hand, female DEP rats displayed higher ACTH levels and a lower CORT response at the 5-min time point compared to their NDEP counterparts, in addition to a faster return of ACTH to basal levels measured at 20 min (Suchecki et al., 2000). These results stand in contrast to the long-term outcomes of maternal deprivation measured in the light/dark box, but possible explanations for these discrepancies include the age of testing (adolescent × adults), the tests employed (Open Field × Light/Dark Box test), and the type of challenge used to stimulate the HPA axis (hypertonic [physiological] × open-field [psychological]).



**Fig. 5.** Percentage of time spent in the light compartment of the light–dark box test of adult rats submitted to maternal deprivation during infancy. Experiments were performed with both males (NDEP:  $N = 12$ ; DEP 3–4:  $N = 11$ ; DEP 11–12:  $N = 12$ ) and females (NDEP:  $N = 9$ ; DEP 3–4:  $N = 12$ ; DEP 11–12:  $N = 9$ ). Females were tested during diestrous, which was confirmed by the presence of leukocytes in the vaginal smear. “Trait” anxiety-like behaviour was initially assessed in the free exploration paradigm, after which the same animals were tested in the light/dark box test to evaluate of “state” anxiety-like behaviour. The apparatus consisted of a Perspex box (50 cm × 20 cm × 20 cm) divided by a small connecting door into a light and a dark chamber of the same size. The dark chamber was entirely black and enclosed with a solid black plastic top. Rats were individually placed into the lit chamber at the beginning of the test and allowed to explore the whole apparatus for 5 min. Time spent in each chamber was recorded. Subsequently, the percentage of time spent in the lit chamber was calculated. The evaluation was performed using a computerized system for animal behaviour tracking (Anymaze, Stoelting Co., Wood Dale, IL, USA). Behavioural data were analysed by one-way ANOVA for each gender. For male rats, there was a main effect of group [ $F(2,31) = 3.60953$ ;  $p < 0.05$ ], and a Newman–Keuls test revealed that the DEP 11–12 group displayed increased anxiety-like behaviour compared to the NDEP group ( $p < 0.05$ ). The impact of maternal deprivation in females was very similar to that in males. One-way ANOVA showed that the groups were different [ $F(2,27) = 4.25434$ ;  $p < 0.05$ ], and the Newman–Keuls test revealed that the DEP 11–12 group spent less time in the lit compartment compared to the NDEP group. Values are expressed as mean ± SEM. (\*) Different from respective NDEP animals ( $p < 0.05$ ).

**Table 3**

Corticosterone release (ng/ml) before (basal levels) and after the light/dark box test. Values are expressed as mean  $\pm$  SEM of 8–11 animals/group/gender.

		Basal levels	Response to light/dark box test
Males	NDEP (N=10)	38.9 $\pm$ 1.5	239.0 $\pm$ 30.9*
	DEP 3–4 (N=10)	24.8 $\pm$ 4.6	240.7 $\pm$ 26.2*
	DEP 11–12 (N=11)	46.3 $\pm$ 11.2	249.4 $\pm$ 22.4*
Females	NDEP (N=9)	51.9 $\pm$ 12.2	406.0 $\pm$ 119.0*
	DEP 3–4 (N=11)	56.9 $\pm$ 15.4	475.5 $\pm$ 52.1*
	DEP 11–12 (N=8)	45.5 $\pm$ 10.7	523.1 $\pm$ 108.6*

After being handled for 5 days, the animal was held on a clean piece of cloth and a small incision was made at the end of its tail with sharp scissors. Application of light pressure along the tail permitted sampling of approximately 50  $\mu$ l of blood that was stored in a tube with EDTA (6% solution). This procedure was carried out before the behavioural test in males and on the previous diestrous in females, at 9:00 a.m. to 11:00 a.m. Animals were decapitated 20 min after exposure to the light/dark box test. Results of males and females were analysed separately by a two-way ANOVA for repeated measures (Group, time-point). For both males [ $F(1,31)=171.99$ ;  $p < 0.01$ ] and females [ $F(1,27)=72.02$ ;  $p < 0.01$ ] there was main effect of time-point, inasmuch as exposure to the light–dark box elicited increased CORT secretion compared to basal values ( $p < 0.05$ ).

\* Different from respective basal levels.

### 3. Discussion and perspectives for future research

Our work in the field of early life stress suggests that early adversity increases emotionality and changes emotional-related behaviours in adult animals. Furthermore, the HPA axis, in some cases, appears to play only a marginal role in the effects of early maternal separation (Catallani et al., 2008; Gujjarro et al., 2007; Kawakami et al., 2007; Tiba et al., 2004, 2008). In all studies conducted by our group, CORT and ACTH levels did not correlate with the behavioural alterations in adulthood, which suggests involvement of other systems that mediate emotional responses. Based on these findings, it is tempting to speculate that the activity of the pituitary–adrenocortical axis is preserved in adulthood, which would make sense in view of the essential importance of proper functioning of this system for survival. Thus, early life stress may lead to long-term, permanent alterations in the response of animals to subsequent stressors, perhaps by altering coping behaviour via mechanisms that include the CRH system in limbic regions involved in the regulation of emotionality. The activity of the hypothalamic component of the axis is definitely important, as it has been shown that mice over-expressing CRH are more anxious in the light/dark box test (van Gaalen et al., 2002). Moreover, numerous studies have reported a hyperactive CRH system in animal models of anxiety-like behaviour (Chung et al., 2005; Coplan et al., 1996; Maciag et al., 2002). Interestingly, a recent study demonstrated that, in a conditional knockout mouse line in which the CRH type 1 receptor is inactivated in the forebrain and limbic structures, but not in the pituitary, anxiety-like behaviour is reduced in the light/dark box test and elevated plus maze tests; this effect was independent of the activity of the HPA system (Muller et al., 2003). Therefore, CRH at hypothalamic/limbic and perhaps brainstem sites may contribute to the mechanisms of neuroplasticity induced by early life stressful stimuli, altering the neuroendocrine and behavioural/affective stress responses permanently (Brunson et al., 2001). We are currently investigating possible changes in CRH immunoreactivity in the amygdala of adult rats submitted to maternal deprivation on PNDs 3 and 11. Moreover, preliminary data indicate that administration of CP154,536, a CRH type 1 receptor antagonist, in LMS mice prevents the expression of alcohol-induced behavioural sensitization (unpublished data).

Other neurotransmitter systems are also involved in anxiety, with the most classical ones being noradrenaline, serotonin and gamma-aminobutyric acid (GABA). In a recent study (unpublished

data), we observed that male and female PND 11 maternally deprived rats displayed low levels of GABA in the hippocampus, a possible mediating locus of anxiety-type behaviour, corroborating our behavioural results. The relationship between HPA axis dysfunction and psychopathologies has been highlighted in the literature, but some papers have reported a subset of psychiatric patients who do not show alterations in the HPA axis (Bloch et al., 2007; Simeon et al., 2007; Thompson et al., 2007). Identification of endophenotypes to help diagnose psychopathologies, thus lowering reliance on personal interpretation and bias, is important, but it is fundamental to bear in mind that physiological parameters other than the HPA axis might mediate behavioural alterations.

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