Aos meus pais

Para o Zé Miguel e para o Bruno

Só o que sonhamos é o que
verdadeiramente somos,
porque o mais, por estar realizado,
pertence ao mundo e a toda a gente.

Bernardo Soares
In "O Livro do Desassossego"

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Abstract

Manipulation of corticosteroid milieu during perinatal period has received much attention due to its implications in pathology later in life. The experience of stressful/traumatic events in early childhood, which triggers the activation of the HPA axis and the permanent increase in corticosteroids levels during critical periods of development, has shown to be associated with cardiovascular, metabolic, immune and psychiatric conditions. Interesting links have indeed, been established between psychopathology and immune dysfunction, although the exact mechanisms by which neuroendocrine and immune system interact, and influence each other, still need further investigation. For that reason, in this collection of studies we investigated the impact in nervous, endocrine and immune systems, of an early stressful event (maternal separation -MS) in two different periods of development: an early period from PND2-15 ($MS_{2\text{-}15}$) and a later period from PND7-20 ($MS_{7\text{-}20}$). Moreover, in order to clarify the cross-talk between those systems an integrated characterization of these animals was performed. In the first study, we assessed if neurological reflexes and somatic milestones were affected by MS, and established possible neurochemical correlates in specific brainstem areas. In order to assess the long-lasting consequences of MS, in a second study we thoroughly characterize adult rats in a battery of behavioural tests. In addition, we assessed the expression of CRF and synapsin-I in several brain regions and cytokines in the brain and in the spleen. Finally, to further analyse the role of the immune system in emotional disorders, we behaviourally characterize transgenic mice models for IL-10. Results show that MS impaired the acquisition of several neurological reflexes, in parallel with increased serotonin turnover, in the vestibular and dorsal raphe nuclei. Analysis of MS animals, in adulthood, revealed a distinct behavioural phenotype; while anxious behaviour and spatial learning abilities were equally affected in $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ (a fact correlated with a persistent hypercoticalism), impaired exploratory behaviour and depressive-like signs were only found in $MS_{2\text{-}15}$ rats. The latter observations in $MS_{2\text{-}15}$ seem to be associated with an increased expression of CRF in the amygdala as well as with an higher expression of pro-inflammatory cytokines in the amygdala (IL1-β) and in the PFC (TNF-α). The effects of $MS_{2\text{-}15}$ were not confined to the CNS; in fact, we also observed a reduced number of T and NK cells in the spleen. Finally, we showed that the differential expression of IL-10 (an anti-inflammatory cytokine) also influenced depressive-like behaviour. Taken together, the present studies demonstrate a time-dependent *imprinting* effect of early life stress/corticosteroids, implicate the imbalance of cytokine

expression on behavioural phenotype, which further support the influence of the immune system on emotional behaviour.

Resumo

A manipulação dos corticosteróides durante o período perinatal tem sido alvo de grande investigação devido às suas implicações em diversas patologias na vida adulta. A experiência de eventos traumáticos, o que desencadeia a activação do eixo HPA e o aumento crónico dos níveis de corticosteróides durante períodos críticos de desenvolvimento, tem demonstrado estar associado a doenças cardiovasculares, metabólicas, imunológicas e psiquiátricas. Foram já estabelecidas ligações entre a psicopatologia e a disfunção imune, embora os mecanismos exacto pelo qual o sistema imunológico e neuroendócrino se influenciam mutuamente necessitam ainda de ser investigados. Por esse motivo, neste conjunto de estudos, investigamos o impacto, nos sistemas nervoso, endócrino e imunológico, de uma experiência traumática precoce (separação maternal-MS), em dois períodos diferentes de desenvolvimento: um período precoce entre o dia 2 e o dia 15 após o nascimento (MS_{2-15}) e um período tardio entre o dia 7 e o dia 20 após o nascimento (MS_{7-20}). Além disso, na tentativa de esclarecer a relação entre estes sistemas foi feita uma caracterização comportamental integrada nestes animais. No primeiro estudo, avaliámos o impacto da separação maternal na aquisição de reflexos neurológicos e em parâmetros somáticos específicos e estabelecemos possíveis correlações neuroquímicas em áreas específicas do tronco cerebral. Num segundo estudo, de forma a avaliar as consequências a longo prazo, caracterizámos animais adultos numa bateria de testes comportamentais. Adicionalmente, foi também avaliada a expressão do CRF e da sinapsina-I em várias regiões cerebrais e de citocinas também no cérebro bem como no baço. Por último, avaliámos também o papel do sistema imunitário em perturbações emocionais através da caracterização comportamental de ratinhos transgénicos para a IL-10. Os resultados mostram que a separação maternal prejudica a aquisição de vários reflexos neurológicos e leva, paralelamente, ao aumento da degradação da serotonina nas áreas vestibular e do núcleo dorsal de rafe. A análise, em adulto, de animais submetidos a MS revelou um fenótipo de comportamento distinto; enquanto o comportamento ansioso e a memória espacial foram igualmente afectados nos grupos MS_{2-15} e MS_{7-20} (um facto correlacionado com uma persistente hipercotisolemia), alterações de comportamento exploratório e sinais depressivos apenas foram evidentes em animais separados no período mais precoce (MS_{2-15}). Estas últimas observações nos animais MS_{2-15} parecem estar associadas com uma expressão aumentada do CRF na amígdala, bem como com o da expressão de citocinas pró-inflamatórias na amígdala (IL1-β) e também no PFC (TNF-α). Os efeitos da separação maternal precoce não estão confinados ao SNC; de facto, foi também

observada uma redução no número de células T e NK. Por último, demonstramos também que a expressão diferencial de IL-10 (uma citocina anti-inflamatória) influencia o comportamento do tipo depressivo. Em resumo, os presentes estudos demonstraram que os efeitos do stress perinatal/corticosteróides são dependentes do período de exposição e implicam alterações dos níveis de citocinas no estabelecimento do fenótipo comportamental, evidenciando a influência do sistema imunológico no comportamento emocional.

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Abbreviations list

ACHT – adrenocorticotrophic hormone

AVP – arginine-vasopressin

CNS – central nervous system

CONT – control animals

CORT - corticosterone

CRF – corticotrophin-releasing factor

CS -corticosteroids

CUS- chronic unpredictable stress

EMP – elevated-plus maze

FS – forced swim

GR – glucocorticoid receptor

HPA – hypothalamus-pituitary-adrenal

IL-10 – interleucine-10

INF- γ –interferon -γ

MR – mineralocorticoid receptor

MS – maternal separation

MWM – Morris water maze

NK –natural killer

OF – open field

PFC –prefrontal cortex

PVN –paraventricular nucleus of the hypothalamus

SEM-standard error of the mean

TNF -α – Tumor necrosis factor α

Chapter 1

INTRODUCTION

1.1 The impact of early life stress

The attention devoted by researchers to the neurobiology of stress has grown since the pioneer works of Hans Selye in the 30s and further encouraged when receptors for adrenal steroids were described in specific areas of the brain (McEwen *et al.*, 1968; Gerlach & McEwen, 1972). However, despite a remarkable expansion of the field (Fig.1), the precise impact of stress as a trigger for pathology in the central nervous system (CNS) is still under investigation.

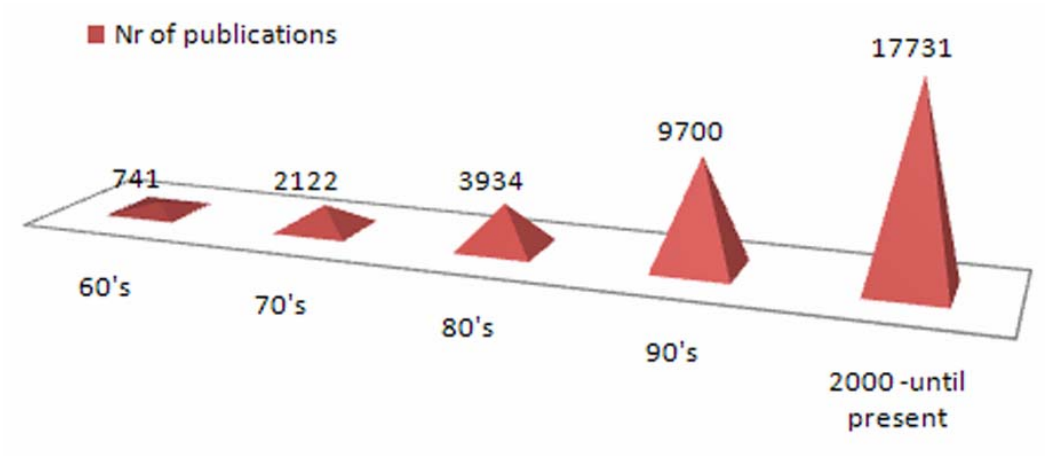

Fig. 1 – Number of publications in each decade, from 1960 until 2008, retrieved by PubMed using the keywords "stress and brain".

A central component of the stress response is the activation of the neuroendocrine system, in particular the hypothalamic-pituitary-adrenal (HPA) axis which, ultimately, triggers the production of corticosteroids (CS) in the adrenal glands. This response promotes adaptation to stressors and, thus, is crucial for survival, by facilitating the mobilization of substrates for energy availability and by the release of chemical mediators (such as catecholamines) that are involved in the autonomic response to stress (e.g. the elevation of blood pressure and heart rate). Interestingly, the components of the stress response operate in two distinct modes (De Kloet *et al.*, 1998). In a *proactive* mode, the CS are responsible for maintaining the basal activity of the HPA axis, promoting coordination of the circadian cycle. In contrast, in the *reactive* mode, stress mediators provide the organism the ability to cope with threatening events as well as to terminate the stress response through highly regulated feedback mechanisms. In fact, to preserve homeostasis, the stress response should be promptly activated when it is needed and efficiently terminated shortly afterwards. Failure to activate this system vulnerabilizes the organism, while excessive or prolonged responses, once initiated, increase the susceptibility to disease.

The control (or its lack) of the stress response will largely determine the impact of stress in the organism. In other words, the stress response can shift from adaptive to maladaptive. Importantly, several intrinsic (genetic) or extrinsic (stochastic) factors determine the individual's response to stressors. Several laboratories have focused their research efforts unravelling the mechanism involved in the control/programming of the stress response (Clark, 1998; Fish *et al.*, 2004; Seckl, 2004; Lesage *et al.*, 2006; Vieau *et al.*, 2007; Darnaudery & Maccari, 2008), and nowadays, it is widely accepted that the HPA axis is highly susceptible to programming during fetal and neonatal periods.

Epidemiological studies clearly evidenced the link between early life events, stress and HPA axis activity on several processes in adulthood, such as the development of hypertension and other cardiovascular disease (Igosheva *et al.*, 2004; Louey & Thornburg, 2005), metabolic disorders (such as diabetes (Lesage *et al.*, 2004)), and also psychopathologies (Heim *et al.*, 1997; Graham *et al.*, 1999; Heit *et al.*, 1999); in common, these conditions are associated to a dysfunction of the HPA axis that, ultimately, leads to increased levels of CS.

The HPA axis, also known as the LHPA axis due to the critical influence of the limbic system in its activity, is a system comprising several sequential *loci* of regulation of the stress response (Sapolsky *et al.*, 1986). In healthy states, they are tightly inter-regulated by precise feedback mechanisms. Briefly, systemic and/or central stimuli, triggered both by physiological or psychological stressors, activate the medial parvocellular region of the paraventricular nucleus (mpPVN) of the hypothalamus that produces CRF (corticotrophin-releasing factor; also designated CRH – corticotrophin-releasing hormone) and arginine-vasopressin (AVP). Although CRF is the primary secretagogue, AVP also act synergistically to stimulate adrenocorticotropic hormone (ACTH) release from the anterior pituitary (AP), particularly in chronic stress conditions (Sawchenko *et al.*, 1993; Herman & Cullinan, 1997; Pinnock & Herbert, 2001). These molecules, released from the median eminence nerve terminals into the hypophysial portal circulation, reach specific receptors on the corticotropic cells of the AP. This leads to the synthesis of the precursor protein proopiomelanocortin (POMC) that is subsequently processed into ACTH. Once released into systemic circulation, ACTH will bind to its receptors located in the adrenal cortex, leading to CS synthesis and release.

When secreted from the cortex of adrenal glands, CS (cortisol in humans; corticosterone in rodents) are able to target several organs. Due to its lipophilic nature, they easily cross the blood-brain-barrier acting within the central nervous system (De Kloet, 2004). There are two types of CS receptors differentially distributed in the brain (Reul & de Kloet, 1985): type I, or mineralocorticoids (MR), are preferentially located in limbic regions such as the septum and the hippocampus (Ahima *et al.*, 1991; Kawata *et al.*, 1998), while type II, or glucocorticoids receptors (GR), are ubiquitously expressed in neurons and glia cells of the brain, including the hippocampal formation, hypothalamus, pituitary, amygdala, bed nucleus of the stria terminalis, nucleus accumbens and cerebral cortex (Fuxe *et al.*, 1985; Ahima & Harlan, 1990; Cintra *et al.*, 1994; Kawata *et al.*, 1998). From this topographical pattern it becomes clear that both the limbic system and the hypothalamus are preferential targets of CS (Sousa & Almeida, 2002; Crochemore *et al.*, 2005; Cerqueira *et al.*, 2007). Noticeably, besides their role in the control of the HPA axis these regions of the brain are also implicated in memory, emotion and learning processes (McEwen & Sapolsky, 1995; Sousa *et al.*, 2000; Cerqueira *et al.*, 2005). It is also important to note that the central actions of the CS are dependent on the level, duration, and timing as well as on the ratio of MR/GR occupation. Indeed, the affinity of both cortisol and corticosterone (Cort) is approximately 10-fold higher for MR, which are known as the high affinity/low capacity corticosteroid receptor system (with a dissociation constant of K_d = 0.5nM) than the for GR (K_d = 5.0nM) (Reul & de Kloet, 1985; De Kloet *et al.*, 1998). Thus, on basal conditions there is a predominant occupancy of MR, while GR are mainly activated when CS levels are high (i.e. at the circadian peak and during periods of stress) (Reul & de Kloet, 1985; De Kloet, 2004). Of notice, GR play an important role in the feedback mechanisms at every HPA axis control *loci*.

It is known that repeated exposure to elevated glucocorticoids levels produces several deleterious effects in the organism (Cerqueira *et al.*, 2005; Dinan, 2005; Swaab *et al.*, 2005); thus, the feedback inhibition system is of the utmost importance to minimize the catabolic, antireproductive and immunosuppressive effects of hypercortisolism. Three different feedback mechanisms have been described in accordance with the time required to inhibit the stress response: fast, intermediate and delayed. The rapid feedback which occurs within minutes does not necessarily require protein synthesis and it is not compatible with the nuclear action of corticosteroids. Indeed, the action of CRF on ACTH release may be inhibited by a rapid effect of

corticosteroids at the cell membrane (Sakakura *et al.*, 1976; Widmaier & Dallman, 1984; Young & Vazquez, 1996). The intermediate feedback mechanism also decreases ACTH release in response to stimulation of the corticotrophic cells, but does not affect ACTH synthesis; however, both CRF synthesis and release appear to be affected by the intermediate corticosteroid action. Intermediate feedback, like fast feedback, apparently does not involve inhibition of total ACTH stores. Delayed feedback, on the other hand, can take several hours to establish and involves the classical genomic steroid mechanism of action; it reduces pituitary ACTH content by decreasing POMC mRNA levels, therefore, inhibiting basal as well as stimulus-induced ACTH secretion. Acting at the transcriptional levels, it also implies changes in the expression of other key molecules on LHPA structures, such as hypothalamic CRF, AVP and hippocampal GRs (Roberts *et al.*, 1979; Akerblom *et al.*, 1988; Cairns *et al.*, 1993).

We have so far described the function and feedback control mechanisms of the mature HPA axis. However, before reaching such highly regulated pattern, this system undergoes several changes, particularly during early post-natal development. In fact, the rodent immature HPA axis is considerably different from that of the adult. There are major differences in structure and function of the brain circuitries involved in its control, as well as at the adrenal level. Until complete development, this system undergoes several maturation and activation processes that represent critical steps to settle the pattern of the adult behaviour stress response. In the first 21 days of life (between birth and weaning), basal CS levels oscillate markedly. At birth, as a consequence of parturition, elevated levels CS are produced by the mother and transferred to the offspring through the placenta; due to the hepatic immaturity of the newborn these high levels are maintained, in circulation, until around PND4 when a decrease is observed. From this time point until the end of the 2nd week the levels of CS remain low. The first studies performed to assess neonatal HPA axis activity identified this stage as a nonresponsive period (Jailer, 1950; Schapiro, 1962). Probably due to the reduced methodological sensitivity, these works were only able to detect low levels of ACTH and no CS. However, in the 80s, further investigation lead to a reappraisal of this concept, re-naming this "silent period" as a stress-hyporesponsive period (SHRP) (Schoenfeld *et al.*, 1980; De Kloet *et al.*, 1988). Curiously, during this time window the HPA axis seems to be hyporesponsive at all central *loci* of control. The factors underlying this quiescence of the HPA axis converge on a blunted pituitary ACTH release, as a consequence of: 1) CRF neurons immaturity (main neuronal input of the pituitary); 2) decreased pituitary peptide

content and 3) decrease sensitivity to CRF stimulus. A decrease in the adrenal gland sensitivity to ACTH has also been postulated.

To better understand the specificities of the stress response development it is crucial to consider the ontogeny of the receptors that are involved in the control of the HPA axis activity, as well as the developmental profile of CRF expression. Given that CRF is not confined to the hypothalamus but is also present in other regions of the brain, in particular the hippocampus, the amygdala and the cortex, where it acts as a neurotransmitter, it has also been implicated in the modulatory feedback of the limbic system to the mpPVN nuclei, where CRF is produced (Herman & Cullinan, 1997). CRF mRNA levels are already detectable by gestational day 17 (E17) in the parvocellular region of the PVN (Grino *et al.*, 1989; Baram & Lerner, 1991) as well as in the median eminence (Bugnon *et al.*, 1982). This mRNA expression, as well as the protein, becomes robust during E18 and E19 decreasing afterwards and reaching adult levels around PND4 (Grino *et al.*, 1989; Baram & Lerner, 1991). In the developing hippocampus, the CRF mRNA expression is low comparable to those found in the hypothalamus (Vazquez *et al.*, 2006). Nevertheless, different studies have shown CRF-immunoreactivity in hippocampal neurons already at PND1 (Yan *et al.*, 1998; Chen *et al.*, 2001), increasing the number of positive neurons throughout development and peaking at PND18 (Chen *et al.*, 2001). Regarding the amygdala low levels of CRF are expressed until PND6, increasing by PND12 and reaching the highest level at PND18 (Vazquez *et al.*, 2006). Although, no consistent data has described the expression of CRF in the PFC, data from Vazquez and co-workers found in the cortex a similar ontogenic pattern to the amygdala (Vazquez *et al.*, 2006).

Moreover, the emergence of CRF receptors in these regions also seems to unveil the functional role of this neuropeptide. In fact, CRF act via two different receptors that show spatial specificity in their distribution in the adult, which is already observed during development. In the developing hypothalamus, $CRF1_R$ show a transient expression in the PVN around the PND2 that is no longer evident throughout development until adult age. In the ventromedial hypothalamus (VMH) $CRF2_R$ are expressed already at E16, even before detectable levels of CRF in the PVN (Grino *et al.*, 1989; Baram & Lerner, 1991); interestingly the parvocellular neurons are born around E16 and express CRF 24h later.

In the pyramidal cell layers of the hippocampus, $CRF1_R$ is present from PND4 and peaks on PND6 (about 300-600% of the adult levels); from this time point to PND12 it declines until it

reaches adult levels (Avishai-Eliner *et al.*, 1996). However, in the dentate gyrus, the pattern is somehow different, since these receptors also emerge on PND4, but do not show any increase until PND12 when they start to increase to reach the level of expression observed in adult animals (Avishai-Eliner *et al.*, 1996). Regarding $CRF2_R$ the pattern of expression is similar in all the hippocampal regions and remains stable from PND1 (when they are first observed) until adulthood (Eghbal-Ahmadi *et al.*, 1998).

In the PFC, the higher levels of $CRF1_R$ expression were found at PND1 decreasing to adult levels until PND12 (Avishai-Eliner *et al.*, 1996). The emergence of $CRF2_R$ occurs earlier, showing a significant expression already at E17 decreasing afterwards to undetectable levels by the 3rd week after birth (Eghbal-Ahmadi *et al.*, 1998).

These observations show a critical regulation of spatial and temporal expression of these receptors implicating them as important targets during early life. It is important to notice the earlier emergence of $CRF2_R$ in hypothalamic regions involved in the autonomic and neuroendocrine functions that are critical throughout life. On the other hand, their expression in higher brain centers is transient (in the PFC) or modest (in the hippocampus). Conversely, $CRF1_R$, appear later during development with higher incidence in the limbic regions known to be involved in the feedback control of the HPA axis. In fact, these different timing in the emergence of CRF receptors suggest specific time windows of sensitivity to stressors.

Concerning pituitary postnatal development, this component of the HPA axis also undergoes marked morphological and functional changes during fetal and early post-natal development. During the first three weeks of the rodent life, the ACTH, content shows a 4-fold increase and, as expected, a similar profile is observed for POMC mRNA levels (Vazquez, 1998). Interestingly, only at late gestation and early postnatal period corticotroph cells in the AP are able to convert POMC in mature ACTH molecules that have the capacity to stimulate CS release. During this period, AP cells already express the type 1 receptor of CRF (implicated in the stress-related behaviour) as well as AVP receptors; however, they are not completely functional until approximately PND10 (Gunnar & Vasquez, 2006) Thus, consistent with the concept of a SHRP, only around the end of the 2nd week after birth, the pituitary exhibits a mature ACTH response to both CRF and AVP (Swanson, 1992; Vazquez, 1998).

There are also important ontogenic changes in the distribution of the CS receptors, as well as differences in their binding capacity, during early post-natal life (Rosenfeld *et al.*, 1993). Regarding hypothalamus, GR start to emerge after birth increasing their expression, specifically in the mpPVN, throughout development reaching adult levels by the end of the 2nd week of life (Rosenfeld *et al.*, 1988). Briefly, during the 1st week of life, GR are highly expressed in the suprachiasmatic nucleus, while after this time-point GR expression becomes more restricted to the PVN (Rosenfeld *et al.*, 1988). Besides these changes in the distribution, there are also differences in their binding capacity. Despite an initial period of high binding capacity (Rosenfeld *et al.*, 1993), it is known that during the first 2 weeks of life GR also show impaired capacity to undergo transformation and/or nuclear translocation, which compromise their action (Rosenfeld *et al.*, 1993). Interesting, is the fact that in parallel to the GR distribution/binding maturation there are important developments in the control of the HPA axis. Indeed, the feedback mechanism system is still lacking in young animals which reveals the immaturity of the hippocampus-mpPVN connection in early life (Vazquez & Akil, 1993).

In the pituitary, a reduced expression of both GR and MR associated with low functionality of the receptors is found during the first week after birth. The levels of MR increase slightly during the second week returning to the previous levels by the third week (Rosenfeld *et al.*, 1990).

In the hippocampus, very low levels of GR are detectable around PND3 (approximately 25% of adult levels) and only at the end of the 3rd week animals reach the adult levels; this pattern correlates with the age animals begin to show an adult-like negative feedback mechanism (De Kloet *et al.*, 1988). The affinity of these receptors for CS also seems to be greater during the perinatal period than later in life (Pryce, 2008), which can also be a compensatory mechanism for this different temporal expression.

Regarding the MR receptors, they are found at the highest concentration in the hippocampal region of the brain. These receptors start to emerge early after birth (around PND1) in the pyramidal cell layer of the Ammon's horn and in the granular neurons of the dentate gyrus (Lawson *et al.*, 1991), but remain at very low levels during the first few days after birth. Their binding capacity rises rapidly thereafter and by the end of the first week, reaches adult levels.

In the PFC, both GR and MR seem to display a stable moderate expression throughout ontogeny (van Eekelen *et al.*, 1991).

As it as been reported for adult animals this spatial and temporal distribution of the corticosteroids receptors likely suggest different roles for MR and GR. Given that GR are mainly

implicated in the stress response, it is of notice their later emergence in areas known to be involved in the negative regulation of the HPA axis. Taken together, these evidence corroborate that the negative feedback mechanisms are not fully functional in the neonate.

Finally, it is of relevance to provide a brief overview of major maturation steps of the peripheric component of the axis. Adrenal glands are also undergoing maturation during this period. In consequence of adrenal immaturity, there is an insensitivity of the adrenals to ACTH stimulation during the 1st week of life. Throughout this period the *zona glomerulosa*, the region in the adrenal cortex where glucocorticoids synthesis occurs, is still undeveloped. Additionally, in vitro studies showed that specific cells of the adrenal medulla, designated as chromaffin cells that are also essential for GCs secretion are still lacking at this time. In fact, although the adrenal cortex and the adrenal medulla are frequently considered two independent functional units of the same organ, several studies have already shown the bidirectional influence of both structures that lead to their optimal function (Carballeira & Fishman, 1980). In what concerns the steroidogenesis occurring in the cortex, it has been proven the ability of the cromaffin cells (medulla-specific celo modulate this process through the expression of specific neurotransmitters (Hinson *et al.*, 1994). In rodents the adrenal medulla is not a well developed region until the end of the 1st post-natal week which is coincident with the period in which zona *glomerulosa* (responsible for GCs secretion) and *zona fasciculata* (responsible for MCs secretion) start to develop quickly. All these facts suggest that corticosteroids synthesis is compromised throughout the first 2 weeks of life, a fact coincident with the SHRP. The ontogenic pattern of the HPA axis, and the main receptors involved in its control is depicted in Fig.2.

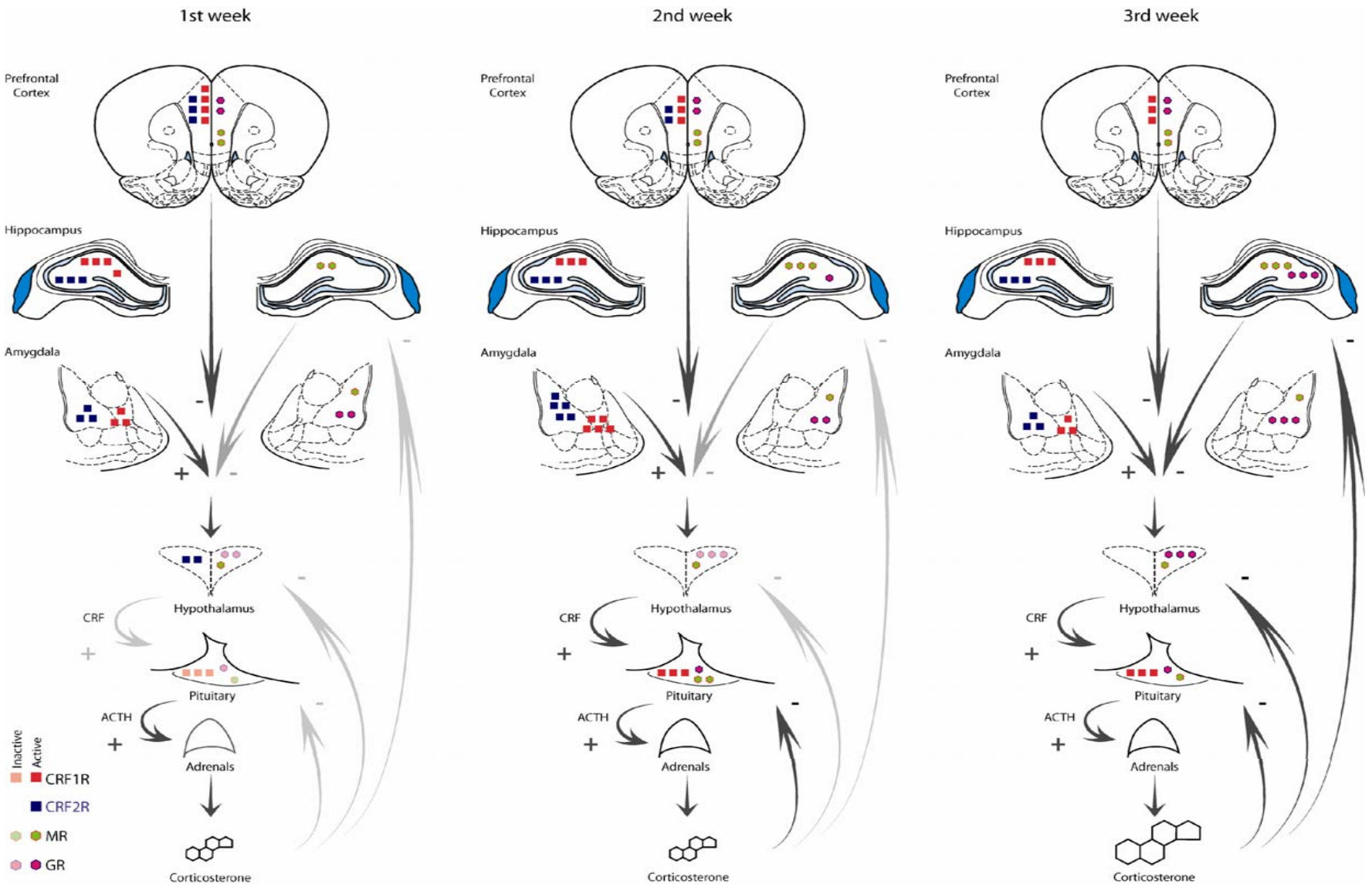

Fig. 2 – Diagrammatic representation of the ontogenic pattern of the rodent HPA áxis during the first 3 weeks of life. In the pre-frontal cortex (PFC), amygdala and hippocampus, the CRF receptors (CRF1 and CRF2) ontogeny is represented in the left side of each structure, while for cortcosteroids receptors (MR and GR) are represented in the right one. The distribution of the receptors is arbitrary within each structure and do not correspond to any specific distribution in the sub-nuckei. Each scheme represents the positive (arrows with +) and negative (arrows with -) feedback mechanisms of the HPA axis in the first, second and third week after birth (from the left to the right); gray arrows represent hypofunctionally, while black arrows identify the normal activity.

1.2 Maternal separation as an early life stress model – implication on future psychopathology

During the perinatal period, CS appear to have mainly organizational and regulatory effects, being required for the maturation of a variety of peripheral tissues. Within the central nervous system, their regulatory action on cellular differentiation of both neurons and glia (O'Banion *et al.*, 1994; Fuxe *et al.*, 1996; Sousa & Almeida, 2002) as well as in the neurotransmitter expression (Puro, 1983) has been demonstrated (for review see (Lauder, 1983)). Not surprisingly, changes in CS levels during this period lead to dramatic morphological, biochemical, physiological and behavioural abnormalities, that are highly dependent on the magnitude of CS changes and on the developmental stage at which the exposure occurs (Levine, 2001; Enthoven *et al.*, 2008). Although it has been shown that the perinatal period comprises a period of attenuated stress response, environmental/rearing manipulations have proven to be effective in disrupting the normal development of this system.

There is accumulating evidence that the neonate can indeed respond to stressors early in life (Walker *et al.*, 1991) which seems to conflict with the notion of a SHRP. The main issue regarding this controversy is probably due to the methodological approach used to evaluate HPA axis activity. In fact, this period has been characterized by adrenal insensitivity with minimal Cort elevations in response to a variety of stressors and failure of mild stressors (such as saline injection or exposure to novelty) to elicit ACTH response (Schoenfeld *et al.*, 1980; Sapolsky & Meaney, 1986; Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992). However, the pituitary has shown to be able to respond to CRF in an age-dependent way (Walker *et al.*, 1986) and to specific stimuli such as bacterial endotoxin (Witek-Janusek, 1988), histamine and ether (Walker *et al.*, 1991) and interleukin-1β (Levine *et al.*, 1994). Thus, although neonatal animals failed to show significant increase in Cort levels they do respond centrally, displaying elevation of ACTH. Walker and co-works have claimed that neonate's stress response is very similar to that of the adult (Walker *et al.*, 1991). However, there are still several differences that should be considered when comparing the adult and neonatal neuroendocrine systems. First, the neonates display a stressor-specific response, meaning that they are responsive to some stimulus, but not to others; second, although they display an ACTH response early in life, the magnitude of this response is age-dependent; and, finally, the adrenal response still show a diminished response when stimulated with the same levels of ACTH compared to adults.

The ability of early life events to modify HPA axis development and subsequent function was first described 5 decades ago (Levine *et al.*, 1957). In that study, Seymour Levine and colleagues, demonstrated that removing pups from their mothers, for few minutes a day, induces a reduced activity of the HPA axis. This manipulation described as *handling* was subsequently shown to be effective in dropping the stress reactivity in response to environmental changes (Caldji *et al.*, 2000; Pryce *et al.*, 2001; Pryce *et al.*, 2003); indeed, handled animals displayed significantly lower increases in CS even when submitted to a subsequent mild stimulation and were able to quickly return to basal levels after stress exposure. Importantly, this response pattern to stress was shown to last until adulthood (Levine, 1957; Weinberg *et al.*, 1978; Meaney *et al.*, 1996). The modified endocrine response of handled animals appears to give them the ability to cope with stressful events leading to a rapid activation/inhibition of the stress cascade, which is a crucial phenomenon to avoid the known deleterious effects of prolonged exposure of CNS to adrenal steroids (Lupien *et al.*, 1998; McEwen, 1998; Cerqueira *et al.*, 2007). Most of these effects have been interpreted considering the alteration in mother-pup interaction upon short periods of separation. They suggest that after returning to their mother's cage the pups would be more licked and groomed than non-handled animals and this would improve their stress response later on.

Based on these differences in maternal care Meaney and colleagues unveilled a possible molecular mechanisms underlying this well adapted stress response (Liu *et al.*, 1997; Francis *et al.*, 1999). They used female animals that had been previously classified, according to their natural-occurring maternal behavior taking into account two different forms of maternal-pup interaction: licking/grooming (LG) and arched-back nursing (ABN) (Liu *et al.*, 1997; Caldji *et al.*, 1998; Francis & Meaney, 1999). Thus, studying the offspring of low LG/ABN and high LG/ABN mothers they found an epigenetic marker on the exon 1_7GR promoter sequence. In a specific region around the binding site for the nerve growth factor-inducible protein A (NGFI-A - a transcription factor known to be induced, in the hippocampus by maternal care) they showed a methylation pattern in low LG/ABN mothers that is absent in the high LG/ABN dams. Moreover, cross-fostering studies clearly demonstrated that this methylation pattern was largely dependent on the rearing conditions, meaning that animals born from low LG/ABN and fostered to high LG/ABN were comparable to high LG/ABN offspring. These results show that the methylation pattern of GR promoter can be programmed by changes in maternal care, independently from the germinative line (Francis *et al.*, 1999).

Beside these studies, animal models of depression induced by social defeat stress have shown to alter BDNF gene expression, by down-regulating two splice variants of the gene: the BDNF-III and BDNF-IV in the hippocampus (Tsankova *et al.*, 2006). In this study chronic stress has lead to increased H3 (histone 3)-K27 dimethylation which is a repressive modification. Interestingly, chronic imipramine treatment is able to induce H3 acethylation, as well as H3-K4 methylation; which represent modifications that promote gene expression.

Since Levine's pioneer studies, different models have been used to study the impact of early life manipulations on the HPA axis maturation and the long-term consequences for psychopathologies. Different postnatal manipulations include longer periods of separation either for 24h at a specific pre-weaning day (maternal deprivation - MD) or for 3 or 6 daily hours for several consecutive days (maternal separation – MS). These protocols of mother/pup relationship disruption have been used as models of childhood trauma due to caregiver neglect. The validity of the models is based on similarities to the human disorder in the following aspects: 1) the etiological factors; 2) the symptomatololy and manifestations; 3) the underlying pathophysiological mechanisms; 4) the response to therapeutic treatment. Indeed, maternal separation in rodents induces long-lasting activation of the HPA axis. As adults, animals submitted to both 3 and 6h/day during the first 2 weeks of life typically show significantly increases of both ACTH and CS plasmatic levels in response to a stressful stimulus compared with non-separated control animals. It has also been shown that maternal separation during 6h leads to decreased GR binding in the hippocampus and the hypothalamus, which are two key regions in the negative feedback mechanism of the HPA axis activity (Plotsky & Meaney, 1993). Furthermore, there is decreased CRF-receptor density in the AP, and increased CRF levels in the median eminence, as well as increased CRF mRNA expression in the mpPVN (Ladd *et al.*, 1996). Quite remarkably, and in agreement with the human disorders, some of the behavioural consequences proved to be reversible with antidepressants (Nemeroff, 1996).

It is important to notice that probably not all the changes in the stress response of early-life stressed subjects should to be attributed to variations in CS/CRF. In fact, during maternal absence, apart from the active sensory stimulation, food and passive contact are also missing to the pups. It has been suggested that milk deprivation may also mediate changes in adrenal sensitivity (Stanton & Levine, 1990), possible through variations in leptin levels. This protein, a critical component of the breast milk, is produced predominantly by white adipose tissue and can

signal the state of energy to the brain influencing food intake and thermogenesis (Hamann & Matthaei, 1996; Misra & Garg, 1996). In the developing rodent, leptin levels are naturally high (Ahima *et al.*, 1998) and the main source of this protein is probably the maternal milk, since leptin levels quickly decline in maternal deprived animals (Walker *et al.*, 2004). Earlier studies found that feeding a high fat diet to the mother could significantly reduce the magnitude of ACTH release in the offspring (Trottier *et al.*, 1998). In developing rats, elevated fat in milk appears to blunt the pup's HPA response, and leptin might be a critical mediator. When pups are administered leptin during the first 10 days after birth and they are tested for ACTH and Cort response to stress, they showed a reduced magnitude of the response (Oates *et al.*, 2000). Walker and colleagues proposed that leptin treatment enhanced glucocorticoid feedback mechanism, which lead to a more efficient HPA axis response, probably by increasing the expression of GR receptors in critical regions such as the hippocampus and PVN (Walker *et al.*, 2004). Leptin and Cort have shown to display inverse relationship during perinatal period, while high levels of Cort are found at the time of delivery, the leptin concentrations decline shortly at birth, increasing afterwards to high levels throughout the suckling period. Taken together, these evidence likely suggest that leptin might suppress basal adrenal production of Cort during development, maintaining a reduced activity of the HPA axis, by inhibiting the stress-induced CRF expression and enhanced glucocorticoid feedback inhibition. In animals separated from their mothers, the levels of leptin are decreased and the appropriate stress response is possibly compromised. Because this effect is probably more pronounced in the maternal deprivation model, we have used for our studies the maternal separation protocol.

Irrespective of the underlying mechanisms, the early life manipulations have long-lasting implications and are probably responsibly for the different vulnerability to stressful events and the susceptibility to later psychiatric disorders (Heim *et al.*, 1997). In fact, individual susceptibility is a very obvious feature in psychiatric disorders; some individuals seem to be more vulnerable to even relative mild adversities while others are able to overcome very difficult situations. Taking these evidence into consideration, it has been hypothesized that perinatal environment could play a critical role in "setting" the individual's stress coping system; most of the evidence supporting this hypothesis derives from maternal separation models. In fact, MS triggers the HPA axis alterations observed in depressed patients, but also the behavioural impairments that characterize depression and anxiety disorders. Once again, it is interesting to notice that some of

these changes can be reversed by antidepressants (MacQueen *et al.*, 2003; El Khoury *et al.*, 2006).

Regarding the neuronal substrates that possibly underlie the long-term consequences of maternal separation, CRF has been one of the main culprits, given its primordial role on emotion and stress-related symptoms (Holmes *et al.*, 2003). Permanent increased on CRF expression induced by maternal separation in brain regions such as the hypothalamus and amygdala were already documented by several studies (Barna *et al.*, 2003; Vazquez *et al.*, 2003; Huot *et al.*, 2004). Since the CRF released from the hypothalamus controls the HPA axis activity, this increase will lead to an hyperactivity of the axis. In parallel with these observations, there is also evidence of impaired GR negative feedback mechanisms. Early life stress seems to reduce the expression of forebrain GRs (Sutanto *et al.*, 1996; Ladd *et al.*, 2004) compromising the normal inhibition of the stress response and several studies have showed the inability of dexamethasone to suppress the adult HPA-axis of early maternal separated animals (Vazquez *et al.*, 1996; Ladd *et al.*, 2004).

Other relevant systems have been also implicated as mediators of the deleterious effects of early life stress. Specifically the serotoninergic pathway is likely to be affected by maternal separation (Vicentic *et al.*, 2006; Arborelius & Eklund, 2007; Bhansali *et al.*, 2007). Given the critical role of this neurotransmitter in anxiety and depression (Gross *et al.*, 2002; Gross & Hen, 2004) it is of relevance the differences observed in the serotonin (5-HT) levels, as well as in the expression of its receptors in specific brain regions. In fact, the observations described in maternal separated animals show decreased 5-HT levels in the hippocampus and reduced expression of the serotonin transporter in the raphe nucleus (Lee *et al.*, 2007); curiosly, in the raphe nucleus others have shown, in maternal separated animals, increased levels of both 5-HT and its metabolite 5-Hydroxyindoleacetic acid (5-HIAA) (Arborelius & Eklund, 2007).

Besides the neuroendocrine and neurotransmitter systems, there are also reports of immunological changes in rodent and non-human primates models of early life stress. Animals submitted to maternal separation seem to display increased vulnerability to inflammatory, allergic and autoimmune diseases (Milde *et al.*, 2004; Kruschinski *et al.*, 2008; Veenema *et al.*, 2008). This increased susceptibility has been associated with a suppressive effect on lymphocyte proliferation and increased number of natural killer cells (Lewis *et al.*, 2000). In addition, it is known that specific stress models, including MS, are able to increase pro-inflammatory cytokines levels (Hennessy *et al.*, 2004; Deak *et al.*, 2005). In other words, there appears to be substantial

evidence linking neuroendocrine and the immunological alterations in early life stress model that might be of relevance for subsequent psychopathology, in particular to depression.

Since the neuroendocrine correlates have been already described in the previous sections, the last part of this introduction will be dedicated to neuro-immune interactions.

1.3 Neuro-immune interactions

The immune and the central nervous systems are the main regulatory systems in the organism having specialized cells to "sense" the environment. Although they have been looked as independent, (some even consider the brain an "immune privileged" organ), there are now very strong evidence to support the interplay of both systems.

The first studies showing the relationship between the central, endocrine and immune systems were published in the 70´s (Solomon, 1969; Solomon *et al.*, 1974). Several reports from (Blalock, 1994; Ader *et al.*, 1995; Altman, 1997; Ader, 2000) unequivocally showed that cells from the immune, neuronal and endocrine systems share the same critical effectors to communicate. This relationship was shown to be bidirectional; in fact, psychological events may influence immunity, but activation of the immune system may also compromise mechanisms related to neuroendocrine and neurotransmitter function. Interestingly, the immune system has been classified as our "sixth sense" (Blalock, 1984). In turn, the CNS has demonstrated to be competent to interpret these signals and undergo plastic changes in regions known to respond also to physical and psychological stressors. In this interplay, we would like to emphasize the role of key molecules such as CS and cytokines (Blalock, 1994; Maier & Watkins, 1998; Blalock, 2005).

Cytokines are low molecular weight proteins crucial to the communication between immune cells. They act as intercellular messengers and induce their effects as soluble or membrane-bound proteins by binding to high-affinity receptors on target cell membranes. Unlike hormones, cytokines usually act over short distances in an autocrine or paracrine fashion. These molecules are pleiotropic, and their effects could be synergistically, redundant and, less frequently, antagonistic. They are involved in very different processes in almost all organs of the body playing important roles in development, growth, healing and maintenance of homeostasis (Granger *et al.*, 2006). The most efficient, but not exclusive, cells producing cytokines, in the periphery, are monocytes and macrophages. The vast family of cytokines comprise different polypeptides

designated as interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), chemokines, and growth and cell stimulating factors (Rothwell, 1999; Elenkov *et al.*, 2005). Based upon their molecular structure and physiological action, cytokines can be classified as pro- or anti-inflammatory. IL-1, IL-6 and TNF-α are examples of pro-inflammatory molecules that act in response to damage recruiting inflammatory components of the immune system to the site of injury. On the other hand, anti-inflammatory cytokines usually dampen the immune response, avoiding the deleterious effects of inflammation; IL-10, IL-4 and IL-13 are examples of anti-inflammatory cytokines.

Conversely to what was initially thought, receptors for these molecules were also found in the central nervous and endocrine systems (Besedovsky *et al.*, 1983; Besedovsky & del Rey, 1989), evidencing their action outside the immune system. Whether these molecules come from the periphery or are produced by nervous cells has been a topic of intense research. Blood-borne cytokines, due to their lipophobic nature, are unlikely to simply cross the blood-brain-barrier (BBB). However, several studies have proposed specialized mechanisms for cytokines to access the CNS. These include: a) the use of specific active transport system (Banks & Kastin, 1991); b) the passage at circumventricular organs where the BBB is weaker (Saper & Breder, 1994); c) or activation of cascades of secondary messenger in the brain parenchyma, after they bind to specific receptors on endothelial cells (Van Dam *et al.*, 1993). Beside these possibilities, other pathways have also been proposed to explain the high interplay of the brain-immune systems. In fact, the *vagus nerve* (the 10th cranial nerve), is considered an alternative and efficient pathway in sending afferent messages from organs where most of the inflammatory/immune responses occurs (such as the spleen, thymus and lymph nodes) directly to the CNS (more specifically to the *nucleus tractus solitarius* located in the brain stem). Indeed, it is already known that cytokine receptors are present in the vicinity of the *vagus nerve* terminals (the paraganglia); after cytokine binding to specific receptors in the paraganglia, there is activation of afferent vagal fibers (Berthoud *et al.*, 1995) that reach the CNS, where they trigger appropriate responses.

Although there are different pathways for cytokines to reach the brain, these chemical mediators are also known to be produced by cells within the CNS (Elenkov *et al.*, 2000; Szelenyi, 2001). Besides expressing receptors for different cytokines, both glia cells and neurons have shown to be able to synthesize and release these molecules (Hopkins & Rothwell, 1995). To add further to the complexity of the neuro-immune interaction it is known that specific neurotransmitters (e.g.

CRF, catecholamines) are implicated in the regulation of cytokine production within the CNS. As an example, CRF regulates the circadian production of IL-1β (Taishi *et al.*, 1997) and TNF-α (Bredow *et al.*, 1997).

However, only in 1986, Besedovsky (Besedovsky *et al.*, 1986) reported for the first time evidence of the relationship between cytokines and the HPA axis activity (mainly through CS). In that study, it was shown that rats injected with IL-1β displayed HPA axis hyperactivity as revealed by increased plasmatic levels of ACTH and CS. This direct consequence was subsequently confirmed by the demonstration that IL-1β activates CRF-containing neurons within the PVN (Berkenbosch *et al.*, 1987). Following these evidence a series of studies highlighted the relationship between cytokine administration and behavioural changes. Administration of IL-1β has shown to elicit anorexia (Hart, 1988; Moldawer *et al.*, 1988), sleep disturbances (Opp *et al.*, 1991), decreased social exploration (Spadaro & Dunn, 1990) and sexual activity (Avitsur & Yirmiya, 1999). These alterations are included in a variety of symptoms that are collectively designated as "sickness behaviour". In fact, sickness behaviour has been described as a disruption in the motivational state of an individual adjusting the organism to cope with infection (Aubert, 1999; Dantzer, 2001). It is characterized by endocrine, autonomic and behavioural changes that are elicited by pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6. Among the diverse symptoms (e.g. nausea, loss of appetite, disrupted sleep and mild cognitive impairments) there are also important subjective feelings such as malaise, lassitude and anhedonia. This motivational state implies important physiological mechanisms that, if prolonged in time and amplified in intensity, can lead to a maladaptive response, and consequently to pathology. The overlap symptomatology between sickness behaviour and depression is obvious; however this similarity is only partial. Whereas sickness is an adaptive response that terminates once the stimulus (e.g. pathogen) has been eliminated, depression usually persists in time even in the absence of the initial trigger.

The first role for cytokines in depression has been proposed by Smith establishing the "macrophage theory of depression" (Smith, 1991); these initial observations were subsequently explored by sereval laboratories and Maes proposed a causal relationship between the immunological profile and the mood state of depressed patients (Maes, 1993). According to this view, depressed patients display enhanced immunity, in the form of increased pro-inflammatory cytokines, which are responsible for clinical features of depression such as HPA axis hyperactivity

and serotoninergic alterations (Maes *et al.*, 1995). More direct evidence of this interplay, arise from studies using immunotherapy in which patients with cancer and viral diseases, treated with INF-α and IL-2 developed the neurovegetative and somatic symptoms of sickness behaviour. Interestingly, only about 30-45% of them also displayed anhedonia, anxiety and cognitive impairments (Valentine *et al.*, 1998; Capuron *et al.*, 2000), showing that cytokine-induced depression can also be determined by individual vulnerability (Capuron & Ravaud, 1999). The latter can be, at least partially, associated to alterations in the serotoninergic pathway (Aberg-Wistedt *et al.*, 1998; Ruhe *et al.*, 2007). In fact, patients that underwent immunotherapy (INF-γ and IL-2) also showed tryptophan depletion (Capuron *et al.*, 2002) and the bioavailability of this amino acid is a limiting element for 5-HT synthesis within the CNS. However it remains to be proved if the decreased circulating levels of this precursor are indeed the cause of the depressive symptoms observed in cytokine treated patients.

Tryptophan can be metabolized by two different enzymes: tryptophan 2,3 dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO) and decreased levels of the amino acid can be due to overactivity of these metabolic pathways. Interestingly, IDO, which is present in macrophages and dendritic cells, is induced by high levels of pro-inflammatory cytokines such as INF-γ and TNF-α. Thus, in the case of immunotherapy as well as other inflammatory disorders, IDO activity is potentiated. Studies using either LPS (Lestage *et al.*, 2002) or *Mycobaterium bovis* (Moreau *et al.*, 2005) treated mice showed that sustained high levels of INF-γ trigger IDO activation, which is associated with "depressive-like" behaviour. However, there are still missing links to establish a clear correlation between IDO activity and changes in the 5-HT metabolism that are predictable of behavioural modulation. Alternatively, tryptophan degradation by IDO can result through the Kynurenin pathway in other products, considered as toxins. It has been demonstrated that quinolinic acid (NMDA agonist) and kynorenic acid (NMDA antagonist) produced in this pathway are involved in nerve cell death and neurotransmission disruption, respectively which are important features likely involved in depressive symptomatology (Muller & Schwarz, 2007). Finally, it has also been proposed a direct role of pro-inflammatory cytokine in the increased brain tryptophan uptake that leads to an higher rate of the 5-HT turnover (Dunn *et al.*, 2005). In fact, INF-α has shown to down-regulate the expression of type 1A 5-HT receptors in non-neuronal cell line, a phenomenon reverted by co-treatment with two anti-depressants (Cai *et al.*, 2005).

Nevertheless, this "serotoninergic hypothesis" is insufficient to explain all the alterations observed in clinical depression. In fact, the HPA axis and, CS in particular, play a fundamental

role in controlling the homeostasis of the organism by restraining the neuroendocrine, but also the inflammatory response to a variety of stimuli, through regulated feedback mechanisms. One of the most reliable "biomarker" of major depression is HPA axis hyperactivity and impaired negative glucocorticoid feedback mechanism. Depressed patients show increased cortisol plasmatic levels (Pariante & Miller, 2001) and exhibit an exaggerated response to ACTH (Holsboer, 2000); these changes, that are thought to be mediated by CRF hypersecretion (Nemeroff, 1996), converge in a GR resistance status.

Interestingly, as it was stated before the interplay is complex, and, depressed patients typically display high levels of pro-inflammatory cytokines (Maes *et al.*, 1995; Sluzewska *et al.*, 1995; Lanquillon *et al.*, 2000; Mikova *et al.*, 2001; Tuglu *et al.*, 2003) that are not suppressed by the elevation of CS. Indeed, these cytokines are able to activate the HPA axis, *per se*, leading also to CRF and AVP production. Depressed patients stimulated to produce IL-1 failed to suppress cortisol levels after a dexamethasone suppression test (Maes *et al.*, 1993). Moreover, cytokines also showed to interfere with GR signaling; given the multiple steps that involves the GR translocation to the nucleus until they are active, there are several possibilities for cytokines to interfere with GR function (Holsboer, 2000). The most relevant phenomena are the protein-protein interactions of GR-NF-kB and GR-GRE (glucocorticoid responsive element) which regulate the GR inflammatory response (McKay & Cidlowski, 1999). Another important property is the impact of cytokine on the expression of GR isoform; there are at least two known isoforms of GR in humans: GR α and GR β (Lewis-Tuffin & Cidlowski, 2006) and pro-inflammatory cytokine were shown to promote the expression of the β isoform that is inactive but able to bind its ligand (Pace *et al.*, 2007). These data together with the ability of cytokine to interfere with GR translocation and function contribute to glucocorticoid resistance. Both the increased cytokine production and the decreased inhibitory feedback mechanism due to glucocorticoid resistance lead to excessive inflammatory state and intensify the stress-response system.

In summary, the neuroendocrine and the immune systems can indeed act synergistically toward adaptation in response to "stressors". Sharing some ontogenic developmental time windows these systems seem to be vulnerable to *imprinting* effects of early traumatic events.

1.4 Aims of the study

Early life stress has been shown to play a critical role on developmental psychopathology having long-lasting implications throughout adulthood. The HPA axis, one of the key elements in the stress response, has been shown to be sensitive to specific stressors especially if they are experienced in the perinatal period. However, the different ontogenic profile of crucial receptors and neurotransmitters in stress-sensitive areas, as well as the existence of the SHRP, constitute critical variables in the establishment of "*temporal windows*" of vulnerability to the stress-programming effects. CS and CRF, produced in response to challenges during neurodevelopment underlie some of the changes that occur at the behavioural, neuroendocrine and immunological level. Indeed, the cross-talk of both the CNS and immune systems is now an indisputable fact and several evidence emphasize the interplay between them in the aetiology of adult pathology. However, the current knowledge in this field has yet many open questions, some of which will be addressed in this thesis.

In summary, the present thesis aim to:

- Characterize the immediate consequences of early life stress on the neurodevelopment (Chapter 2).
- Evaluate the long-term behavioural and neuroendocrine consequences of exposure to stress in two different early life periods (Chapter 3).
- Determine the immunological substrates through which early life stress can underlie differential behavioural outcome (Chapter 3).
- Clarify the role of an anti-inflammatory cytokine in mood disorders, and its possible implications in mood disorders (Chapter 4).

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Chapter 2

Mesquita AR, Pêgo JM, Summavielle T, Maciel P, Almeida OFX & Sousa N.

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NEURODEVELOPMENT MILESTONE ABNORMALITIES IN RATS EXPOSED TO STRESS IN EARLY LIFE

A. R. MESQUITA,[a] J. M. PÊGO,[a] T. SUMMAVIELLE,[b] P. MACIEL,[a] O. F. X. ALMEIDA[c] AND N. SOUSA[a]*

[a]*Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*

[b]*Neurobehavior Unit, Institute for Molecular and Cellular Biology, University of Porto, 4150-180 Porto, Portugal*

[c]*Neuroadaptations Group, Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, D-80804 Munich, Germany*

Abstract—Manipulation of the corticosteroid milieu by interfering with the mother–newborn relationship has received much attention because of its potential bearing on psychopathology later in life. In the present study, infant rats that were deprived of maternal contact between the 2nd and the 15th postnatal days ($MS_{2–15}$) for 6 h/day were subjected to a systematic assessment of neurodevelopmental milestones between postnatal days 2 and 21. The analyses included measurements of physical growth and maturation and evaluation of neurological reflexes. Although some somatic milestones (e.g. eye opening) were anticipated, $MS_{2–15}$ animals showed retardation in the acquisition of postural reflex, air righting and surface righting reflexes, and in the wire suspension test; the latter two abnormalities were only found in males. A gender effect was also observed in negative geotaxis, with retardation being observed in females but not males. To better understand the delay of neurological maturation in $MS_{2–15}$ rats, we determined the levels of various monoamines in different regions of the brain stem, including the vestibular area, the substantia nigra, ventral tegmental area and dorsal raphe nuclei. In the vestibular region of $MS_{2–15}$ rats the levels of 5-HT were reduced, while 5-HT turnover was increased. There was also a significant increase of the 5-HT turnover in $MS_{2–15}$ animals in the raphe nuclei, mainly due to increased 5-hydroxyindoleacetic acid (5-HIAA) levels, and an increase of 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the ventral tegmental area (VTA) of stressed females. No significant differences were found in the immunohistochemical sections for tyrosine and tryptophan hydroxylase in these regions of the brain stem. In conclusion, the present results show that postnatal stress induces signs of neurological pathology that may contribute to the genesis of behavioral abnormalities later in life. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: maternal separation, neurological reflexes, vestibular area, monoaminergic systems.

*Corresponding author. Tel: +351-253604806; fax: +351-253604809.
E-mail address: njcsousa@ecsaude.uminho.pt (N. Sousa).

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; HPA, hypothalamic–pituitary–adrenal; HPLC/EC, high performance liquid chromatography, combined with electrochemical detection; HVA, homovanillic acid; $MS_{2–15}$, maternal separation between the 2nd and the 15th postnatal days; PND, postnatal day; SHRP, stress hypo-responsive period; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area; 5-HIAA, 5-hydroxyindoleacetic acid.

Early stressful/traumatic experiences are believed to play a major role in the development of a number of neuropsychiatric disorders (Roceri et al., 2002; McEwen, 2003; Pryce et al., 2005). Making an appropriate response to stress is a crucial adaptive prerequisite for optimal performance in a variety of circumstances over the lifetime. In contrast, inappropriate responses to stress are associated with impaired growth and development and may, ultimately, trigger endocrine, metabolic, autoimmune and psychiatric disorders; some of these disorders may only become manifest later in life, depending on other factors such as genetics and subsequent environmental stimuli. The activity of the hypothalamic–pituitary–adrenal (HPA) axis lies at the core of the stress response. Several studies have demonstrated that the organization of the HPA axis is influenced by the corticosteroid milieu early in life (Matthews, 2000; Meaney, 2001; Weinstock, 2001; Welberg and Seckl, 2001; Schneider et al., 2002; Sloboda et al., 2002; Oliveira et al., 2006), while others have suggested a link between HPA dysfunction resulting from early life events and anxiety and depression (De Kloet et al., 1998; McEwen, 2003).

Glucocorticoid secretion in rats is initiated during the fetal period and adult-like levels of corticosterone are seen during late gestation. However, there is a marked reduction of corticosterone secretion during the first days of life, and corticosterone levels remain low until postnatal week 2 (Sapolsky and Meaney, 1986; Rosenfeld et al., 1992). Moreover, this period is characterized by a quiescence of the HPA axis insofar that rats aged 4–14 days show a marked blunting of their adrenocortical response to stress; in accordance, this developmental window has been designated as the "stress hypo-responsive period" (SHRP) (Schapiro, 1962; Rosenfeld et al., 1992). Additionally, studies by Levine (Kuhn et al., 1990; Levine et al., 1991) have shown that the normal glucocorticoid negative feedback mechanisms in the brain and pituitary are also dampened during the SHRP.

According to some authors, the SHRP constitutes a protective mechanism, ensuring low and stable levels of glucocorticoids during early postnatal development. In fact, different lines of research demonstrate that the exposure to high levels of glucocorticoids, during the neonatal period leads to irreversible reduction of the brain weight (Huang et al., 1999), decreased DNA content in the brain (Velazquez and Romano, 1987), impaired neuronal myelination (Dunlop et al., 1997), widespread reduction of dendritic spines (Antonow-Schlorke et al., 2003) and inhibition neural precursor cell proliferation in brain areas that show postnatal neurogenesis (Scheepens et al., 2003). All these changes

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are thought to underlie alterations in social behavior and learning processes, and are believed to be caused by elevations in glucocorticoid levels (Flagel et al., 2002; Neal et al., 2004). Further, a recent study (Ellenbroek et al., 2005) has shown that acute maternal deprivation can also lead to neurological defects (e.g. in negative geotaxis, a primitive neurological reflex).

The main objective of this study was to examine how maternal deprivation in early life influences the timing of acquisition of a variety of somatic functions of rats and sensorimotor reflexes. Our behavioral analysis was complemented by measurements of monoamine metabolism in different regions of the brain stem implicated in the genesis/regulation of these reflexes.

EXPERIMENTAL PROCEDURES

Animals

Female primigestous Wistar rats (Charles River, Barcelona, Spain) were maintained under standard laboratory conditions under artificial 12-h light/dark cycle: lights on from 8:00 a.m. to 8:00 p.m., and an ambient temperature of 22 °C; food and water were available *ad libitum*. The day on which a female rat showed a vaginal plug was designated as embryonic day 0 and the day of delivery as postnatal day (PND) 0. Males were removed from the cage when a vaginal plug was confirmed. Nest material was provided to each dam, which was singly housed. No bedding changes were performed on the last days of pregnancy. Litters were delivered on gestation day 22; the size of each litter was adjusted to 10 (n=5 male and n=5 female).

All experiments were conducted in accordance with European regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation. Every effort was made to minimize the number of animals used and their suffering.

Early life stress protocol

Pups from five different litters (n=20; 10 males and 10 females) were separated daily from their mothers between 9:00 a.m. and 3:00 p.m. and placed in an incubator at 35 °C. After 360 min of separation, pups were returned to their home cages. This protocol was applied from PND 2–15 (MS_{2-15}).

Control animals (five different litters; n=20; 10 males and 10 females) were left undisturbed with their mothers until weaning. All animals were weaned at PND 21.

Maternal behavior during the period of maternal separation was analyzed by two independent raters through video analysis of three periods (30 min each) during different daytimes. The following parameters were assessed: non-nutritive contact, licking, nursing posture, self-grooming, eating, resting, rearing and horizontal activity. The length of the period in which the mother was feeding the pups was also estimated.

Somatic parameters

Animals were weighed daily. Other parameters measured included ano-genital distance, the day of eye- and ear-opening, incisors eruption and fur appearance.

Neurological reflexes

The evaluation of neurological reflexes was performed daily, starting at 9:00 a.m., always by the same experimenter (A.R.M.) blind to experimental treatment, during the PND 2 to PND 21. The pups were left in the same room as the mother during separation, under soft white light.

Surface righting reflexes

The neonate was placed in the supine position and the time needed to turn over and restore its normal prone position was recorded (maximum: 30 s). Complete acquisition of the reflex was assumed when the animal could rotate 180° around its longitudinal axis.

Air righting reflex

The neonate was held on its back 30 cm above a soft surface before being released. The position in which the animal reached the soft pad was recorded. The reflex was considered to be achieved when the neonate landed on the surface with all four paws.

Wire suspension test

A metal bar was suspended 50 cm above a soft surface. The animal was held and its forepaws were allowed to touch the bar. Complete acquisition of the reflex was assumed when the animal was able to grasp the bar. This test lasted 120 s in total.

Negative geotaxis

The animal was placed on a grid, tilted 45° to the plane, with its head facing downwards. Animals that could rotate a full 180°, face up, and that could climb the grid within a maximum time of 30 s were considered to have fully acquired this reflex.

Postural reflex

Neonates were placed in a small box and shaken left and right, up and down. Those animals that could maintain their original position in the box by extending all four limbs were assumed to have acquired this skill.

Neuroendocrine analysis

Blood samples from pups were collected at PND 21 during the kill between 1:00 and 3:00 p.m.; the interval between transferring animals from their undisturbed environment to decapitation was kept below 60 s.

Blood samples from mothers were collected from the tail on the last day of the separation protocol. Serum corticosterone levels from both the mothers and the pups were assessed by radioimmunoassay (RIA), using ImmuChem^{TM} Corticosterone-^{125}I kits (MP Biomedicals, LLC, Orangeburg, NY, USA). The minimum detectable dose with 7.7 ng/ml.

Neurochemical determinations

Levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and norepinephrine were assayed by high performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson instrument (Gilson, Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 μM; 7.5 cm$\times$4.6 mm; flow rate: 1.0–1.5 ml/min; Supelco, Bellefonte, PA, USA). The vestibular regions, dorsal raphe nuclei, substantia nigra (SN) and ventral tegmental area (VTA) were rapidly dissected on PND 21, using a microscope and following orientation marks provided by (Paxinos and Watson, 2005) (see Fig. 1 for vestibular region illustration). Samples were snap-frozen by immersion in liquid nitrogen until HPLC-EC analysis. Tissues were homogenized, and 50 μl aliquots were injected directly into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l) and Na-EDTA (40 mg/l).

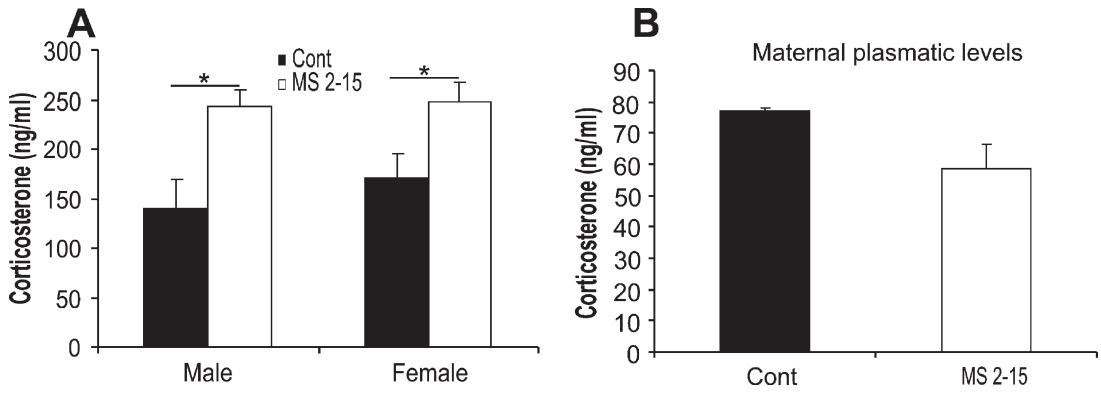

Fig. 1. Serum corticosterone levels of both male and female control (Cont) and maternal separated (MS_{2-15}) pups (A) and from Cont and MS_{2-15} mothers (B). Values are mean±S.E. * $P \leq 0.05$ (analyzed by Student's *t*-test).

Immunohistochemical analysis

Another subset of animals was prepared for tyrosine hydroxylase (TH) and tryptophan hydroxylase immunocytochemistry. Brain sections which included the SN, VTA and raphe nuclei were treated with 3% H_2O_2 in PBS to eliminate endogenous peroxidase activity and blocked with 4% bovine serum albumin (BSA, Sigma-Aldrich, Sintra, Portugal) in PBS. Sections were then incubated overnight at 4 °C in rabbit anti-TH (1:2000) and mouse anti-tryptophan hydroxylase (1:2000) (Biomol International, Plymouth Meeting, PA, USA) with antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA, USA) and diaminobenzidine (DAB: 0.025% and 0.5% H_2O_2 in Tris–HCl 0.05 M, pH 7.2). Specimens were lightly counterstained with hematoxylin. Analysis of the immunohistological sections was performed by optical densitometry and qualitative scoring (0–5 score, by two independent blind raters).

Data analysis

Data on physical maturation and reflex acquisition were statistically analyzed by the Fischer's exact (χ^2) test, and are expressed as the percentage of animals achieving a particular response. Body weight curves were analyzed by repeated measures ANOVA. Whenever appropriate post hoc comparisons were performed using Tukey test. Student's *t*-test was used to evaluate the neurochemical and neuroendocrine data. In all cases, statistical significance was set at $P \leq 0.05$.

Concerning the maternal behavior, the three daily 30 min observations were summed and a mean score was calculated per week. As each behavior was scored as present (1) or absent (0), week scores range between 0–3. These values were analyzed by the Fischer's exact (χ^2) test.

RESULTS

Effects of experimental procedures in maternal behavior

Maternal behavior was not significant affected by the separation of the pups as can be observed in Table 1. The only significant difference was related with the feeding time of the pups, which was increased during the observation period in maternal separated groups, probably as a compensation for the 6 h period of maternal separation (36.1 min for MS and 21.7 min for Cont).

Effects of maternal separation on basal corticosterone levels in pups and mothers

Statistical analysis showed that maternal separation significantly increased basal levels of corticosterone in both male ($P \leq 0.01$) and female ($P \leq 0.05$) pups (Fig. 1A). Conversely, serum corticosterone levels of dams in which pups were separated for 6 h were not different from those in which separation was only for a brief period (Fig. 1B).

Stress exposure early in life alters acquisition of development milestones

Although the body weight of male animals submitted to maternal separation was lower than that of controls (Fig. 2A) ($P < 0.05$), their body weight gain was only transiently affected, reaching control rates by the time of weaning.

Table 1. Maternal behavior parameters scored throughout the 2 weeks after birth

Group	Non-nutritive contact	Licking	Nursing posture	Self-grooming	Eating	Drinking	Resting	Rearing	Horizontal activity
Week 1									
Cont	0.33±0.11	2.00±0.24	2.33±0.33	1.67±0.30	1.00±0.20	1.17±0.33	1.17±0.42	1.33±0.24	1.33±0.30
MS_{2-15}	0.43±0.20	1.86±0.26	2.86±0.14	1.57±0.37	0.86±0.46	0.86±0.46	1.29±0.42	1.00±0.37	1.33±0.39
Week 2									
Cont	0.00±0.00	1.83±0.30	1.83±0.30	1.50±0.42	1.50±0.22	2.17±0.30	1.00±0.30	2.17±0.36	2.17±0.30
MS_{2-15}	0.11±0.21	1.44±0.44	2.33±0.33	1.11±0.33	1.11±0.36	1.11±0.42	0.56±0.40	1.11±0.49	1.33±0.42

Values are means±S.E. of scores.

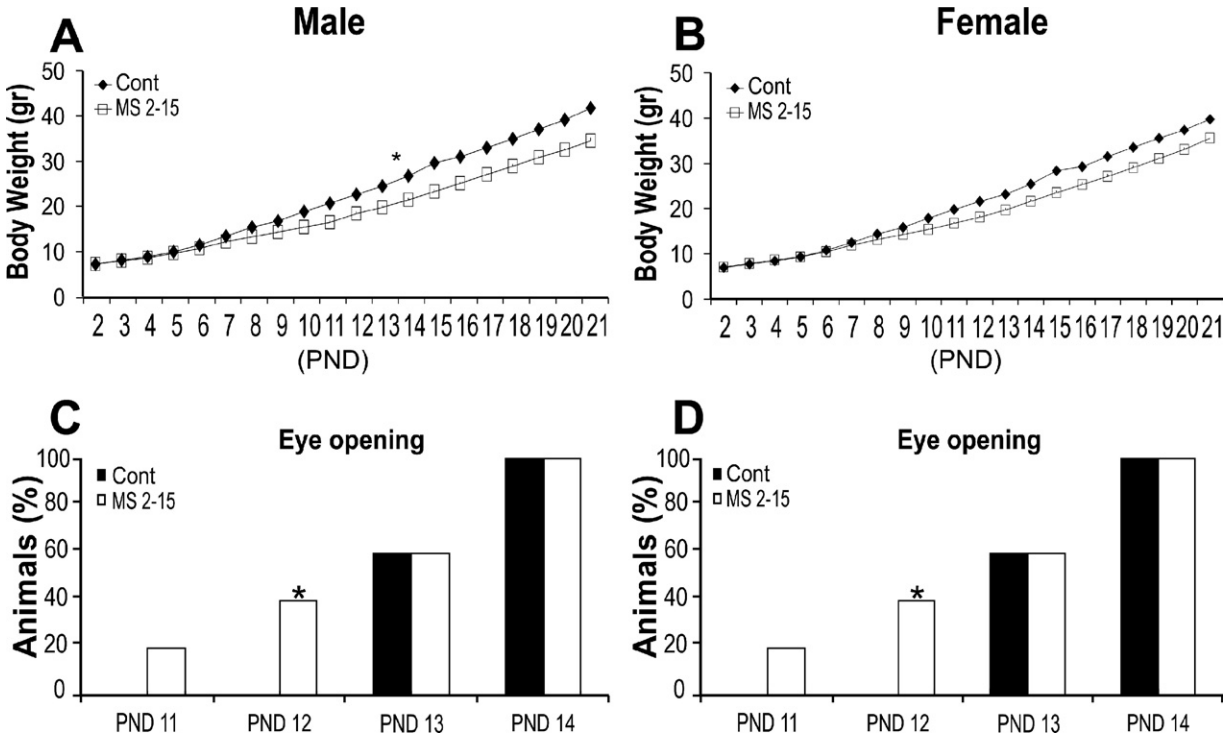

Fig. 2. Effects of maternal separation on physical growth and maturation evaluated with specific parameters. Body weight (in grams) measured along the 21 PND in male (A) and female (B) animals; values are means±S.E. (C, D) Age of eye opening of male and female animals, respectively. Bars represent the percentage of rats with opened eyes. * $P \leq 0.05$.

Maternal separation did not significantly affect body weight in female animals (Fig. 2B).

Ano-genital distances were not altered by the maternal stress paradigm in either of the sexes (data not shown).

Of the parameters examined to judge physical maturation, eye opening proved to be the most markedly influenced by maternal separation, with 40% of the MS_{2-15} animals showing eyelid separation at PND 12, i.e. significantly earlier than control animals ($P<0.05$). However, these group differences were not found at PND 14 when all animals showed fully open eyes (Fig. 2C and D). No gender differences were observed in this parameter.

No significant differences were found in ear-opening, day of eruption of incisors or in fur appearance in both males and females (data not shown).

As compared with controls, male MS_{2-15} animals showed significant differences in time of acquisition of a number of neurological reflexes. Specifically, whereas 60% of control animals were displaying the *air righting reflex* at PND 11, only 20% of MS_{2-15} animals had acquired this innate response at this age ($P<0.05$). This reflex was fully manifest in all control animals at PND 12, but was seen in only 40% of MS_{2-15} animals ($P<0.01$). By PND 13, animals in both groups had acquired the air righting reflex (Fig. 3A). Time of acquisition (Fig. 3B), as well as latency time (data not shown) of the *surface righting reflex* did not differ between control and MS_{2-15} animals. Maternal separation resulted in a retardation of *postural reflexes* at three test times ($P<0.001$ at PND 11, PND 12 and PND 13), and MS_{2-15} animals showed their first full responses at PND 14 (Fig. 3C). The test of *negative geotaxis* revealed that 40% of male MS_{2-15} animals had acquired this reflex (including turning to their upright position and grid climbing) by PND 5; in this respect, MS_{2-15} animals showed an advancement of acquisition of this skill, as compared with control animals. The between-groups differences were maintained until PND 7 ($P<0.05$ at PND 5, PND 6 and PND 7) (Fig. 3D). In the *wire suspension* test, a significant difference was observed in the animal's ability to sustain its own body weight at PND 4 ($P<0.05$); 20% of MS_{2-15} animals demonstrated this ability, as compared with 80% of control animals who could sustain their weights while holding onto the bar (Fig. 3E); however no differences were found in the latency time to fall (data not shown).

Female animals displayed similar neurodevelopmental profiles to those found in males. In the *air righting reflex,* a significant difference between control and MS_{2-15} animals was observable as early as PND 9 (40% of control vs. 0% MS; $P<0.05$ from PND 9–11 inclusive), but these differences disappeared at PND 12 (Fig. 3F). Acquisition of the *surface righting reflex* was also retarded in MS_{2-15} animals; 80% of control animals vs. 20% of MS_{2-15} animals, could turn onto their prone position on PND 2 ($P<0.05$) (Fig. 3G); however, once acquired the latency time was not significantly different (data not shown). The temporal profile of acquisition of the *postural reflex* was similar to that observed for male animals, with a retardation in the acquisition of this reflex in MS_{2-15} animals at PND 11, 12 and 13 ($P<0.01$), and disappearance of these between-group differences by PND 14 (Fig. 3H). At PND 7, 40% of control

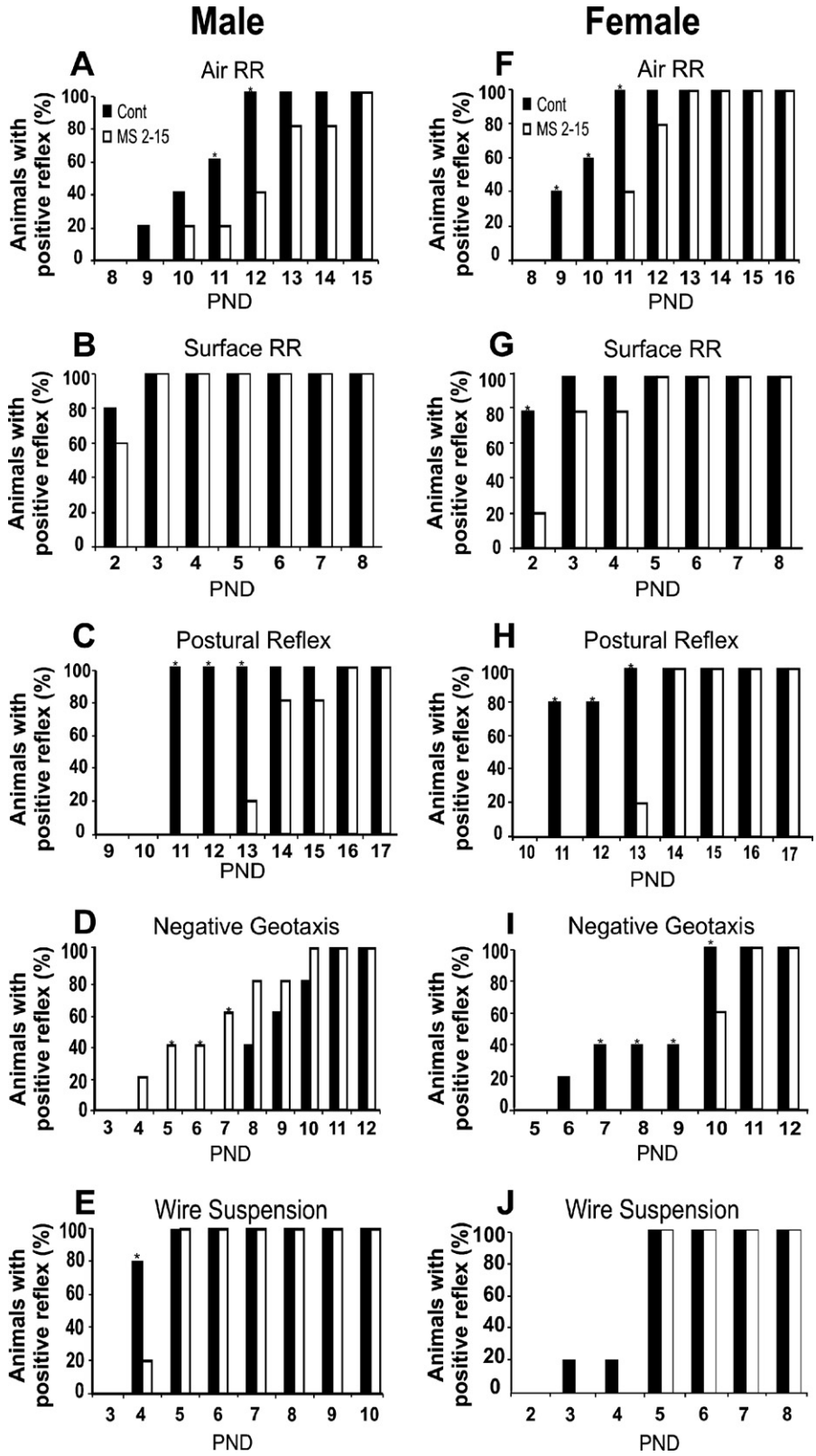

Fig. 3. Influence of maternal separation in the acquisition of neurodevelopmental milestones in male (A–E) and female (F–J) control (Cont) and maternal separated (MS_{2-15}) animals along the 21 PND. (A, F) Development of air righting reflex in rats. (B, G) Acquisition of the surface righting reflex. (C, H) Development of the postural reflex. (D, I) Acquisition of the negative geotaxis response. (E, J) Acquisition of the wire suspension response. All values are percentage of animals presenting a positive reflex. * $P \leq 0.05$ (analyzed by Fischer's exact (χ^2) test).

animals, and none of the MS_{2-15} animals, showed *negative geotaxis*; this difference between the two groups was maintained until PND 10 ($P<0.05$ from PND 7–10) (Fig. 3I). No statistically significant differences were observed between control and MS_{2-15} animals in performance of the *wire suspension* test, both in terms of acquisition as well as in the latency to fall (Fig. 3J).

Impact of maternal separation in neurotransmitter systems in different brain stem regions

MS_{2-15} female animals displayed decreased 5-HT levels and increased 5-HIAA levels in the vestibular area (Fig. 4, for the illustration of the dissection of vestibular area, and Fig. 5B). The same trend was observed in males although it did not reach statistical significance (Fig. 5A). However, maternal separation also increased 5-HT turnover in both genders (Fig. 5C and D) ($P<0.05$). In this region of the brain stem, we also found a significant increase in the levels of DOPAC of male MS_{2-15} animals (Fig. 5A) ($P<0.05$). No differences were found in dopamine turnover (Fig. 5E and F).

In dorsal raphe nuclei, the major area of 5-HT production, no differences were observed in 5-HT concentration (Fig. 6A and B); however, increased levels of 5-HIAA were

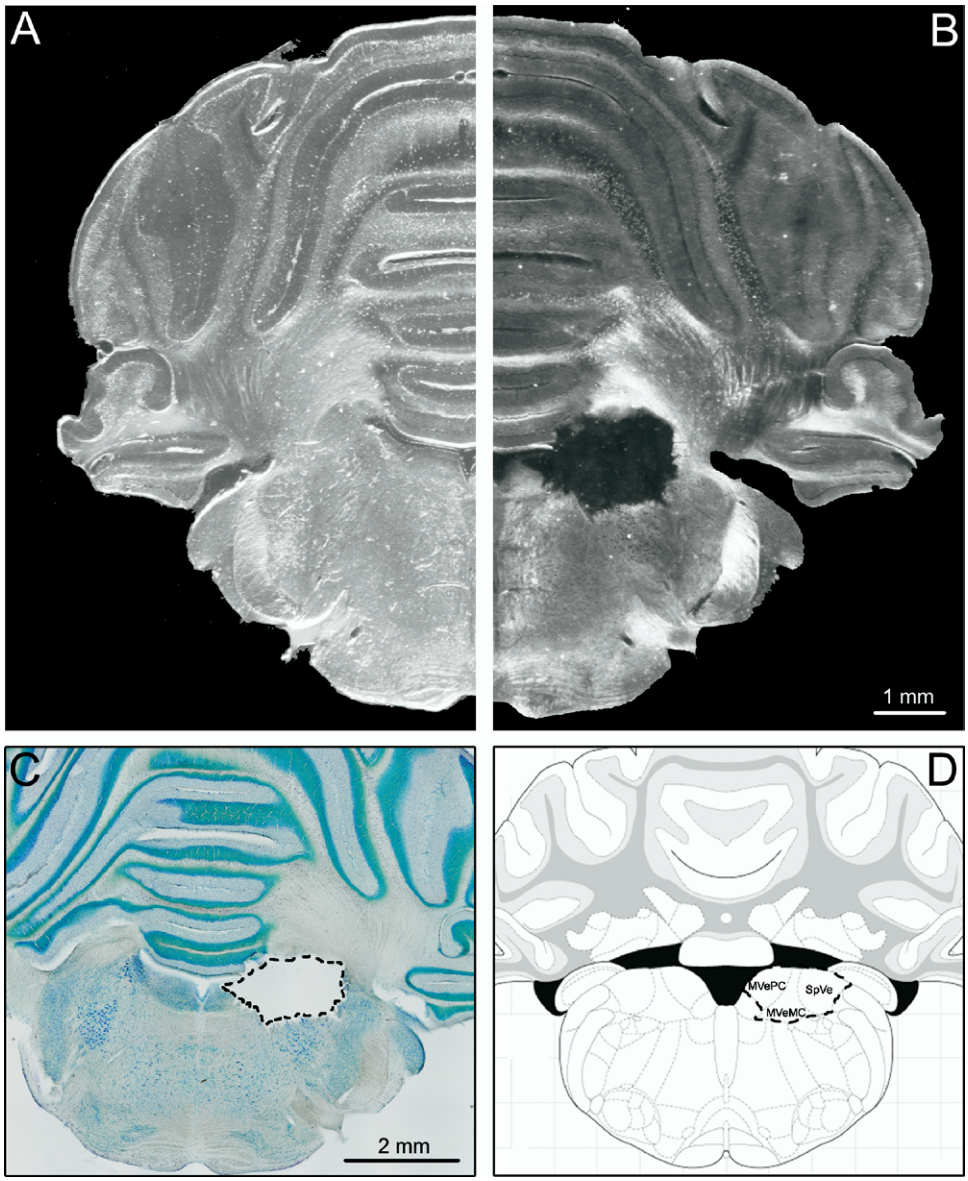

Fig. 4. Illustrations of the dissected vestibular area used for neurochemical analysis. Non-stained photomicrographs illustrating a coronal section of the rat brain before the dissection (A) and after vestibular region dissection (delineated area) (B); scale bar=1 mm. (C) Photomicrographs of Giemsa-stained coronal sections of the rat brain illustrating a dissected vestibular area (right hemisphere) and an intact one (left hemisphere); scale bar=2 mm. (D) Schematic representation of a coronal section of the rat brain (adapted from Paxinos and Watson, 2005) showing the equivalent dissected vestibular area (delineated area): MVePC, parvicellular medial vestibular nucleus; MVeMC, magnocellular medial vestibular nucleus; SpVe, spinal vestibular nucleus.

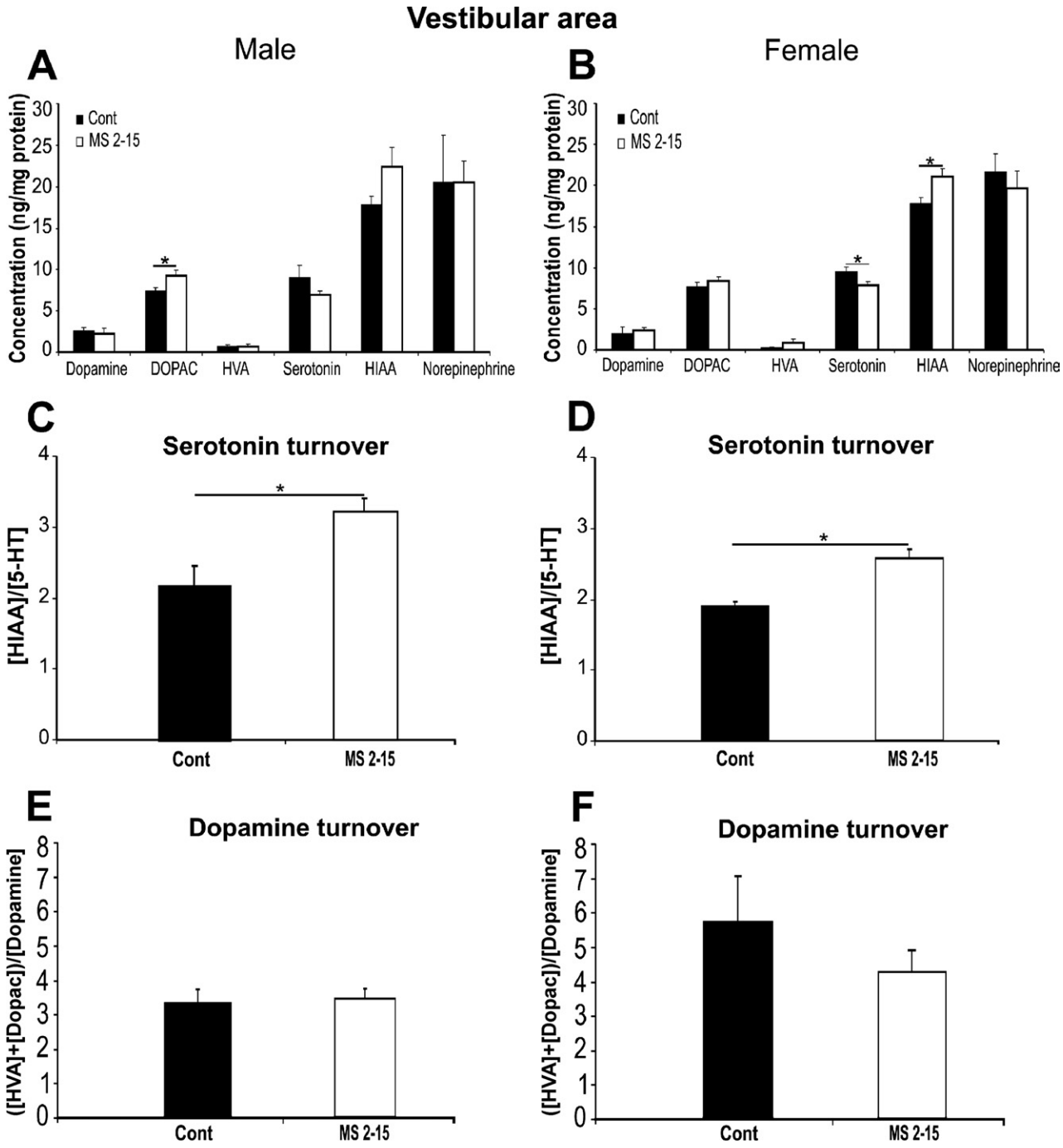

Fig. 5. Neurochemical analysis by HPLC of the vestibular region of control (Cont) and maternal separated (MS_{2-15}) animals. Concentration of each neurotransmitter (ng/mg of protein) in both male (A) and female (B) animals. 5-HT turnover assessed by the ratio 5HIIA/5HT, in both male (C) and female (D) animals. (E, F) Dopamine turnover assessed by the ratio between dopamine metabolites and dopamine concentrations in both male and female animals, respectively. All values are mean±S.E. * $P \leq 0.05$.

found in this area in females, but not in males, MS_{2-15} compared with Cont (Fig. 6C and D) ($P \leq 0.05$). Similarly to the vestibular area, 5-HT turnover was also increased in the dorsal raphe nuclei by maternal separation in both genders (Fig. 6E and F) ($P \leq 0.05$). No differences were found in dopamine or norepinephrine levels in these nuclei.

In order to assess dopaminergic transmission we also analyzed the levels of this neurotransmitter, and its metabolites, in the VTA and the SN (Fig. 7). Data revealed only a significant increase in DOPAC concentration in the VTA in MS_{2-15} female when compared with their respective controls (Fig. 7F).

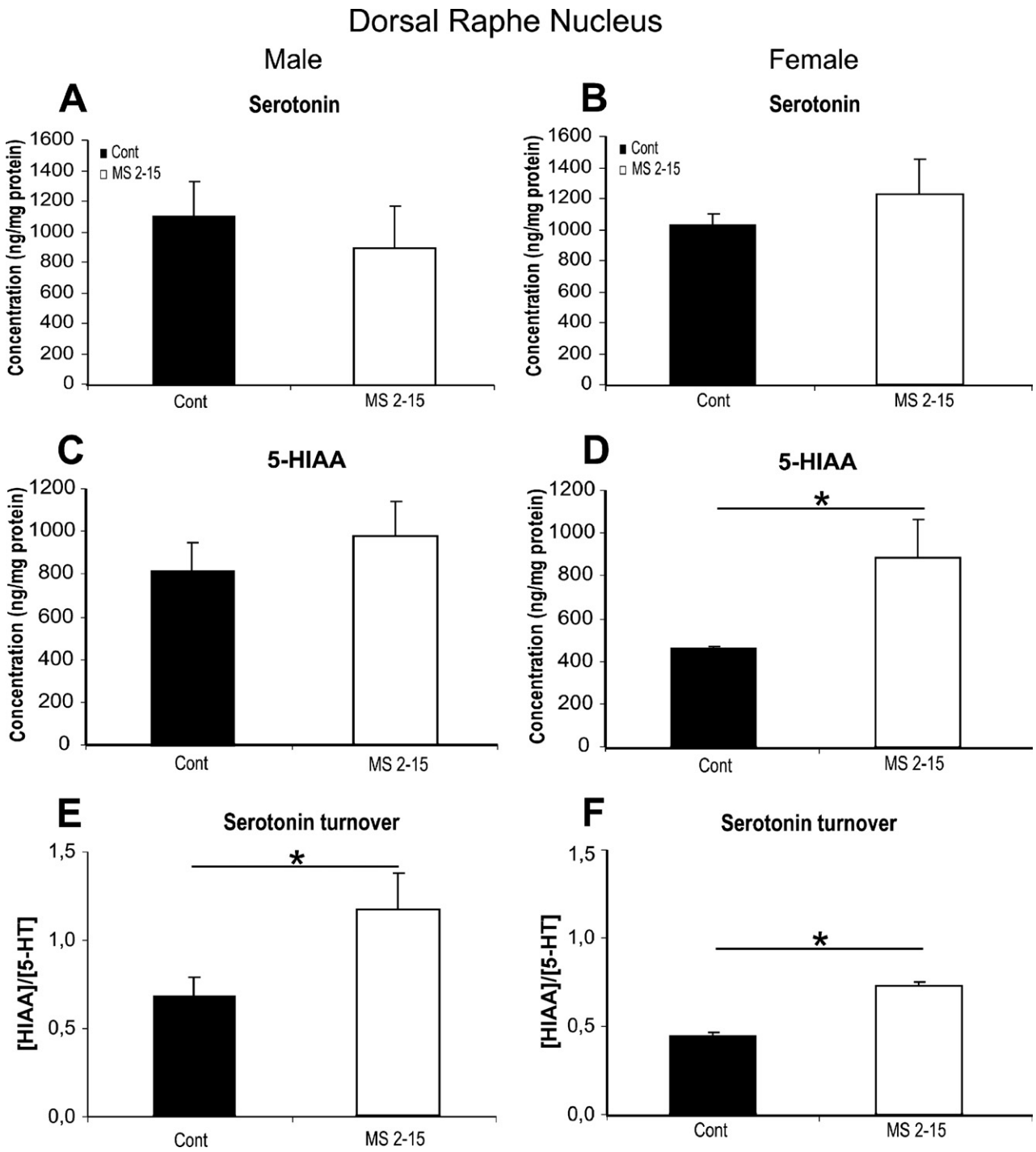

Fig. 6. Neurochemical analysis by HPLC of the dorsal raphe nucleus of control (Cont) and maternal separated ($MS_{2–15}$) animals. 5-HT concentration (ng/mg of protein) of both male (A) and female (B) animals; (C, D) 5-HIAA concentration (ng/mg of protein) in male and female, respectively; (E, F) 5-HT turnover assessed by the ratio between HIIA and 5-HT concentrations in male and female animals, respectively. All values are mean±S.E. * $P \leq 0.05$.

Overall, the analysis of the immunohistochemical sections failed to show any significant different in the expression of TH and tryptophan hydroxylases between Cont and $MS_{2–15}$ (Fig. 8).

DISCUSSION

Neurodevelopmental assessment typically includes analysis/scoring of physical growth and maturation and neuro-

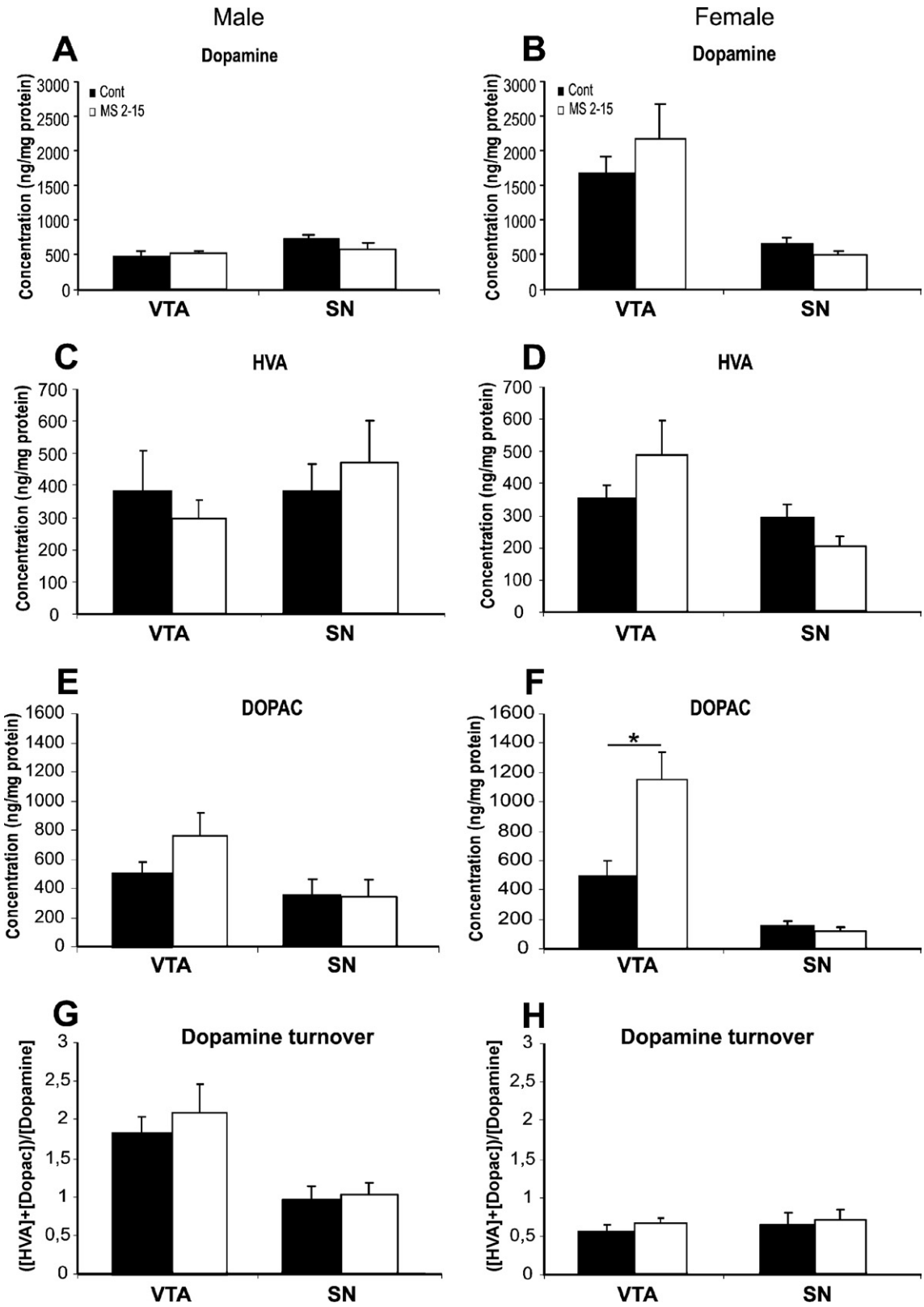

Fig. 7. Neurochemical analysis by HPLC of the VTA and SN of control (Cont) and maternal separated (MS_{2-15}) males (A, C, E and G) and female (B, D, F and H) animals. (A, B) Concentration of dopamine (ng/mg of protein); (C, D) HVA concentration (ng/mg of protein); (E, F) DOPAC concentration (ng/mg of protein). (G, H) Dopamine turnover assessed by the ratio between dopamine metabolites (DOPAC+HVA) and dopamine concentrations. All values are mean±S.E. * $P \leq 0.05$.

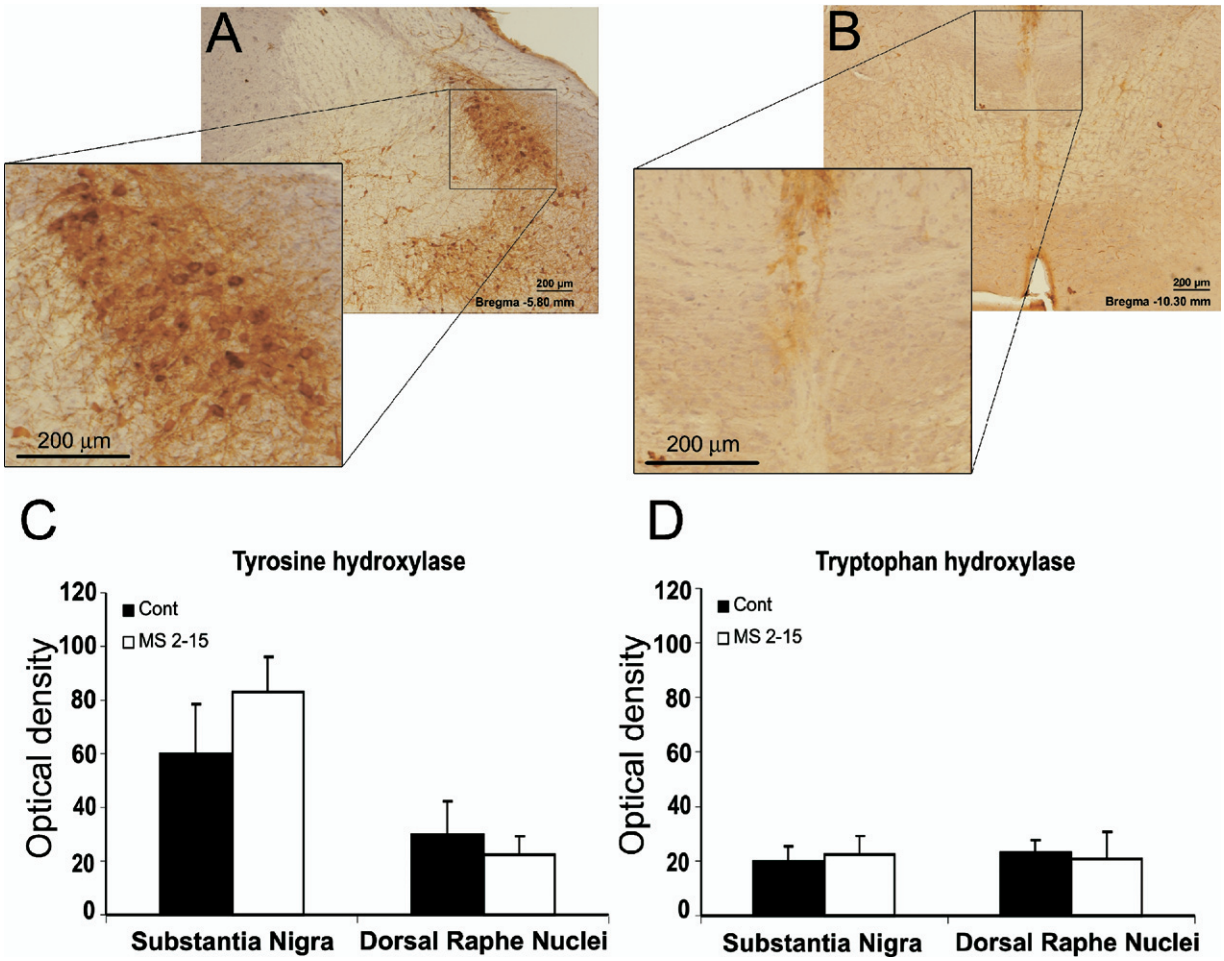

Fig. 8. Representative photomicrographs of (A) TH-positive immunocytochemistry in the SN and (B) tryptophan hydroxylase positive- immunocytochemistry in the dorsal raphe nucleus; scale bar=200 μm; distance from the bregma −5.80 mm (A) and −10.30 mm (B). Optical density measurements of TH (C) and tryptophan hydroxylase (D) in both SN and dorsal raphe nucleus; values are represented in arbitrary units.

logical reflexes (Spear, 1990; Sousa et al., 2006). Maturational parameters such as timing of ear and eye opening, teeth eruption, body weight and ano-genital distance measure the physical maturation. Dynamic tests of sensorimotor development, such as negative geotaxis, postural and righting reflexes, reflect maturation of vestibular function (Altman and Sudarshan, 1975; Khan et al., 2004), whereas the wire suspension, regarded more as a "static" test that depends upon visual afferents, allows assessment of balance control mechanisms (Perrin and Perrin, 1996).

Among the maturation parameters assessed in this study, differences were most marked in terms of eye-opening, with MS_{2-15} animals showing a significant advancement in the timing of this feature. Although contrasting with those of a recent study involving acute maternal deprivation (Ellenbroek et al., 2005), our observations are consistent with previous studies describing an acceleration of sensory development by synthetic glucocorticoids (dexamethasone or betamethasone) (Gramsbergen and Mulder, 1998; Neal et al., 2004). In this respect, it is important to recall that glucocorticoids are prescribed during late gestation to accelerate fetal lung maturation in some 10% of risk pregnancies (Crowley, 1995; Crane et al., 2003). Other direct catabolic effects of high corticosterone levels (in our case a result of maternal separation) probably contribute to our observations of a transient decrease in body weight in male MS_{2-15} rats. Although it is plausible that thermoregulatory problems might also have contributed to the transient slowing down of the growth curve, this seems an unlikely explanation since the MS pups were maintained at maternal temperature throughout the separation procedure.

MS_{2-15} animals displayed delayed acquisition of postural and righting reflexes, indicating that MS during the SHRP can impair development of motor skills. The latter reflexes measure the development of dynamic postural adjustments that require the integrity of muscular and motor function and adequate acquisition of symmetrical coordination between left and right sides of the body (Dierssen et al., 2002). Consistent with the view that early life stress can have a major impact on the development of the nervous system, we also observed retardation in the acquisition of wire suspension, suggesting impeded maturation of tactile and fine motor pathways and according with the

view that early life stress is a powerful modulator of nervous system development. Finally, data from the tests of acquisition of negative geotaxis are somewhat difficult to interpret: while this reflex occurred significantly earlier in male MS_{2-15}, female MS_{2-15} animals showed a delay, as compared with control animals. Despite reports of gender differences in neuronal maturation (Olesen and Auger, 2005) that might be underpinning this finding, others have questioned the validity of this test in defining neurological milestones, especially at angles greater than 10° (Kreider and Blumberg, 2005; Motz and Alberts, 2005).

The results of this study demonstrate that corticosteroid/stress treatment during early postnatal life has two important neurodevelopmental outcomes that may have a bearing on psychobiological development. Corticosteroids/stress during early life accelerate some somatic milestones while delaying the acquisition of neurological reflexes. The latter appear to result from the deleterious actions of corticosteroids on serotoninergic transmission, both in the vestibular region and dorsal raphe nuclei. These findings complement and extend previous work on the damaging effects of corticosteroids on the HPA axis and its ascending regulatory pathways (De Kloet et al., 1998; De Kloet and Oitzl, 2003); they raise the possibility that neurodevelopmental abnormalities in the hippocampus–HPA axis and brain stem act in concert to contribute to psychopathology in later life. Like in the hippocampus, corticosteroid receptors are expressed in these brain stem regions (Cintra et al., 1991; Cameron and Dutia, 1999). Corticosteroid receptors are known to be involved in the developmental programming of central catecholaminergic and serotoninergic activity, aberrations of which can lead to long-lasting functional abnormalities (Slotkin et al., 1992; Kreider et al., 2005). The vestibular region receives serotonergic enervation which is implicated in normal vestibular function (Cransac et al., 1996; Gil-Loyzaga et al., 1997). The present observation of increased 5-HT turnover in the vestibular area of MS-treated animals indicates reduced availability of 5-HT at vestibular post-synaptic receptors, which might be indicative of an altered vestibular function. Interestingly, vestibular information has been suggested to be important for hippocampal function (O'Mara et al., 1994; Russell et al., 2003; Zheng et al., 2003; Brandt et al., 2005; Smith et al., 2005) and, thus, might represent another pathway through which early postnatal stress influences hippocampal-dependent functions later in life.

MS is known to modulate forebrain serotoninergic transmission (Gartside et al., 2003; Vicentic et al., 2006). To the best of our knowledge, this is the first report of MS-induced increases in the turnover of 5-HT in the vestibular area. In addition, we show that an increased 5-HT turnover in the dorsal raphe nuclei of MS animals, even thought no significant changes were detected in the levels of 5-HT nor in the immunohistological analysis of tryptophan hydroxylase in this nucleus. These effects of MS can result from the increased levels of corticosteroids observed in MS_{2-15} animals, a view supported by previous work on PND 7–9 rats (Kreider et al., 2006), as these hormones are known to modulate 5-HT(1A) autoreceptors (Bellido et al., 2004; Slotkin et al., 2006); another plausible alternative is through the stress-induced activation of corticotrophin-releasing factor-1 receptors, that are known to modulate serotoninergic transmission from the raphe nucleus (Valentino et al., 2001). Whatever the underlying mechanism, we demonstrate that maternal separation programs the serotoninergic system, and transiently impairs the acquisition of innate reflexes; the former, due to their implications on the enervation of multiple limbic brain regions, may contribute to the long-lasting effects of MS on emotionality and cognition.

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Chapter 3

Mesquita AR, Roque S, Silva R, Nóbrega C, Nunes-Alves C, Carvalho IM, Palha JA, Correia-Neves M & Sousa N

The effects of early-life separation display specific temporal specificity

(Manuscript in preparation)

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The effects of early-life separation display specific temporal specificity

A.R. Mesquita, S. Roque, R. Silva, C. Nóbrega, C. Nunes-Alves, I.M. Carvalho, J.A. Palha, M. Correia-Neves & N. Sousa*

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal

*Corresponding author

Nuno Sousa, MD, PhD

Life and Health Sciences Research Institute (ICVS)

School of Health Sciences

University of Minho, Campus de Gualtar, 4710-057 Braga

Portugal

e-mail: njcsousa@ecsaude.uminho.pt

Tel: +351-253604806

FAX: +351-253604809

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Abstract

Maternal separation (MS), an early stressful event, has been demonstrated to be a trigger for neuropsychiatric disorders later in life. However, the mechanisms underlying this link are still largely unknown. In this study we compared in several different dimensions two different temporal periods of 6 hours of MS with undisturbed animals (Cont). In order to characterize the behavioural phenotype of these animals later in life we evaluated them at 3 months of age in a battery of behavioural tests. Both $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ displayed an anxious phenotype when evaluated in the elevated plus-maze as well as spatial memory deficits in the Morris water maze. However, in the forced swimming test, only $MS_{2\text{-}15}$ animals showed a depressive-like behaviour. To characterize the impact of MS in the regulation of the HPA axis, we determined the plasmatic levels of basal corticosterone, thymus weight and the CRF expression at weaning and at 3 months of age. In MS groups there were signs of hypercorticalism. In addition, at weaning we found increased CRF in the PFC, amygdala and in the hypothalamus of $MS_{2\text{-}15}$; interestingly at 3 months of age the CRF levels were increased only in the amygdala of $MS_{2\text{-}15}$ animals. To search for possible correlates underlying the differential impact of early life stress we decided to investigate the expression of IL-1beta, TNF-alpha and IL-10 in different brain regions and in the spleen. CNS measurements, using real-time PCR, revealed that $MS_{2\text{-}15}$ triggers a sustained impact in central cytokine expression showed by the increased expression of IL-1beta and TNF-alpha in the amygdala and PFC, respectively. Additionally, flow cytometry, performed to asses the number and type of different immune cells populations, showed a reduced number of the NK, CD4 and CD8 positive cells in the spleen of $MS_{2\text{-}15}$ compared with controls and $MS_{7\text{-}20}$.

Taken together, these findings demonstrated that animals exposed to early life stress display deficits in cognition, anxiety and mood later in life. These behavioural changes are paralleled by altered control of the HPA axis as well as by changes in the immunological profile. Noticeable is the observation of regional and temporal specificity in the programming effects of this manipulation.

Introduction

The programming effects of early life events in different systems of the organism became increasing relevant, since its implications in the pathogeny of several neuropsychiatric, cardiovascular and immune disorders later in life (Barker, 2004; 2007). In fact, exposure to early life stressful events (mostly mediated by changes in corticosteroid *millieu*) has been shown to have long-lasting consequences on emotional and cognitive behavior (Graham *et al.*, 1999; Aisa *et al.*, 2007; Garner *et al.*, 2007; Lee *et al.*, 2007), but also with changes in the neuroendocrine system (Meaney *et al.*, 1989; Liu *et al.*, 1997; Lehmann *et al.*, 2002). Most of the studies have used maternal separation (MS) as a developmental disruptor, because this manipulation is able to interfere with the maturation and activity of the HPA axis (Clarke, 1993; Plotsky & Meaney, 1993; Slotten *et al.*, 2006). Although the neuroendocrine mechanisms, and its consequences for psychiatric disorders, have been explored in the last decades, the programming effects on other regulatory systems, namely the immune system has been remarkably underappreciated. Even though, recently, some studies have highlighted that perinatal stress triggers long-term disturbances in the immune system (Coe *et al.*, 2002; Gotz & Stefanski, 2007; Vanbesien-Mailliot *et al.*, 2007).

The neuroendocrine and the immune systems are the two major regulatory systems of the body. Although they have been regarded as autonomous and independent their functional *cross-talk* is now indisputable (Ader *et al.*, 1995; Ader, 2000). Of notice, the hypothalamic-pituitary-adrenal (HPA) axis is one of the key elements in this interplay, as it regulates the release of glucocorticoids, which are potent immunomodulators (for review see (Webster Marketon & Glaser, 2008)). Although glucocorticoids have been primarily used as anti-inflammatory drugs, there is growing evidence that the interaction between corticosteroids and the immune response is much more complex than a simple immunosuppressive effect. Indeed, during a stress response, the hormonal changes may alter the expression pattern of several cytokines (Elenkov, 2004) and influence the cell fate of specific immune cells (Matyszak *et al.*, 2000). In addition, pro-inflammatory cytokines are able to hyperactivate the HPA axis (Maes *et al.*, 1993) an important hallmark of several psychiatric disorders (e.g. depression) (Leonard, 2007b; a; Cerqueira *et al.*, 2008; Kim *et al.*, 2008). Thus, it is not surprising that one consistent finding in depressed patients is the presence of an altered immunological and hormonal profile. More specifically, the mood disturbance is often accompanied by changes in the number of

lymphocytes and natural killer cells as well as by increased levels of circulating pro-inflammatory cytokines and glucocorticoids, which clearly compromises the immune function of those patients (Irwin, 1999; Maes, 1999).

Given the fact that the vulnerability to depression in adults might be influenced by adverse events experienced early in life, we thought of interest to assess if MS occurring in two different developmental "windows" (between the 2nd and the 15th - $MS_{2\text{-}15}$ or between the 7th and the 20th – $MS_{7\text{-}20}$) would compromise differentially the neuroendocrine response and understand the role of possible immune mediators in these long-lasting consequences.

Material and Methods

Animals

Wistar Han pregnant females (n=27) were singly housed in standard rat cages and maintained under standard laboratory conditions with artificial light/dark cycle of 12/12 hours, lights on at 8:00 a.m., temperature of 22°C and with food and water *ad libitum*, until delivery. On day 22 of pregnancy (E22) the females delivered and the litters were separated in 3 different experimental groups (6 litters per group): 1) maternal separation group ($MS_{2\text{-}15}$) 2) maternal separation group ($MS_{7\text{-}20}$) and control group (Cont). Litters were normalized to eight animals.

Separation procedure

Pups from six different litters were daily separated from their mothers between the 2nd and the 15th post natal days ($MS_{2\text{-}15}$) and kept in an incubator at 37°C. After 360 min of separation (from 9.00 am to 3 pm) the pups returned to their home cages. The same procedure was applied to other six litters which constituted the $MS_{7\text{-}20}$ group; however, in the later, the period of separation occurred between the 7th and the 20th post-natal days.

Pups from control litters were left undisturbed with the dam until the weaning day (P21).

Two animals of each litter were sacrificed at weaning for CRF determination in different brain regions (see details below). Body weight was determined weekly.

Behavioural tests

At 3 months of age, male animals (n = 28, derived from different litters) were behavioural evaluated in a battery of tests.

Open-field (OF)

Animals were individually tested during 5 min in an open-field arena consisted on a white square base (43.2 cm x 43.2 cm) surrounded with acrylic transparent walls (ENV – 515; MedAssociates, VT, USA). Illumination was provided by a white bright light. The session started with the animal placed in the center of the arena and, using a system of two 16 beam infra-red arrays connected to a computer, different parameters were recorded: (a) time spent in the central area (a measure of anxious-like behaviour); (b) total distance travel (a measure of general locomotor activity) (c) number and duration of rears, manually recorded by two experimenters independently (a measure of exploratory activity).

Elevated-plus maze (EPM)

Animals were individually tested for 5 min in an experimental apparatus consisted of a plus-shaped platform elevated 72.4 cm above the floor. The maze consisted of two opposing arms (50.8 x 10.2) closed by a 40,6 cm-high side walls and two open arms (50.8 x 10.2) with no walls. Illumination was provided by a white bright light. The session started with the animal placed in the junction of the four arms and the following parameters were recorded, using an infra-red beam system connected to a computer: (a) time spent in the open arms; (b) entries into open arms (c) entries into the closed arms (which corresponds with the overall activity).

This test reflects a conflict between the rat's exploratory activity and its innate fear of height and exposed areas. Thus, the amount of time spent and number of entries in the open arms show a negative correlation with anxiety-like behaviour.

Forced Swimming Test (FST)

Learned helplessness, as a measure of depression-related behaviour, was assessed using the forced swimming test (FST). For this test, animals were placed in acrylic cylinders filled with water (25° C) to a depth such the animals had no solid support for their rear paws. After a 10 min pre-test session, animals were rested for 24h before being subjected to the actual test which lasted for 5 min. At the end of each test session, animals were dried and place under a heating lamp (15 min.) before return to their home cages. The cylinders were filled with fresh water after each trial. A video camera was used to record test sessions; video recordings were later scored by an investigator blind to the experimental details. Time of immobility and latency to immobility were computed.

Morris water maze (MWM)

This test was performed using a black tank with 170 cm in diameter and 50 cm deep, filled with water and maintained at 25°C. The pool was divided in quadrants by imaginary lines and a black platform (12 cm diameter, 30 cm high) was located in a specific quadrant 2 cm below the surface of the water. Mild light was used and extrinsic clues were placed in the room walls. A *place learning task* was performed in order to asses the ability of each animal to find the hidden escape platform. Animals were individually tested for 4 consecutive days (4 trials per day) and the platform was placed in the center of one arbitrary quadrants. Each trial began with the animal

facing the wall in the quadrant immediately at right of the one where the platform has been placed, completing a clockwise rotation in the remaining trials every day. The starting point is the same for all the animals and the duration of each trial is 120 sec. If the rat did not find the platform within this period, the experimenter guided the animal to the platform where it remains for 30 seconds. Between each trial all animals were placed in a clean cage and they were dried before continuing the session.

The path swum to find the platform was recorded using a video camera fixed to the ceiling of the room and connected to a video-tracking system (Viewpoint, France).

Gene expression analysis

At 3 months of age, male animals (n = 32) submitted to the same treatment procedures described previously were sacrificed by quickly decapitation. The brain was removed and different areas were dissected using a microscope and following orientation marks provided by (Paxinos & Watson, 2005). Samples were snap-frozen by immersion in liquid nitrogen for posterior total RNA extraction. The spleen and the thymus were also removed, weighted and used for flow cytometry analysis; half of the spleen was also frozen for gene expression analysis.

Total RNA was isolated from the hippocampal formation, pre-frontal cortex, extended amygdaloid complex, hypothalamus and spleen using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA was then reverse transcribed into first-strand complementary DNA using the superscript first-strand synthesis system for reverse-transcription polymerase chain reaction (PCR) (Invitrogen) according to the manufacturer's instructions.

The expression levels of CRF, synapsin-I, IL-1β, TNF-α and IL-10 were assessed by real-time PCR using the iQ5 real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The iQ Syber Green Supermix was used according to the manufacturer's instructions. The cycling parameters were 1 cycle of 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, annealing temperature was 56 °C for *all genes analysed* for 20 s and 72 °C for 30 s. Single acquisitions were done at the end of each annealing step.

Using the same procedure, we analyzed the expression levels of CRF in the extended amygdaloid complex, PFC, hippocampal formation and hypothalamus at PND21 of Cont and $MS_{2\text{-}15}$ (n=12).

Primers: IL-1β *fw* -5'- CAC CTC TCA AGC AGA GCA A – 3' and *rev* 5' – ACG GGT TCC ATG GTG AAG TC – 3'; IL-10 *fw* 5' – GCC AAG CCT TGT CAG AAA TGA - 3' and *rev* 5' – TTT CTG GGC CAT GGT TCT CT – 3'; TNF-α *fw* 5' – GTG ATC GGT CCC AAC AAG GA 3' and *rev* 5' – AGG GTC TGG

GCC ATG GAA – 3'; CRF *fw* 5' – GGA GCC GCC CAT CTC TCT 3' and *rev* 5' – TCC TGT TGC TGT GAG CTT GCT – 3'; Synapsin- I *fw* 5' CAG GGT CAA GGC CGC CAG TG 3' and *rev* 5' CAC ATC CTG GCT GGG TTT CTG 3'; HPRT *fw* 5' – GCA GAC TTT GCT TTC CTT GG – 3' and *rev* 5' – TCC ACT TTC GCT GAT GAC AC 3'.

Endocrine evaluation

Radioimmunoassay

Blood samples were always collected at sacrifice between 1:00 and 3:00 p.m.; the interval between transferring animals from their undisturbed environment to decapitation was kept below 60s. Serum corticosterone levels were assessed by radioimmunoassay (RIA), using ImmuChemTM Corticosterone-125I kits (MP Biomedicals, LLC, Orangeburg, NY, USA). The minimum detectable dose is 7.7 ng/ml.

Immune cells phenotyping

Flow cytometry analysis

For the cell phenotypes determination, using flow cytometric analysis, $5x10^5$ cells were used from each individual animal and incubated with a specific antibody for 20 minutes at 4 °C. Cell surface markers were analysed using anti-CD4 (W3/25) FITC, anti-CD8 (OX-8) Pe, anti-CD11 b/c (OX-42) FITC, anti-CD45RA (OX-33) biotin (Caltag, CA, USA), anti-CD161 Alexa 647 (Biologend, San Diego CA, USA), anti-CD45RA (OX-33) Pe, anti-CD134 (OX-40) biotin, anti-granulocytes (RP-1) Pe (BD Pharmingen, San Diego CA, USA). All cells were fixed with 2% formaldehyde after staining. The analysis of the cell populations was based on the acquisition of 50,000 events using FlowJo software on a FACscalibur flow cytometer (Becton Dickinson, NJ, USA).

Statistical analysis

Data from the OF and EPM tests were analysed by one-way ANOVA. Whenever the homogeneity of variances was not achieved a variable transformation was performed. In the OF, we submitted the variable "percentage of time spent in the center" to a LOG_{10} transformation, while in the EPM both the "number of entries" and the "time spent in the open arms" were transformed by square root. The FST was analyzed by Independent-samples *t-test*..

The Morris water maze task was analysed using repeated measures ANOVA on the average results of the time and distance of the four trials in each day.

Data regarding the gene expression assay using real-time PCR were analyzed by one-way ANOVA. Specific variable transformation was performed in the extended amygdala for synapsin I and CRF (Log_{10}), in the hippocampus for, synapsin I (Log_{10}) and for CRF (Square root) and in the hypothalamus for CRF (Log_{10}) in order to achieve homogeneity of variances.

Regarding the immunological assessment, data was analysed using one-way ANOVA, and a variable transformation was performed for CD8 (Log) and for NK a non parametric test (Kruskal wallis) was also performed.

All the results are expressed as mean ± standard error of the mean (SEM) and statistical significance was considered for p values ≤ 0.05. Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago/IL).

Results

Behavioural data

Depressive-like, exploratory and locomotor behaviour are dependent of the age of exposure to MS

Regarding the depressive phenotype evaluated with the FST, a differential effect was observed in earlier and later maternal separation groups: while $MS_{2\text{-}15}$ displayed a significant increase in the immobility time (t = 2,74 and $p \leq 0{,}05$), no differences were observed between $MS_{7\text{-}20}$ and respective controls animals (Fig 1).

In the OF, regarding the total distance travelled in the arena, a reduced spontaneous locomotor activity was observed in $MS_{2\text{-}15}$ compared to controls ($F_{(2,27)}$ = 7,24 and $p \leq 0{,}01$) (Fig 2A).

Concerning the number and the duration of rearings, a measurement of exploratory behaviour, a significant effect of maternal separation ($F_{(2,27)}$ = 9,87 and $p \leq 0{,}001$ and $F_{(2,27)}$ = 4,10 and $p \leq 0{,}05$, respectively) was found. Multiple comparison analysis demonstrated a decreased exploratory activity of $MS_{2\text{-}15}$ animals compared to Cont ($p \leq 0{,}001$ and $p \leq 0{,}05$ for number and duration of rearings, respectively) (Fig 2B).

The OF test demonstrated that the percentage of time spent in the center of the arena was significantly affected by maternal separation ($F_{(2,27)}$ = 6,21 and $p \leq 0{,}01$). In fact, multiple comparisons by Tukey post-hoc test showed that $MS_{2\text{-}15}$ animals spent significantly less time in the central area compared to Cont group (Fig 2C).

MS at both ages triggers an anxious-phenotype and spatial memory impairments

In contrast to behaviour in the FST and OF in which there was a temporal "window" of susceptibility to MS, data from the EPM and MWM was affected by early life stress independently of the age of the procedure. Analysis of the EPM revealed a significant effect of MS in anxious-like behaviour assessed by the number of entries ($F_{(2,27)}$ = 13,72 and $p < 0{,}001$) (Fig 3A) and the percentage of time spent in the open arms ($F_{(2,27)}$ = 49,25 and $p < 0{,}001$) (Fig 3B). Post-hoc comparisons demonstrated a marked effect of earlier ($MS_{2\text{-}15}$) ($p < 0{,}001$ and $p \leq 0{,}01$) and later maternal separation ($MS_{7\text{-}20}$) ($p < 0{,}001$ and $p \leq 0{,}001$) in both parameters compared to Cont animals. Maternal separation groups were not different.

In the Morris water maze, a significant effect of maternal separation was observed in spatial learning. In fact, both $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ ($F_{2,23}$ = 4,58 $p < 0{,}05$) displayed increased latency times when compared to Cont animals (Fig 4).

Endocrine evaluation

MS permanently increases Cort levels

In order to determine the biological efficacy of the maternal separation protocol we assessed the basal corticosterone levels at PND 21; statistical analysis revealed increased basal corticosterone levels in $MS_{2\text{-}15}$ when compared to normal reared animals (t = 3,2 and $p \leq 0,01$) (Fig 5A). Accordingly, measurements in adult animals, showed a significant reduction in the thymus weight of both stressed-exposed groups compared with controls ($F_{2,32}$ = 4,8 and $p \leq 0,05$) (Fig 5B). Basal corticosterone levels were still increased in adult $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals ($F_{2,21}$ = 11,99 and $p \leq 0,001$) (Fig 5C).

Gene expression analysis

MS alters the pattern of CRF and Synapsin I expression

In order to evaluate the central control of the HPA axis, we assessed the CRF expression in specific stress-sensitive areas: extended amygdala, PFC, hippocampus, and hypothalamus.

CRF expression analysis, at weaning day, revealed a significant effect of maternal separation on the expression levels of this neurotransmitter in specific areas of the CNS (Fig 6). In fact $MS_{2\text{-}15}$ animals showed a considerable increase in the PFC (t = -2,99 and $p \leq 0,05$) (Fig 6B) and hypothalamus (t = -3,89 and $p \leq 0,01$) (Fig 6D) and, although not statistical significant, there is a border line effect in the extended amygdala as well (t = -2,12 and p = 0,06) (Fig 6A). In the hippocampus we failed to detect any difference between $MS_{2\text{-}15}$ and Cont (Fig 6C).

In adult animals, the expression of CRF in the extended amygdaloid complex, a region known to be highly implicated in emotional behaviour, was shown to be vulnerable to early life stress ($F_{(2,31)}$ = 3,8 and $p \leq 0,05$) (Fig 6E). However, this effect seems to be differential according with the period of maternal separation. While $MS_{2\text{-}15}$ show a trend for increase expression of CRF in the amygdala, $MS_{7\text{-}20}$ show decreased levels of CRF expression ($p \leq 0,05$) compared to controls in this region of the brain (Fig 6E). No significant differences in CRF expression were observed in the PFC, hippocampus and hypothalamus at 3 months of age (Fig 6F, G and H, respectively). Interestingly, the synaptic marker, synapsin I display a similar profile, showing increased levels in

the amygdala in $MS_{2\text{-}15}$ compared with latter separated animals. No differences in the other brain regions were observed (Fig 7).

Maternal separation influences cytokine expression

In order to establish possible immunological correlates underlying the differences in the depressive-like and exploratory behaviours observed between $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals we analysed the immunological profile of early life stressed and Cont animals, assessing the expression levels of pro- and anti-inflammatory cytokines both within the central nervous system (extended amygdala, PFC, hippocampus and hypothalamus) and in the spleen.

Interestingly, measurements of central cytokine levels showed regional specificity of cytokine expression. Indeed, earlier maternal separation significantly increased the expression of IL-1β in the amygdala ($p \leq 0{,}05$) compared with $MS_{7\text{-}20}$ (Fig 8) and TNF-α in the PFC ($p \leq 0{,}01$) compared to controls (Fig 9). No statistical differences were observed in the hippocampus (Fig 10). Regarding the hypothalamus we found decreased levels of IL-1β in $MS_{2\text{-}15}$ comparatively to $MS_{7\text{-}20}$ ($p \leq 0{,}05$ (Fig 11A). TNF-α levels were also increased in $MS_{7\text{-}20}$ when compared with both $MS_{2\text{-}15}$ and controls ($p \leq 0{,}001$ and $p \leq 0{,}01$, respectively) (Fig 11B). No differences were observed in the peripheral levels of cytokines assessed in the spleen (Fig 12).

Immunological assessment

Early maternal separation influences different populations of immune cells

Although no changes were observed in the cytokine expression, data from the flow cytometry analysis revealed a significant effect of earlier maternal separation in the percentage of specific cells population in the spleen. Analysis of data revealed a major effect on the percentage of $CD4^+$ ($F_{2,32}$=4,19; $p < 0{,}05$) and $CD8^+$ ($F_{2,32}$= 13,47; $p < 0{,}01$) T cells of earlier MS. Indeed, $MS_{2\text{-}15}$ displayed a significant reduction of $CD4^+$ cells compared either with Cont and $MS_{7\text{-}20}$ ($p \leq 0{,}05$). Regarding $CD8^+$ cells the reduction was only significant when compared with Cont values ($p \leq 0{,}001$) (Fig 13A). NK were also diminished in $MS_{2\text{-}15}$ comparatively with Cont group ($p < 0{,}005$). Although there is a reduction in both CD4 and CD8 T cells the ratio CD4/CD8 is increased ($F_{2,32}$= 6,02; $p < 0{,}01$), while T/B cell ratio is decreased ($F_{2,32}$= 4,63; $p < 0{,}05$) in $MS_{2\text{-}15}$ animals compared to controls ($p \leq 0{,}01$ for both parameters) (Fig 13 A(a)). Interestingly, earlier maternal separation also lead to increased number of T cells expressing the stimulatory molecule CD134

($F_{2,32}$= 4,54; $p < 0,05$) (which is a marker of T cell activation) when compared with control animals ($p < 0,05$) (Fig 13A).

Although it was observed a significant effect on thymus weight in both $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$, no changes were observed in percentage of the different population of cells in this organ (Fig 13B).

Discussion

The present study reports the behavioural, neuroendocrine and immunological consequences of early life MS in two different "developmental windows". The earlier period of maternal separation, occurring between $PND_{2\text{-}15}$, mimics the most widely used period in literature and assesses the impact of a severe stress exposure in an immature developing brain. However, we also assessed the impact of a later period MS that was delayed by 5 days (from PND 7-20) to allow a similar time length of exposure to stress without overcoming weaning (PND21). Data revealed that both periods of MS induced a sustained overactivation of the HPA even in "resting conditions" (assessed by the basal levels of corticosterone and thymus weight) that is associated with an anxious phenotype and an impaired performance in spatial reference memory task. In contrast, performance in the FST and in the OF later in adulthood was only altered in those animals exposed to the early period of MS a fact coincident with the central and peripheral immunological alterations observed.

In accordance with literature, we observed herein that MS activates the HPA axis most likely through the increased production and release of CRF, one of the main central regulators of the HPA axis. In fact, the mRNA expression of CRF, shortly after MS, was increased in the extended amygdala, PFC and hypothalamus; this observation, particularly in the hypothalamus, is likely to stimulate the "descendent" cascade of the HPA axis events that ultimately leads to increased circulating levels of Cort. Interestingly, and since both the CRF and Cort measurements were performed at weaning, i.e. one week after the termination of MS, one might conclude that the increased Cort levels failed to restrain CRF in the hypothalamus. However, this is not surprising, given that the negative feedback mechanism of the stress response is not mature until the end of the third post-natal week (Vazquez, 1998; Pryce, 2008), or eventually through an altered (decreased) expression of GR in the PVN, PFC and hippocampus (van Oers *et al.*, 1998; Avishai-Eliner *et al.*, 1999).

Interestingly, the impact of MS in the control of the HPA axis is preserved throughout life; this effect occurs even without exposure to stressful conditions and seems to be sustained in time (as revealed by the thymic atrophy observed in both MS groups). However, at 3 months of age despite the increased Cort levels, the pattern of CRF expression was only significantly altered in

the extended amygdala; in this brain region, there was a trend for an increase CRF production in $MS_{2\text{-}15}$ rats but a decrease in $MS_{7\text{-}20}$ animals. In the hypothalamus, there was a slight tendency for an increase in CRF expression in MS groups, but this determination is not sufficient to assure differences in the expression of this peptide in the parvocellular component of the paraventricular nucleus, where the secretion of CRF is known to promote the release of ACTH in the pituitary. Additional studies are undergoing, using laser microdissection, to specifically analyse this point. In addition, it is important to emphasize that CRF is not the only neurotransmitter regulating the activity of the HPA axis, and other regulators might display an altered expression; among these regulators is vasopressin, that it known to have a significant role in the control of the HPA axis, particularly in conditions of sustained activation of the axis (de Goeij *et al.*, 1991; Herman *et al.*, 1992; Bartanusz *et al.*, 1993).

Irrespective of the underlying mechanisms, the basal activity of the HPA axis of MS animals is enhanced. Such state of hypercorticalism is known to influence several behaviours, including anxiety (Pego *et al.*, 2008) and memory (Sousa *et al.*, 2000). In accordance with the literature, we confirmed that both MS groups displayed a hyperanxious phenotype (Kalinichev *et al.*, 2002) as well as impaired performance in the spatial memory task (Aisa *et al.*, 2007). Whereas anxiety and spatial memory were independent from the period in which MS had occurred, performance in the FST and OF revealed a higher susceptibility of $MS_{2\text{-}15}$ animals for "depressive-like" behaviour and changes in locomotor and exploratory activity. In common the performance in these tasks is highly dependent of motor performance and, thus, it is tempting to speculate that the exposure to stressful conditions, in the first week of life, is likely to impact on the development of brain circuits related to motor performance. In support of this assumption, is our previous observation of delayed neuronal milestones acquisition in the $MS_{2\text{-}15}$ animals (Mesquita *et al.*, 2007). More speculative, but not mutually exclusive, is the hypothesis that the differential expression of CRF in the amygdala might be implicated in these distinct performance of MS groups. Indeed, $MS_{2\text{-}15}$ group display increased CRF levels in the extended amygdala when compared to $MS_{7\text{-}20}$. Moreover, the opposite changes observed in the levels of this neurotransmitter in the amygdala in MS groups are paralleled by similar changes in the expression of synapsin I, a synaptic protein involved in vesicle trafficking/docking in the synapse, thus suggesting that there is an altered pattern of neuronal activity in this brain region as a result of the different temporal exposure to MS. Importantly, some studies correlate the increased

activation of the amygdala with depressive behaviour, in both rodents (Salm *et al.*, 2004) and humans (Monk *et al.*, 2008). To further explore this finding, we plan to use a pharmacological approach, using CRF1R antagonists, which are drugs with antidepressant properties that might revert the behavioural phenotype observed in $MS_{2\text{-}15}$ rats. Of notice, is also the remarkable decrease in the expression of this synaptic protein in the hypothalamus of $MS_{2\text{-}15}$ animals, and its preservation in the $MS_{7\text{-}20}$ group; this strongly indicates a huge impact on the overall synaptic activity in the hypothalamus if the animals are exposed to stress during the first week of life.

The action of cytokines in the brain is still enigmatic; while some studies suggest that these molecules might exert neurodegenerative effects (especially at higher doses), others unequivocally demonstrate that, within a certain physiological range, these molecules can act as growth factors in several systems (Bernardino *et al.*, 2008; Li *et al.*, 2008). Therefore, the elevation in IL-1β expression in the amygdala of $MS_{2\text{-}15}$, might correlate with an increased activity of this region of the brain in these animals, namely in the CRF neurotransmission. This fact should not be underappreciated since CRF have shown to directly stimulates leukocytes to produce immunoregulatory pro-opiomelanocortin (POMC)-related peptides (β-endorphin, ACTH, and α-melanocyte-stimulating hormone) (Smith *et al.*, 1986) and to secrete IL-1β (Kavelaars *et al.*, 1989; Singh & Leu, 1990). Because this cytokine can activate vagal afferents which are efficient pathways in sending peripheral signals directly to the CNS, this is a likely via to increase central pro-inflammatory cytokine expression (Laye *et al.*, 1995; Hosoi *et al.*, 2000). However the production of IL-1β within the CNS should not be discarded, in vitro studies have demonstrated the ability of CRF to stimulate the release of another pro-inflammatory cytokine, namely TNF-α, by cultured rat microglia (Wang *et al.*, 2003). The release of pro-inflammatory cytokines in the $MS_{2\text{-}15}$ that is observed both in the amygdala and PFC (in this case is TNF) can indeed influence the behavioural profile observed in these animals. Several studies have already implicated those cytokines in "sickness behaviour", which is described as changes in the motivational state of an individual (Aubert, 1999; Dantzer, 2001), including decreased social interaction (Spadaro & Dunn, 1990) and exploratory activity (Avitsur & Yirmiya, 1999). Moreover, the overlap between those symptoms and the present observation of decreased exploration in the OF and increased passive attitude in a learned helplessness paradigm such as the FST is difficult to dissociate and possibly highlights a specific developmental window to this *imprinting* effect. To further strength this link, it would be valuable to study, these experimental groups under conditions of stress, to

observe if the neuroendocrine and behavioural alterations described in the MS groups would, or not, accentuate.

Our results, showing a pro-inflammatory profile in $MS_{2\text{-}15}$ animals, that is not displayed by Cont or $MS_{7\text{-}20}$ animals is of notice as the increase in the expression of these molecules has been implicated in altered neurotransmission (Leonard, 2005; Muller & Schwarz, 2007; O'Connor *et al.*, 2008). More specifically, there is evidence that increased levels of pro-inflammatory cytokines trigger an increase in serotonin turnover and tryptophan degradation leading to the generation of toxic compounds such as quinolinic acid (NMDA agonist) and kynurenic acid (NMDA antagonist) through the kynurenin pathway (Muller & Schwarz, 2007). Interestingly, we have already evidences that $MS_{2\text{-}15}$ displayed an increased rate of serotonin degradation, at least in the vestibular area at PND21 (Mesquita *et al.*, 2007).

In what regards the expression of cytokines in different brain regions, there is an additional observation that merit to be commented. The significant decrease of IL-1β and TNF-α in the hypothalamus of $MS_{2\text{-}15}$ rats. If one takes into consideration the remarkable decrease in the expression of synapsin-1 (which is a reliable marker of synaptogenesis (Moore & Bernstein, 1989)) in the same brain region, it is reasonable to admit that these molecules might be working as trophic factors (or better in this case, as the lack of its trophic effects).

Another relevant observation of the present study is the impact of $MS_{2\text{-}15}$ in the immune profile at the periphery. The regulation of thymocyte growth and differentiation is a highly regulated mechanism that involves the action of several hormonal stimuli. Thymic-derived lymphocytes are selected based on the ability of their receptors (TCRs) to associate with major histocompatibility complex (MHC). Given that very high and very low affinity of TCRs for self antigen/MHC are potentially harmful for the organism, since high affinity can lead to autoimmunity and low affinity to incompetent function, only cells with intermediate avidity will survive. Interestingly, corticosteroids are the key molecules for apoptosis induction (Gonzalo *et al.*, 1993) of these ineffective thymocytes. However, most of the differentiation process occurs at birth/early life period coincident with low Cort levels produced by the adrenals. In order to overcome this situation it is now known that the thymus is also able to produce glucocorticoids (Vacchio *et al.*, 1994), as well as CRF (Aird *et al.*, 1993; Ekman *et al.*, 1993; Muglia *et al.*, 1994) and ACTH

(Batanero *et al.*, 1992). According to our results, the decreased levels of mature thymocytes observed in the spleen in $MS_{2\text{-}15}$ animals is probably a consequence of hypersecretion of corticosterone occurred during the first week after life, a period consistent with the higher rate of thymocytes selection (Vacchio *et al.*, 1994). In contrast, the stress initiated only from PND7 did not influence thymic-cells development, probably because this differentiation period was already finished. As a consequence of this altered cellular profile, there might be a compromise of the normal immune function leading to increase susceptibility to infectious or allergic diseases already suggested in some studies (Wright, 2007). Interestingly, and in further support of this assumption, $MS_{2\text{-}15}$ animals also show increased number of cells expressing CD134, a marker of T cell activation.

In conclusion, MS triggers a sustained overactivation of the HPA axis that is paralleled by cognitive impairments and an anxious phenotype. However, the consequence on locomotion, exploratory behaviour and learned helplessness seem to occur only if the exposure to stress occurs in the first week of life. Similarly, the impact on neuronal activity and in several immunological parameters seems to be striker if the MS occurs early in life, which might be explained by a disruptive effect on developmental processes that are taking place in the first week of life. Importantly, a better knowledge of the programming effects of stress during early life is of great relevance to appreciate different susceptibility to neuropsychiatric and immune disorders later in life.

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Figures

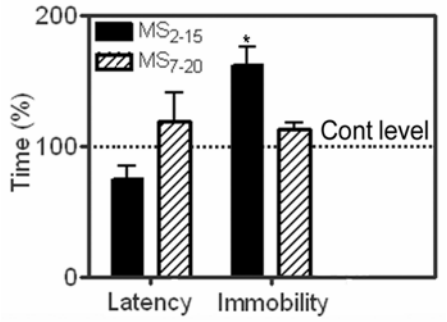

Fig. 1 – Results from the Forced Swimming test. Depressive-like behaviour was assessed using the latency and immobility times expressed as percentage of control levels (dotted line). * represents a significant increase in the immobility time of $MS_{2\text{-}15}$ animals compared to controls ($p < 0,05$).

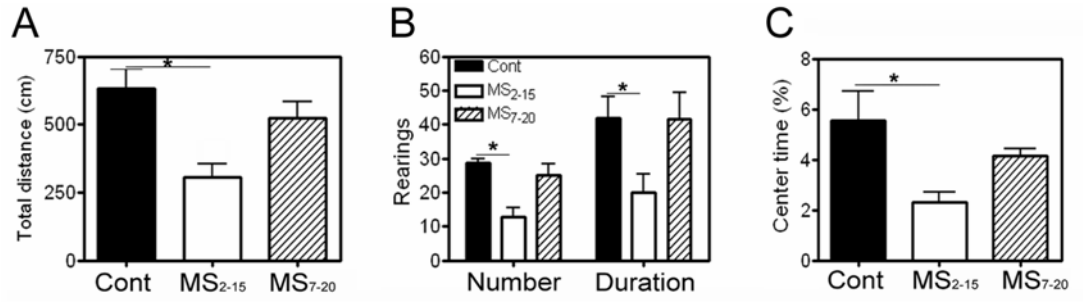

Fig. 2 - Locomotor and exploratory activity assessed in the Open-Field. For the total distance travelled in the arena (**A**) as well as for the number and duration (seconds) of vertical exploratory activity (rearings) (**B**); results are expressed as mean ± SEM. The time spent in the center of the arena (**C**) is expressed as percentage of total time. * $p < 0,05$.

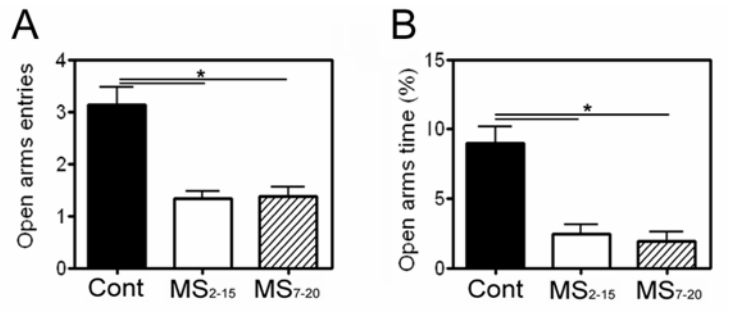

Fig. 3 - Anxious-like behaviour measure in the Elevated Plus Maze. A significant decrease in the number of entries made in the open arms (**A**) as well as in the percentage of time spent in the open arms over the total time (**B**). Results are expressed as mean ± SEM * $p < 0,05$.

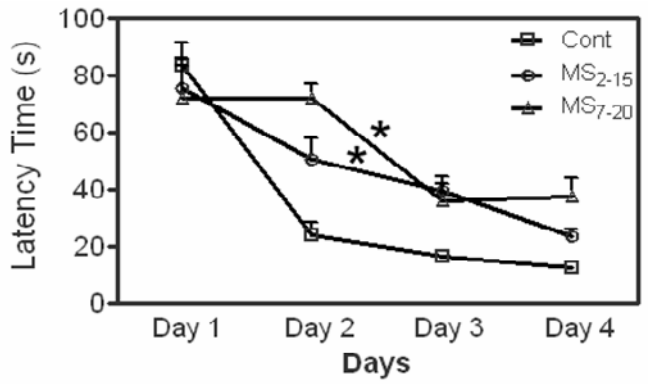

Fig. 4 - Spatial memory assessment in the classical Morris water maze. Latency time swam (seconds) was recorded for the four trials in each day. Results are expressed as mean of the four trials ± SEM. * $p <$ 0,05.

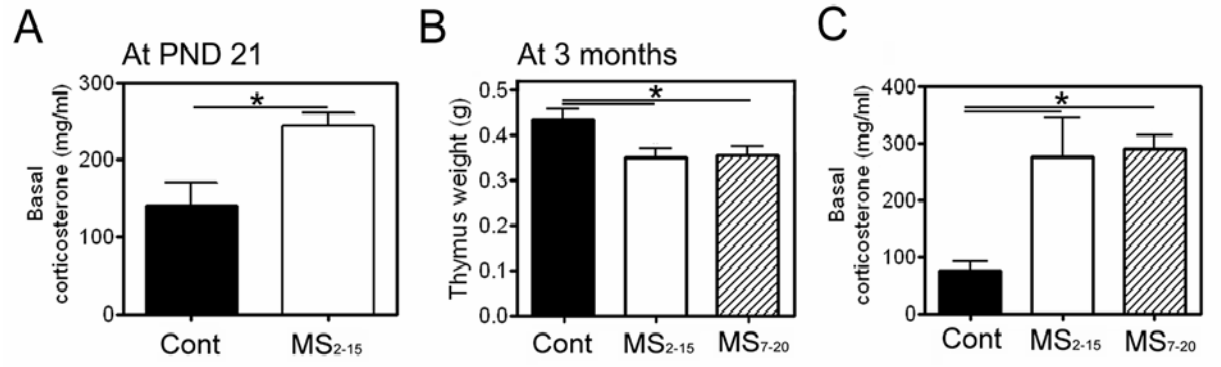

Fig. 5 - Biometric parameters. Corticosterone measurements by radioimmunoassay in Cont and $MS_{2\text{-}15}$ animals, at weaning (**A**). Thymus weight (in grams) of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$. (**B**) Corticosterone measurements by radioimmunoassay in adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals (). Results are expressed as mean ± SEM. * $p <$ 0,05.

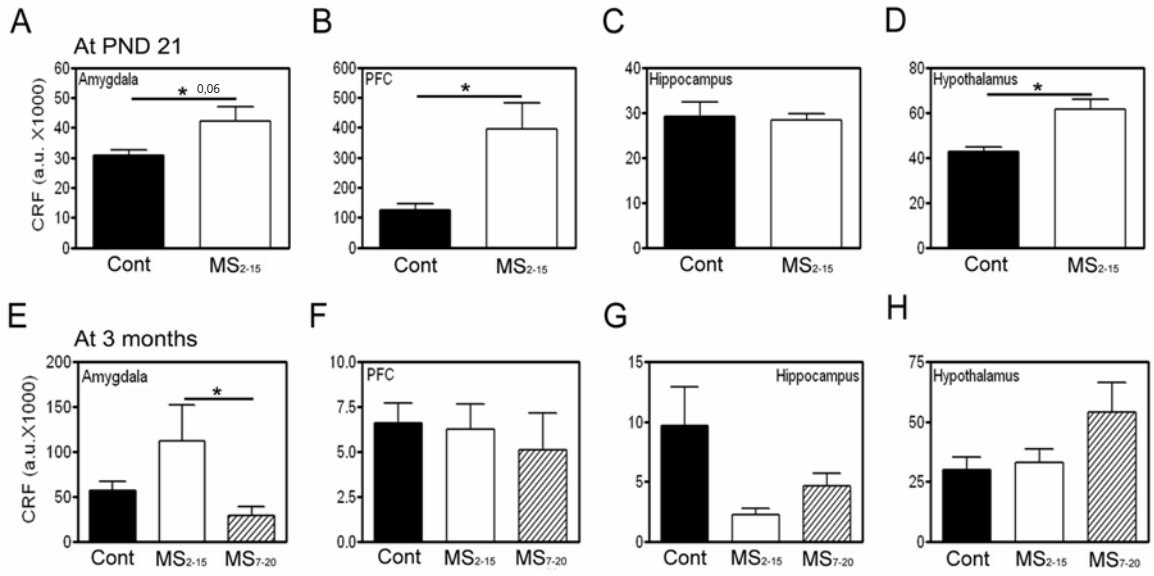

Fig. 6 - Real-time PCR analysis of CRF in the extended amygdala (**A** and **E**), pre-frontal cortex (**B** and **F**), hippocampus (**C** and **G**) and hypothalamus (**D** and **H**) of Cont and MS animals. Upper panel represents measurements performed at post-natal day 21 in Cont and $MS_{2\text{-}15}$ (**A**-**D**), while **F**-**H** are measurements performed in adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$. CRF expression is normalized for HPRT (housekeeping gene) and are expressed as mean ± SEM. * $p < 0{,}05$.

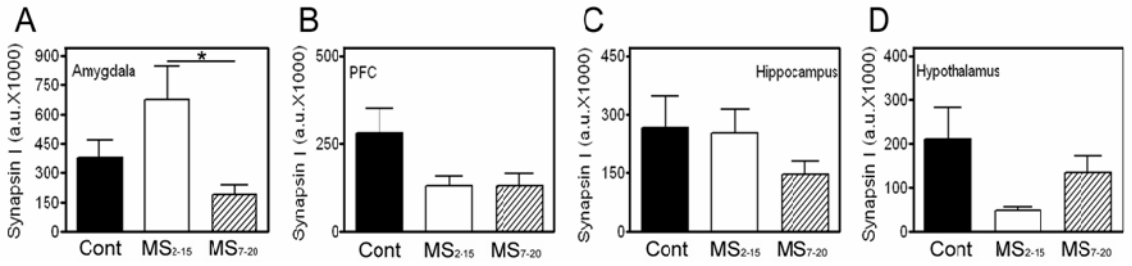

Fig. 7 - Real-time PCR analysis of Synapsin I in the extended amygdala (**A**), pre-frontal cortex (**B**), hippocampus (**C**) and hypothalamus (**D**) of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Synapsin-I expression is normalized for HPRT (housekeeping gene). Results are expressed as mean ± SEM. * $p < 0{,}05$.

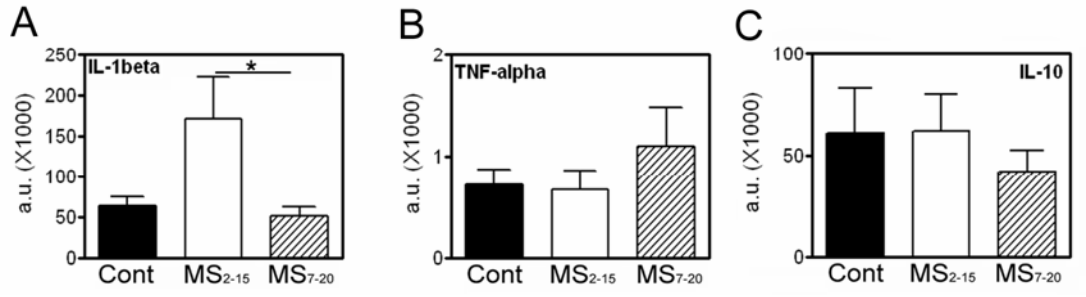

Fig. 8 - Real-time PCR analysis of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in the extended amygdala of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Gene expression was normalized for HPRT. Results are expressed as mean ± SEM. * $p < 0{,}05$.

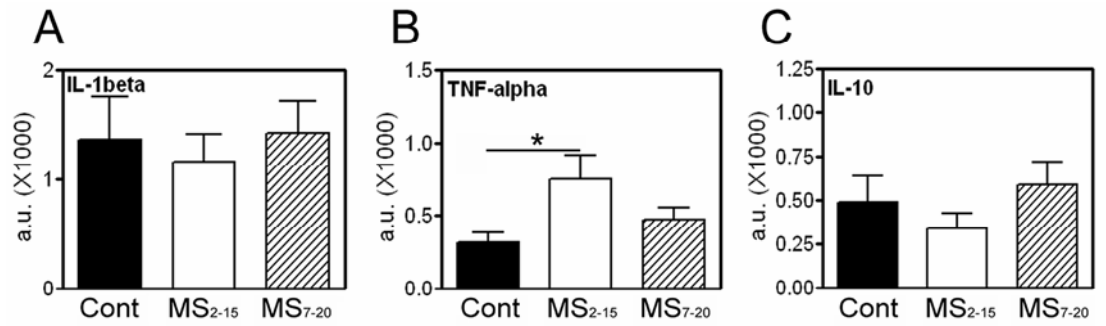

Fig. 9 - Real-time PCR analysis of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in the pre-frontal cortex of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Gene expression was normalized for HPRT. Results are expressed as mean ± SEM. * $p < 0{,}05$.

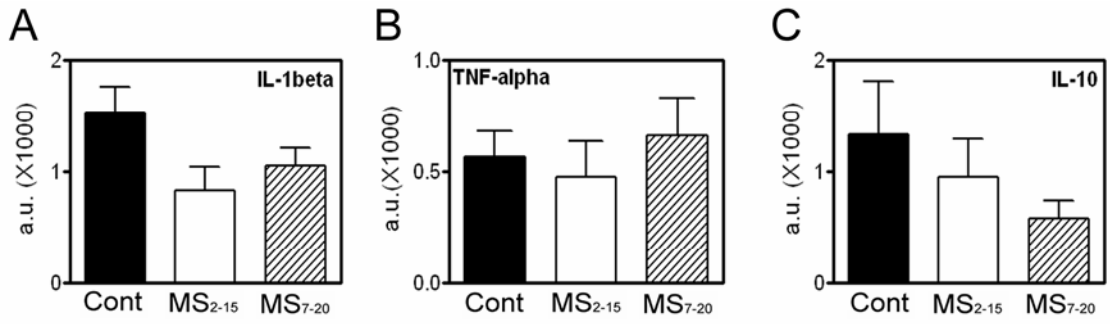

Fig. 10 - Real-time PCR analysis of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in the hippocampus of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Gene expression was normalized for HPRT. Results are expressed as mean ± SEM. * $p < 0{,}05$.

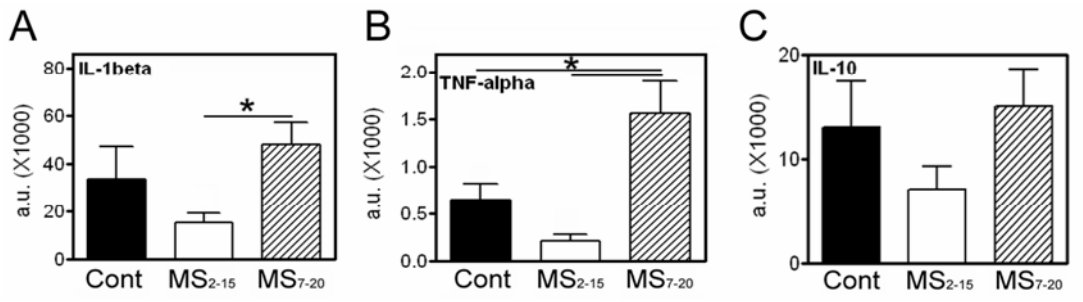

Fig. 11 - Real-time PCR analysis of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in the hypothalamus of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Gene expression was normalized for HPRT. Results are expressed as mean ± SEM. * $p < 0{,}05$.

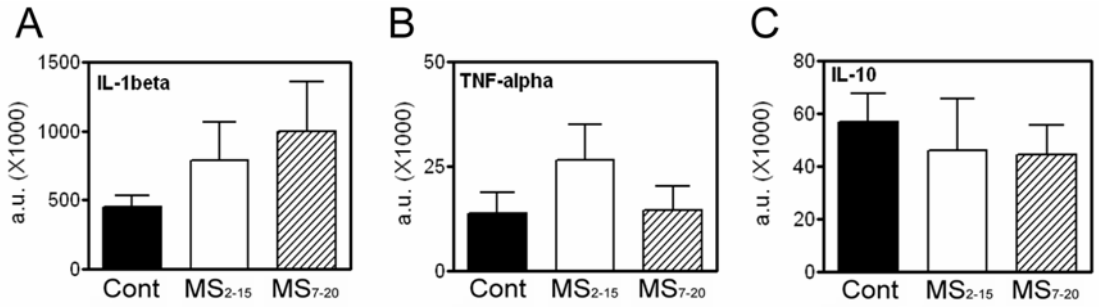

Fig. 12 - Real-time PCR analysis of IL-1β (**A**), TNF-α (**B**) and IL-10 (**C**) in the spleen of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Gene expression was normalized for HPRT. Results are expressed as mean ± SEM. * $p < 0{,}05$.

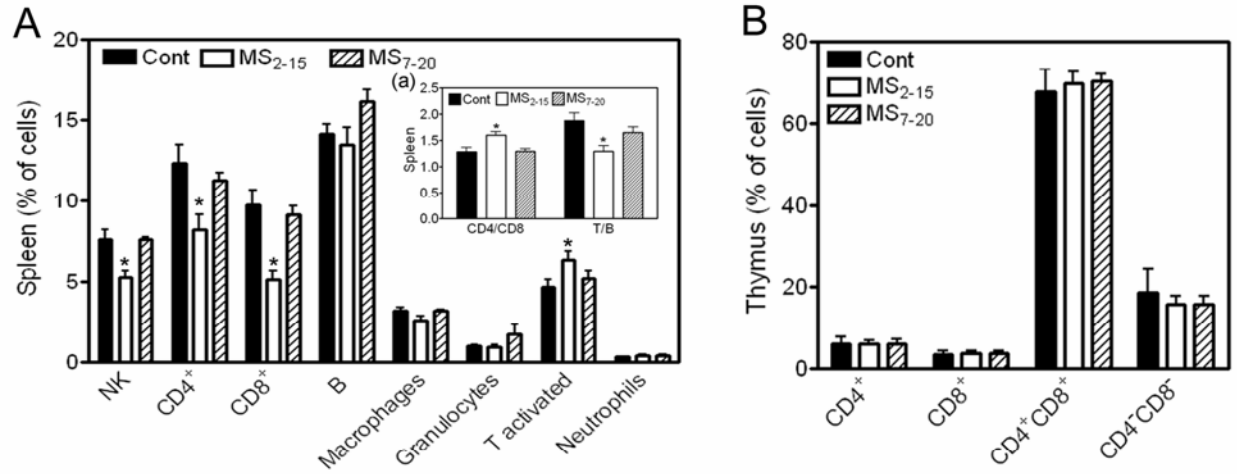

Fig. 13 - Flow cytometry analysis of the spleen (**A** and **(a)**) and the thymus (**B**) of adult Cont, $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ animals. Results are expressed as percentage of cells represented as mean ± SEM. * $p < 0{,}05$.

Chapter 4

Mesquita AR, Correia-Neves M., Roque S, Castro AG,. Vieira P, Pedrosa J, Palha JA. & Sousa N.

IL-10 modulates depressive-like behavior

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IL-10 modulates depressive-like behavior

Ana Raquel Mesquita [a], Margarida Correia-Neves [a], Susana Roque [a], António Gil Castro [a,b], Paulo Vieira [b,1], Jorge Pedrosa [a], Joana Almeida Palha [a], Nuno Sousa [a,*]

[a] *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[b] *Instituto Gulbenkian de Ciência, 2781 Oeiras, Portugal*

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Abstract

The role of pro-inflammatory cytokines in psychiatric disorders has been the focus of great research attention in recent years. Paradoxically, the same is not true for anti-inflammatory cytokines. In the present study, we assessed the behavioral profile of animals with altered expression of the anti-inflammatory cytokine IL-10.

We performed a battery of tests to assess anxiety, depressive-like and cognitive behaviors in mice overexpressing IL-10 (PMT10) and IL-$10^{-/-}$ animals; in the later mice we also tested the behavioral effect of IL-10 administration.

In the forced-swimming test, IL-$10^{-/-}$ females displayed increased depressive-like behavior; importantly, this phenotype was reverted by the injection of IL-10. Moreover, mice overexpressing IL-10 presented a decreased depressive-like behavior. Despite the presence of a similar trend, male animals did not reach significant differences in depressive-like behavior. Assessment in the open-field showed that the absence of IL-10 decreased the percentage of time spent in the center of the arena in both male and female mice, while male animals overexpressing IL-10 revealed an opposite behavior. For both sexes, imbalance in IL-10 levels did not affect spatial reference memory.

In conclusion, variations in IL-10 expression are associated with an altered depressive-like behavior, but do not influence cognitive performance. Interestingly, IL-10 imbalance produced more profound behavioral changes in females than in male animals. This is in accordance with clinical data demonstrating an increased susceptibility of women to mood disorders, suggesting an interplay between anti-inflammatory cytokines and sexual steroids.
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Keywords: Anti-inflammatory cytokines; Depression; Anxiety; Cognition; Sexual steroids

1. Introduction

The cross-talk between the central nervous system (CNS) and the peripheral immune system has been a subject of great research interest in recent years. Since the discovery of cytokine receptors in both glial cells and neurons (Araujo et al., 1989; Breder et al., 1988; Cunningham and De Souza, 1993; McGeer and McGeer, 1995). The influence of cytokines in brain function, resulting either from signaling by cytokines produced peripherally or within the CNS (mainly by microglia and astrocytes) (Araujo et al., 1989; Breder et al., 1988; Cunningham and De Souza, 1993; McGeer and McGeer, 1995), has been characterized. Several studies have highlighted the role of cytokines in neuropathogenesis, particularly in mood disorders (Papanicolaou et al., 1998; Schiepers et al., 2005; Yirmiya et al., 2000). Indeed, one of the most consistent observations in the field is the increased levels of pro-inflammatory cytokines in depression (Lanquillon et al., 2000; Maes et al., 1995b; Mikova et al., 2001; Sluzewska et al., 1995; Tuglu et al., 2003).

In an attempt to integrate the above described findings, it has been proposed that the release of pro-inflammatory cytokines (mostly IL-1β, IL-6, IFN-γ, TNF-α) is a major

* Corresponding author. Tel.: +351 253604806; fax: +351 253604809.
E-mail address: njcsousa@ecsaude.uminho.pt (N. Sousa).
[1] Present address: Institut National de la Santé et de la Recherche Médicale (INSERM) U 668, and Unité du Développement des Lymphocytes, Institut Pasteur, Paris, France.

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determinant for the behavioral, neuroendocrine and neurochemistry alterations associated with depressive disorders (Maes et al., 1993). Whether these changes in cytokine expression are the cause or consequence of depression is still a matter of dispute, but the demonstration that the immune dysregulation precedes the development of depression (Yirmiya et al., 2000) is of notice. In support of the role of cytokines in mood disorders are the observations of early depressive symptoms in patients receiving interferon and IL-2 therapy (Capuron et al., 2000); interestingly, subsequent studies have also shown that these depressive symptoms can be relieved by the administration of antidepressants (Musselman et al., 2001; Yirmiya et al., 2001). The role of cytokines in mood disorders is further strengthened by the demonstration that pro-inflammatory cytokines are able to activate the hypothalamus–pituitary–adrenal (HPA) axis (Crane et al., 2003). The overactivation of the HPA is one of the most recognized findings in mood disorders and considered to be an important trigger of these psychopathologies (Carroll et al., 1968; Plotsky et al., 1998). Moreover, increases in both IL-1β (Linthorst et al., 1995; Merali et al., 1997; Shintani et al., 1993; Song et al., 1999) and TNF (Brebner et al., 2000; Hayley et al., 1999) have been associated with alterations at the neurochemical level, predominantly in the serotoninergic system (Dunn et al., 1999), in several brain regions known to be implicated in depression. As a consequence, animals exposed to increase levels of pro-inflammatory cytokines display signs of depressive-like behavior (De La Garza, 2005; Dunn et al., 2005) and increased anxiety (Silverman et al., 2007).

While several reports implicate pro-inflammatory cytokines in behavior, less is known on the influence of anti-inflammatory cytokines. Among the few studies on the subject, administration of IL-10 prior to LPS injection has been shown to revert the behavioral effects of LPS injection (Bluthe et al., 1999), including the effects upon mobility, rearing activity and social exploration and interaction (Leon et al., 1999; Nava et al., 1997; Smith et al., 1999). Several authors have hypothesized that the behavioral effects of IL-10 are a consequence of an inhibitory effect on IL-1, INF-γ and TNF production and not a direct effect of this anti-inflammatory molecule; in fact, it is known that IL-10 is important on the down-modulation of these pro-inflammatory cytokines (Fiorentino et al., 1991; Harvey et al., 2006; Moore et al., 2001). However, more recently, it was demonstrated that IL-10 administration to animals without exposure to inflammatory challenge induces increased motor activity and abnormal exploratory patterns (Harvey et al., 2006), which indicates that this cytokine might directly influence behavior. In this study, we aimed to further investigate whether manipulation of IL-10 levels could modulate behavior. To achieve this goal, we examined the behavioral profile of IL-10$^{-/-}$ and transgenic mice that overexpress this anti-inflammatory cytokine (PMT10); moreover, to further assess the influence of this cytokine in modulating behavior, we analyzed the effects of IL-10 administration to IL-10$^{-/-}$ mice.

2. Methods and materials

2.1. Animals

The IL-10$^{-/-}$ animals on a Balb/c background were bred in our animals facilities from a breeding pair provided from Dr. A. O'Garra (National Institute for Medical Research, London, UK). For behavioral characterization of these knock-out animals, we used 40 IL-10$^{-/-}$ (23 females and 17 males) on a Balb/c background and 31 wild-type Balb/c (17 females and 14 males) as respective controls. All animals were genotyped by PCR. As some IL-10$^{-/-}$ animals develop spontaneous inflammatory bowel disease, analysis of bowels was carefully performed throughout the experimental period and diarrheic animals were excluded from this study.

PMT10 animals on a C57BL/6 background were produced by Drs. P Vieira and AG Castro. A mouse IL-10 cDNA sequence was cloned in the p169ZT vector (Sousa et al., 2002), which carries the sheep metallothioneinc (MT) Ia promoter (Peterson and Mercer, 1986), a β-globin splice site and the SV40 polyadenylation signal. The resulting vector (pMT-IL10) was injected in C57BL/6 eggs and transgenic founders were identified by PCR using MT-specific primers. The presence of the transgene was confirmed by Southern blot analysis encompassing the sheep MT-promoter. PMT10 mice were bred at our animal facilities. In this experiment, ten PMT10 animals and ten wild-type C57BL/6 as respective controls were used. IL-10 overexpression was induced by giving a 2% sucrose solution with 50 mM of zinc sulfate to animals ad libitum. As the IL-10 promoter is associated with a metalloprotein, the presence of zinc in this solution induces its activation. Wild type animals were also supplied with the same drinking solution.

Serum levels of IL-10, which range between 3 and 5 ng/ml, could be measured 3 days after induction and remained stable while the animals were drinking the zinc solution. IL-10 was never detected in the serum of non-transgenic littermates or in non-induced transgenic mice. IL-10 overexpression was induced 1 week before the behavioral testes were initiated.

All mice were kept in an animal facility in a 12 h light:12 h dark cycle, with food and water available ad libitum. Males and females were kept separately. At 3 months of age all animals were behaviorally tested between 10 a.m. and 5 p.m. in a counterbalanced order; IL-10$^{-/-}$ mice were compared to wild-type Balb/c, whereas PMT10 animals were compared to wild-type C57BL/6 mice. At the end of the experiment, animals were sacrificed by decapitation; decapitation was performed by trained operators. All experimental procedures were conducted in accordance to the European Communities Council Directive, 86/609/EEC.

2.2. IL-10 injection

Another subset of 20 females and 16 males of IL-10$^{-/-}$ mice were used in a supplementary experiment in which

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they were injected with recombinant mouse IL-10 (R&D systems, Minneapolis, USA). Half of these animals received a daily i.p. injection of 40 ng of IL-10 for 11 days (6 days prior the behavioral analysis followed by 5 more days concomitant with the behavioral assessment), while control animals were injected with a saline solution.

2.3. Cytokine measurements

Serum levels of INF-γ were measured by a two-side sandwich ELISA with the anti-IFN-γ specific affinity-purified mAbs (R4-6A2 as capture and biotinylated AN-18 as detecting mAbs), and a standard curve was generated with known amounts of IFN-γ (Peprotech, Rocky Hill, NJ, USA). The sensitivity of the assay was 16 pg/ml.

Quantification of TNF-α was done by ELISA (Duo Set; R&D Systems, Minneapolis, USA). The sensitivity of the assay was 32 pg/ml.

Serum levels of IL-10 were determined by ELISA (Quantikine; R&D Systems, Minneapolis, USA). The sensitivity of the assay was 4 pg/ml.

2.4. Behavioral analysis

2.4.1. Open-field test

Animals were placed in the center of the arena (43.2 × 43.2 cm transparent acrylic walls and white floor) and their position was monitored and recorded by a two 16-beam infrared system (MedAssociates, Vermont, USA), over a period of 5 min. Time in the predefined central and peripheral areas was recorded and used to evaluate anxious-like behavior, while the total distance traveled assessed spontaneous activity. The number and duration of the "*rearings*" (vertical activity) were also recorded as a measure of exploratory behavior.

2.4.2. Elevated-plus maze

Animals were placed in a elevated-plus maze (EPM) apparatus consisting of two opposite open arms (50.8 × 10.2 cm) and two opposite closed arms (50.8 × 10.2 × 40.6 cm) raised 72.4 cm above the floor (ENV-560, MedAssociates, Vermont, USA). The number of entries and the time spent in each arm was registered by an infrared system over a total test period of 5 min.

2.4.3. Forced-swimming test

Animals were placed in a cylinder (diameter: 37 cm; 55 cm of height) filled with water (25 °C) to 35 cm depth such as that they had no solid support for the rear paws nor for the tail. Animals were tested in a 5 min session 24 h after being exposed to the test for the same time period. Only the second session was recorded and later scored by two independent researchers which were blind to the experimental conditions. Time of immobility and latency to immobility were computed and used as a measure of depressive-like behavior.

2.4.4. Morris water maze

This maze consisted of a tank (diameter: 170 cm; depth: 50 cm), divided into quadrants by imaginary lines and filled with opaque water to a depth of 31 cm. During testing, a platform (12 × 12 cm; invisible to the mice) was placed in the same quadrant during five consecutive days. Each test session consisted of four trials which lasted in maximum for 2 min. Time spent swimming to reach the hidden platform was recorded and used to evaluate learning and memory performances.

2.5. Statistical analysis

All the results from the behavioral tests are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago/IL). The effect of gender and IL-10 expression, per se, in the Open Field and the forced swimming tests were analyzed by independent samples *t*-test, while the overall effects were studied by two-way analysis of variance (ANOVA).

Data from the Morris water maze task was analyzed using a repeated measures ANOVA analysis throughout the 5 days test. Each day is a mean of the four consecutive trials. All behavioral results are expressed as means ± SE and statistical significance was considered for p values $\leqslant 0.05$.

3. Results

3.1. Biometric parameters

Analysis of the relative weight of thymus and adrenal glands from IL-$10^{-/-}$ animals compared with wild-type animals showed a significant reduction of the thymus in both male and female mice ($t = 3.9$; $p \leqslant 0.001$ and $t = 2.9$; $p < 0.05$, respectively) (Fig. 1A and B). Two-way

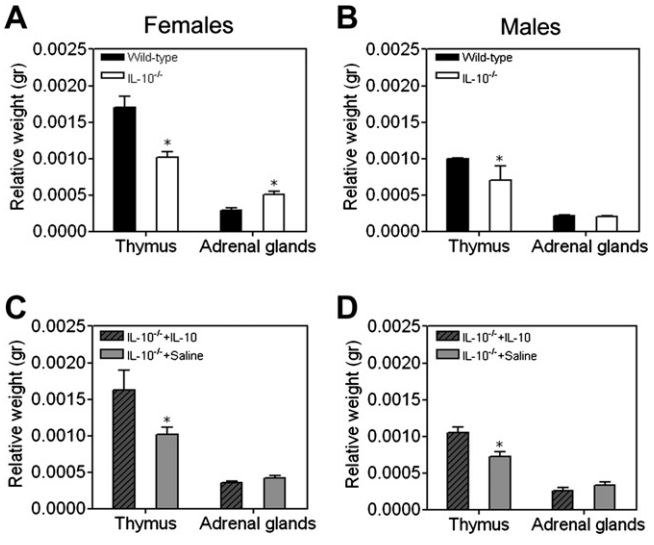

Fig. 1. Thymus and adrenal glands weight relative to the body weight of Wild-type and IL$10^{-/-}$ females and males (A and B) and IL-$10^{-/-}$ receiving IL-10 (IL-$10^{-/-}$ + IL-10) and IL-$10^{-/-}$ receiving saline (IL-$10^{-/-}$ + Saline) animals (C and D). Values are means ± SE and $^{*}p < 0.05$.

ANOVA revealed a gender effect on adrenals weight ($F_{1,27} = 24.1$; $p < 0.001$), with interaction between gender and IL-10 factors. Interestingly, IL-10 administration reverted the atrophy of thymus in IL-$10^{-/-}$ mice ($t = -3.1$; $p < 0.05$ for females and $t = -3.5$; $p < 0.05$ for males) (Fig. 1C and D).

Regarding the adrenals weight, a significant increase was found in females IL-$10^{-/-}$ ($t = -4.5$; $p < 0.001$) (Fig. 1A). Once more, IL-10 administration showed to be effective in restoring the adrenals weight in IL-$10^{-/-}$ females ($t = 2.5$; $p < 0.05$; Fig. 1C). No statistical significance was found in males (Fig. 1B and D). Two-way ANOVA showed a gender effect on both biometric parameters ($F_{1,31} = 13.8$; $p < 0.05$ for thymus and $F_{1,31} = 9.3$; $p < 0.05$ for adrenal glands).

3.2. IL-10 production influences depressive-like behavior in female mice

In the forced-swimming test (FST) we evaluated the ability of mice to cope with a stressful and inescapable situation (learned helplessness). In this test, animals displaying decreased latency to immobility and longer immobilization periods are considered to have increased helplessness, which is a sign of depressive-like behavior. When tested in the FST, IL-$10^{-/-}$ female showed decreased activity ($t = 6.1$, $p < 0.001$) during the 5 min of the test and stopped swimming earlier than wild-type mice ($t = 3.3$ $p < 0.005$ (Fig. 2A). No significant differences were observed between male animals (Fig. 2B). Two-way ANOVA revealed a significant effect of gender, being the females more affected than males in latency to immobility ($F_{1,67} = 14.0$, $p \leqslant 0.001$) and activity times ($F_{1,67} = 5.2$, $p \leqslant 0.05$).

Interestingly, IL-10 administration was able to reverse the depressive-like phenotype of IL-$10^{-/-}$ females, in as much as after administration of this cytokine there was an increased activity time namely when compared with animals receiving saline injection ($t = -2.9$, $p < 0.05$) (Fig. 2C). Although this difference was only observed in females, a similar trend was also found in male mice (Fig. 2D).

Further supporting the anti-depressant action of IL-10, PMT10 female mice showed increased activity time when compared with their respective counterparts ($t = -5.5$; $p \leqslant 0.001$), which reveals a decreased susceptibility to depressive-like behavior (Fig. 2E). Although male animals showed a similar behavioral pattern, no statistical significant differences were observed in any of the parameters analyzed (Fig. 2F). Again, two-way ANOVA showed a significant effect of gender in the activity time ($F_{1,16} = 6.5$, $p \leqslant 0.05$), being the females more vulnerable to IL-10 imbalances.

3.3. Variations in IL-10 levels affect anxious-like behavior in the open-field

The open-field test (OF) was performed to assess general locomotor activity and exploratory behavior. In terms of

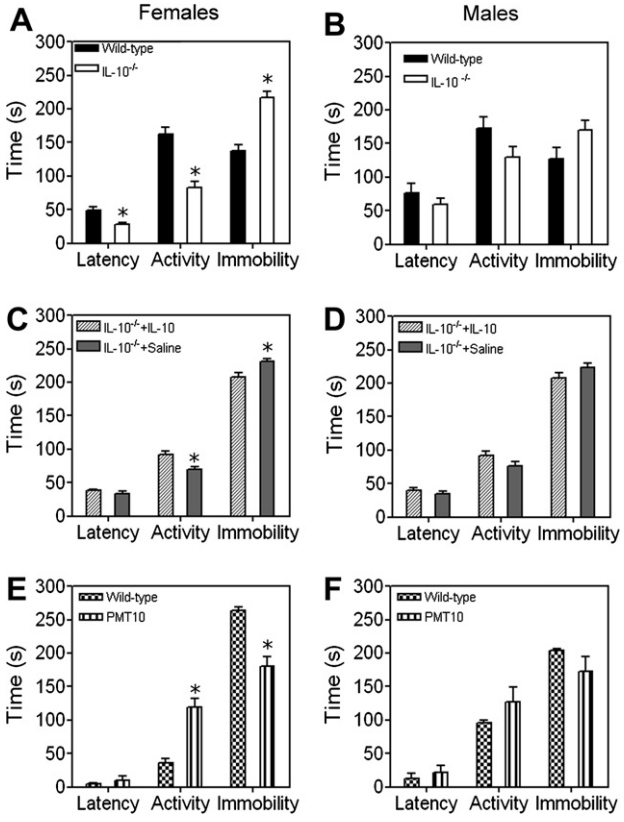

Fig. 2. Depressive-like behavior assessed with the forced-swimming test (FST). IL-$10^{-/-}$ females displayed increased immobility time and decrease latency to immobility compared with their wild-type controls (A). No significant effect was observed in male mice (B). Administration of IL-10 was only able to increase activity time in female IL-$10^{-/-}$ mice, when compared with IL-$10^{-/-}$ mice receiving saline injection (C and D). Female animals overexpressing IL-10 (PMT10) revealed an increase activity time in the FST (E). The same tendency was obtained for male PMT10 animals (F). Values are means ± SE and $^{*}p < 0.05$.

spontaneous activity, assessed by the total distance traveled throughout the 5 min of the test, no differences were observed between IL-$10^{-/-}$ and wild-type animals. Moreover, both IL-10 administration and overexpression failed to induce any change in this behavior both in males and females.

Analysis of the exploratory behavior in terms of animals' vertical activity (number and duration of rearings) revealed a significant increase in both measurements in IL-$10^{-/-}$ female mice compared to their wild-type counterparts (Fig. 3A). No significant differences were observed in male animals, although there was a trend for increased exploratory behavior in IL-$10^{-/-}$ animals (Fig. 3B). Both the administration and the overexpression of IL-10 failed to show any effect in vertical activity (Fig. 3C, D and E, F), despite a tendency for reduction after IL-10 administration in male IL-$10^{-/-}$ mice.

Although the OF is not the most widely used test to assess anxious-like behavior, the percentage of time spent in center of the arena over the total time provides an indicative measure of anxiety-behavior (Boguszewski and Zagrodzka, 2002; Ramos et al., 1997; Simen et al., 2006).

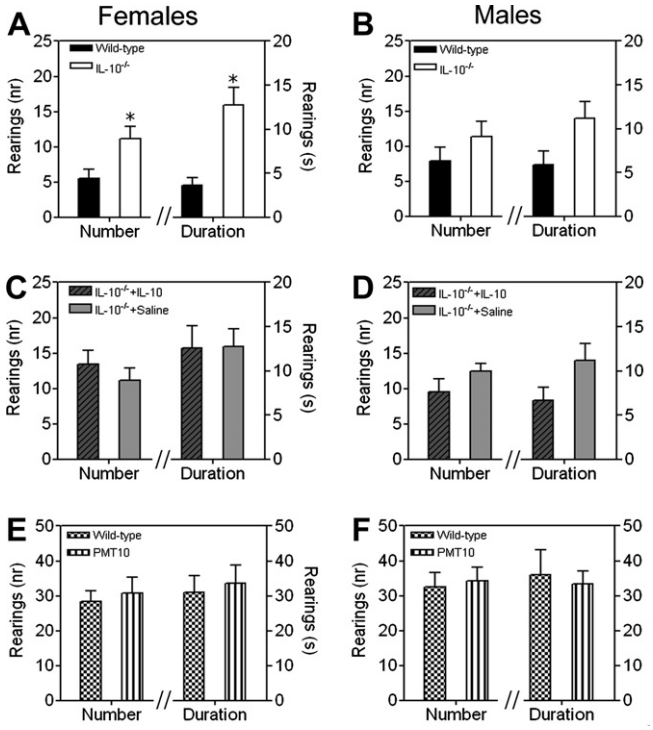

Fig. 3. Vertical activity assessed by the number and duration of rearings in the open-field test (OF), showed increased exploratory behavior in IL-$10^{-/-}$ female but not in males (A and B); IL-10 administration did not change rearing activity neither in male or female mice (C and D). No significant differences in rearing activity were observed between PMT10 and respective control animals (E and F). Values are means ± SE and $^{*}p < 0.05$.

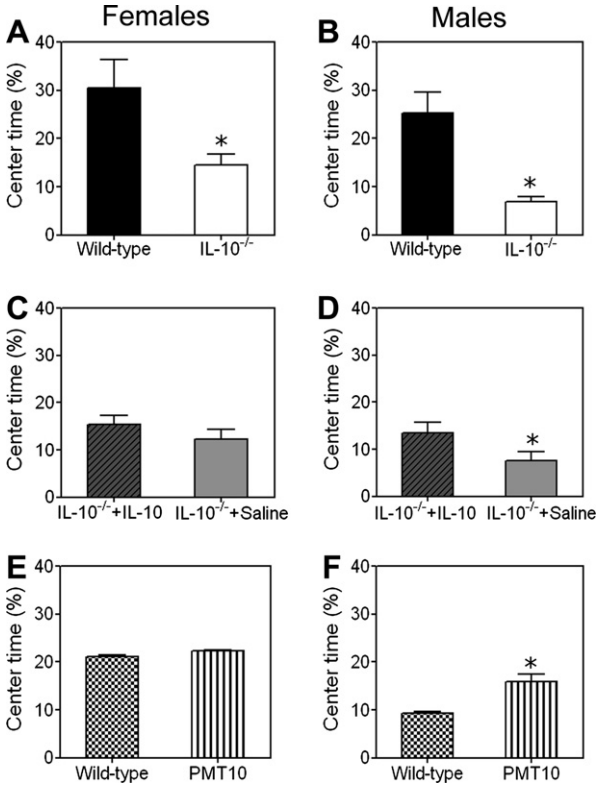

Fig. 4. Anxious-like behavior assessed by the time spent in the center of the OF arena, showed increased anxiety in IL-$10^{-/-}$ (A and B). However, administration of IL-10 only reversed the phenotype in IL-$10^{-/-}$ male (C and D). Male PMT10 spent more time in the center than control animals a sign of decreased anxious-like behavior (E and F). Values are means of the percentage of time spent in the center over the total time ± SE and $^{*}p < 0.05$.

Analysis of this parameter demonstrated that both female and male IL-$10^{-/-}$ mice spent significantly less time in the center than in the periphery when compared to wild-type animals ($t = 2.6$, $p < 0.05$, $t = 4.0$, $p \leqslant 0.001$, respectively) (Fig. 4A and B).

Interestingly, the IL-10 treatment was able to reverse the IL-$10^{-/-}$ mice phenotype, in males, resulting in increased time spent in the center of the OF arena ($t = -2.9$, $p < 0.05$). Two-way ANOVA revealed a gender effect in this parameter ($F_{1,32} = 4.6$, $p < 0.05$) (Fig. 4C and D).

The same trend was observed for PMT10 animals which spent significantly more time in the center of the OF, even though differences were only significant in males ($t = -4.3$, $p \leqslant 0.05$; Fig. 4E and F).

However, care must be taken in the interpretation of these results as no differences were found in the EPM in neither IL-$10^{-/-}$ nor in PMT10 mice when compared with respective control animals (data not shown).

3.4. IL-10 production did not affect hippocampal-dependent spatial memory

To investigate whether changes in IL-10 "milieu" influence hippocampal-dependent learning and memory, the Morris water maze (MWM) test was performed. No differences were found in both male and females regarding the time and the distance swam to find the hidden platform (Fig. 5), thus showing that neither the overexpression nor the absence of IL-10 seem to affect spatial memory.

3.5. Changes in IL-10 expression did not induce detectable changes in TNF-α and IFN-γ levels

In order to investigate whether genetic manipulation of IL-10 expression influences the production of two relevant pro-inflammatory cytokines, the serum levels of TNF-α and INF-γ were measured. No differences were observed between PMT10 and IL-$10^{-/-}$ and their respective wild-type controls as determinations for both cytokines in the serum were below the detection levels (16 pg/ml for TNF-α and 32 pg/ml for INF-γ).

4. Discussion

By studying emotional behavior in mice lacking or over-expressing IL-10, we herein show that this anti-inflammatory cytokine influences depressive-like behavior. Mice lacking IL-10 displayed signs of depressive-like behavior, assessed by immobilization time and latency to immobility, when compared to their strain-matched wild-type counterparts. In contrast, both PMT10 and IL-$10^{-/-}$ receiving this

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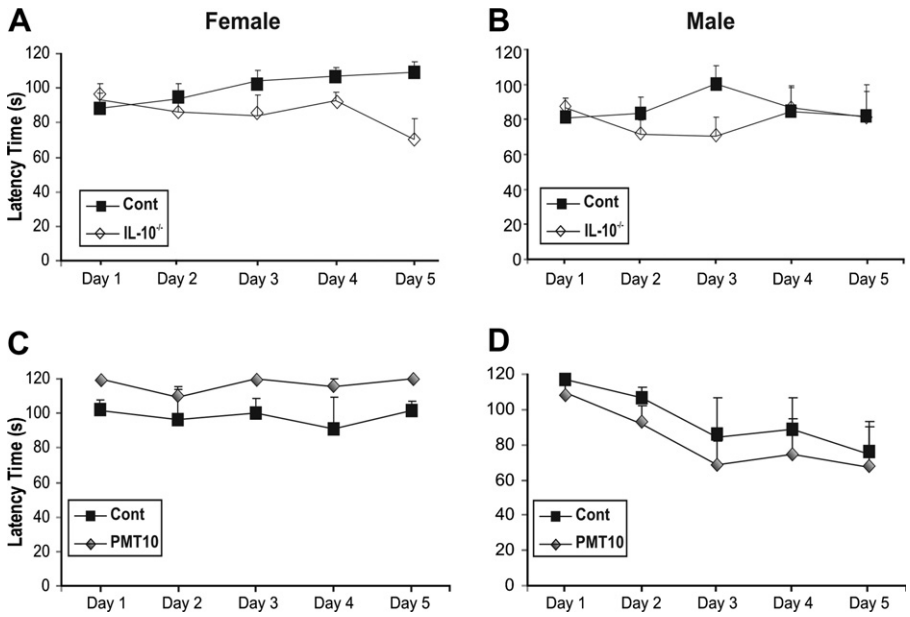

Fig. 5. Spatial memory evaluation in the classical Morris water maze (MWM) paradigm failed to show any significant difference between IL-$10^{-/-}$ and wild-type animals for both female (A) and male (B). The same results were observed for PMT10 animals (C and D). Values are expressed as means of the four trials/day ± SE and $^{*}p < 0.05$.

cytokine displayed an opposite behavioral phenotype. Remarkably, variation in IL-10 levels affected more profoundly females, which correlates with the recognized higher susceptibility of women to depression. Taken together, these results reveal, for the first time, that this anti-inflammatory cytokine is an important mediator in depression.

This observation adds to the so-called "cytokine hypothesis of depression", that was built on the evidence that pro-inflammatory cytokines have a trigger effect on the pathogenesis of depression (Maes et al., 1995a; Maes et al., 1993). This effect was proposed to be mediated by neuroendocrine and neurotransmitter systems involved in vulnerability to affective disorders (Maes, 1999). In this respect it has been demonstrated that IL-1β and TNF-α stimulate the expression/release of corticotrophin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus (Hayley et al., 2001; Tilders and Schmidt, 1998), the control center of the HPA axis and alters the turnover of norepinephrine and serotonin (5-HT) in the hypothalamus, amygdala, prefrontal cortex, and hippocampus (Ando and Dunn, 1999; Brebner et al., 2000; Dunn et al., 1999; Hayley et al., 1999). Further evidence for the role of pro-inflammatory cytokines in depression was gathered from studies with TNF receptors (TNFR) knock-out mice, in which it was shown that both TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice were more active in the FST than wild-type animals (Simen et al., 2006). Importantly, the mechanisms underpinning the behavioral changes in these mice models are similar and include alterations in neurotransmission in regions of the brain implicated in emotional behavior. Of notice, is also the evidence of the immunomodulatory effects of antidepressants that act preferentially in the noradrenergic and serotoninergic system. Kubera and co-workers (Kubera et al., 2001) demonstrated the ability of different drugs to decrease the levels of INF-γ, while increasing the levels of the anti-inflammatory cytokine IL-10. These data were also corroborated by studies using other antidepressants in stimulated human blood cells (Maes et al., 1999).

While the involvement of pro-inflammatory cytokines in many aspects of depressive illness is now indisputable, we clearly demonstrate in this study that anti-inflammatory cytokines also influence emotional behavior in rodents. Work from Bluthe and collaborators (Bluthe et al., 1999) had already suggested that IL-10 administration could abrogate the behavioral effects of LPS injection in sickness behavior in rats. It was postulated that IL-10 inhibits the expression of pro-inflammatory cytokines (IL-1, INF-γ and TNF-α) produced in response to LPS and its behavioral consequences, increasing the duration of social interaction. The present data reveals that variations in IL-10 expression influence mood behavior, even in the absence of detectable variations in the serum levels of INF-γ and TNF-α. This fact, however, does not exclude the possibility of an inhibition of pro-inflammatory cytokines secretion in the CNS; in accordance, IL-10 deficient mice have increased brain levels of TNF-α and IL-6 (Agnello et al., 2000).

Another relevant issue raised from our study is the increased susceptibility to variations in IL-10 expression in females. Of relevance is the fact that the same gender effect is observed in the clinical practice, where the increased susceptibility to depression in women in conditions of decrease estrogen secretion (e.g., premenstrual, during the postpartum period and perimenopausally) is

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well-known (Osterlund et al., 2005). This increased susceptibility has been attributed to fluctuations in estrogen secretion. In fact, evidence derived from experimental and clinical studies demonstrated an important role of (decreased) estrogens in the pathogenesis of depression but also in the production and bioactivity of a variety of cytokines (Nordell et al., 2003; Suuronen et al., 2005). Estrogens depletion increases the levels of pro-inflammatory cytokines (Bismar et al., 1995), and studies using murine microglia cells showed that estrogens are able to increase IL-10 levels (Dimayuga et al., 2005). Accordingly, our results suggest that in animals that cannot express IL-10, the possible protective effects induced by estrogens are lost since IL-$10^{-/-}$ female mice displayed increased signs of depressive-like behavior when compared with male animals. In contrast, the higher levels of IL-10 present in PMT10 animals were probable bolstered by estrogens, potentiating their protective effects and anti-depressive effects in female mice. Moreover, clinical studies showed that estrogens not only trigger antidepressant-like actions (for review, see (Halbreich and Kahn, 2001)) but also improve the therapeutic action of antidepressants (Soares et al., 2001), specially of those acting in the serotoninergic system(Chang and Chang, 1999; Estrada-Camarena et al., 2006; Lu and Bethea, 2002). Besides the influence of estrogens, other sexual steroids, such as testosterone, might also be implicated in the gender difference observed in depressive-like behavior after IL-10 imbalances. In fact some human and animal studies have shown that testosterone increases IL-10 expression (Liva and Voskuhl, 2001; Malkin et al., 2004) and reduces the expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (D'Agostino et al., 1999). Taken together, these data highlight a possible interaction between sexual steroids and cytokines actions.

The association between anxiety and depressive disorders is well-known, and there are several common factors involved in both conditions (Cameron, 2006; Cameron et al., 2004; Gulley and Nemeroff, 1993). Therefore, the exploration of anxiety-like signs in experimental models influencing depressive-like behavior becomes relevant. Interestingly, data from the open-field test suggested that IL-$10^{-/-}$ animals display a hyperanxious phenotype both in males and females. However, this phenotype could not be confirmed in the EPM, a more robust test to assess anxiety-like behavior. A possible explanation for these paradoxical findings could be the increased exploratory behavior evinced by IL-$10^{-/-}$ animals. As the EPM is based on the conflict between the innate exploratory behavior and fear of height and exposed environments, it is likely that the increased tendency for exploration in IL-$10^{-/-}$ might be a confounding effect in this behavioral paradigm and blunt the hyperanxious phenotype. In further support of this view are the findings of decreased anxiety evinced by PMT10 mice. Further studies, using other behavioral tests, are needed to better explore the influence of IL-10 levels in anxiety behavior both in basal and under stressful condition (an approach currently under study in our laboratory).

While the influence of IL-10 on anxiety behavior needs further experimental work, the present data rule out any influence of this anti-inflammatory cytokine in reference memory. In fact, the hippocampus-dependent task used to assess spatial reference memory failed to reveal differences between the performance of both genetically modified mice models and their respective wild-type controls. These findings are of relevance, in the sense that they reveal the specificity of the influence of IL-10 levels in affective/mood conditions.

In conclusion, our behavioral data demonstrate that IL-10, an anti-inflammatory cytokine, is an important molecule in the modulation of depressive-like behavior. This finding calls for a reappraisal of the "cytokine hypothesis of depression", in the sense that imbalances of both pro- or anti-inflammatory cytokines might modulate mood behavior. Furthermore, the present observations might be of relevance in all those conditions (autoimmune, malignant and infectious disease) associated to polymorphisms of the IL-10 family gene clusters in which depression seems to be more prevalent (Nery et al., 2007; van Boxel-Dezaire et al., 1999; Zorzon et al., 2001).

Conflict of interest

There are no financial or other conflicts of interest.

Contributors

Mesquita AR, Correia-Neves M and Sousa N designed the study, wrote the protocol, analyzed the data and wrote the first draft of the manuscript; Mesquita AR performed all behavior experiments and Roque S the ELISA procedures; Pedrosa J and Palha JA discussed the results and provided very important comments for the final version of the manuscript; Castro G and Vieira P produced the PMT10 animals and also provided important comments for interpretation of results. All authors contributed to and have approved the final manuscript.

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Chapter 5

DISCUSSION

The work developed and presented in this dissertation was designed to analyse the programmatic effect of early life events on the aetiology of mood disorders. Both early and long-lasting behavioural and neuroendocrine consequences were described in this study, having into account the impact of stress exposure during specific "temporal windows" of rodent's development. In addition, possible immunological mediators of the behavioural end-phenotypes induced by stress early in life were identified. Together, the data presented herein can give new insights into the aetiology of several disorders in which the bi-directional cross-talk of the nervous, endocrine and immune systems is of relevance. Throughout this short discussion, the most significant clinical implications of our work will be explored, even if only speculative, as the discussion of the results obtained was already explored in the previous Chapters.

5.1 Animal model: developmental/ontogenic considerations

The individual stress response is highly variable and even subjects with similar genetic background (e.g. identical twins) react differently when submitted to identical stressful situations. Besides their immediate consequences, traumatic events during childhood have shown to influence the susceptibility to pathology later in life; this is probably related with the altered profile of responsiveness to adversities, which can have implications in later psychopathology. The crucial importance of the developmental period in the shaping of the functioning of the brain is not novel. In humans, the theory of attachment highlights the role of parental caregiving as a critical factor in shaping personality (Egeland & Farber, 1984; Sroufe *et al.*, 1999). Parental loss or neglect in early developmental periods interferes with the normal development of the child and can indeed programme the organism to adult pathology (Zeanah *et al.*, 2003). Several disorders, affecting different physiological systems have indeed been attributed to unfavourable experiences during perinatal life, ranging from cardiovascular to metabolic diseases (Edwards *et al.*, 1993; Phillips *et al.*, 1998).

In order to mimic an early adverse situation in which the interaction between the progenitor and its offspring is disrupted, we used an animal (rat) model of MS. However, translation of data obtained in rodents to humans is not trivial. It is crucial to understand the different species-specific developmental time periods regarding the maturation of the different systems. The rat is considered an *altricial* specie characterized by very immature offspring, with eyes and ears

closed at birth and virtually no hairs on the body. In contrast, primates such as the Reshus macaques, for instance, are classified as *precocial* species since they usually have a single offspring highly mature at delivery, with eyes and ears open at birth and advanced motoric abilities. The nature of human development further confuses any comparison with experimental models. Regarding the immaturity of motor skills, the human newborn should be classified as *altricial* specie, however the relatively highly developed brain as well as several perceptual systems clearly places the human neural development in a *precocial* category (Verley, 1977; Clancy *et al.*, 2001).

Despite the different levels of maturation at birth, mostly due to the gestation time required by each specie, it is possible to establish homologies between rodents and humans. Of notice is the fact that the developmental periods are measured in a timeline of days in rodents *versus* months/years in humans, meaning that any intervention performed in rats in a short period of time will correspond to longer processes in humans. Regarding our model of MS, and given the fact that the 1st week after birth, in rodents, is usually translated in the last trimester of human gestation and immediate perinatal period, we are conceptually mimicking the disruption of mother-offspring relationship in a stage of functional maturation of the CNS that is comparable to the perinatal period in the human neonate (ranging from late gestation to early neonatal period). Having into account these factors, our MS model is a powerful model to study the *imprinting* effects of stress/corticosteroids and their implications in psychopathology. Indeed, this model triggers a permanent activation of the HPA axis as well as the anxious phenotype typically described in humans with a history of traumatic childhood events (Kendler *et al.*, 1992; Nicolson, 2004).

The vulnerability of the organism to different stimuli is highly dependent on the type, intensity and, importantly, the period in which the exposure occurs. It is clear that experiencing a particular stimulus during the ontogenic maturation of a specific system will increase the impact of that stimulus as it produces an immediate effect but, in addition, triggers programming actions that might have future implications. Having into account these temporal factors, in this dissertation we decided to assess the consequences of the same stressor (MS) in two different "temporal windows". The earlier period of MS, occurring between PND 2-15, comprises the so-called stress hypo-responsive period (SHRP), is the most widely used period of early life stress

models in literature, allowed us to assess the impact of a severe stress exposure in an immature developing brain. Additionally, we also evaluated the impact of a later period of MS that was delayed by 5 days (from PND 7-20) to allow a similar duration/intensity of the stressor without overcoming weaning (PND21); this later period allowed us to assess the impact of stress in a less immature brain and, thus, to understand how vulnerable is the nervous, endocrine and immune system in a different development period.

5.2 The impact of MS on neurodevelopment

Remarkably, when we started this project, there were few studies in the literature evaluating the immediate neurodevelopmental profile in this animal model. Thus, the first aim of this dissertation was to evaluate the acquisition of neurological milestones in animals exposed to MS. Since at PND7 some innate reflexes are already acquired, we assessed them only in $MS_{2\text{-}15}$. The results of our study (Chapter 2) demonstrated that stress during early postnatal life has important neurodevelopmental consequences that can be viewed as predictors of impaired psychobiological development. In fact, MS was shown to accelerate some somatic parameters (such as eye opening) while delaying the acquisition of neurological reflexes. The latter occurs in parallel with deleterious actions of corticosteroids on serotoninergic transmission, both in the vestibular region and dorsal raphe nuclei, highlighting the role of stress/corticosteroids in modulating the development of the central nervous system. These observations can have considerable impact if we consider some human studies in which chronic stress during pregnancy also led to deficits in orientation in neonates (Rieger *et al.*, 2004). Interestingly, and since our model is based on the assumption of elevated levels of corticosteroids in a period of the rodent's life that overlaps the last trimester of human gestation, it is of relevance to compare our data with that of children submitted to antenatal corticotherapy, for prevention of chronic lung disease of prematurity. In this case, children exposed to high levels of corticoids display poorer motor skills and deficits in coordination (Yeh *et al.*, 2004) as well as lower rate of walking and achievement of other developmental milestones (Stott, 1973). All these evidence legitimize our model as a useful tool to explore the mechanisms underlying those deleterious consequences, which might help to develop clinical strategies to intervene in these children.

As we have shown, MS impact on neurodevelopment is associated with an increased serotoninergic turnover in brainstem regions important for the neurological reflexes acquisition;

however, these neurochemical changes are likely to affect the function of other limbic regions such as the hippocampus due to the crucial connections between several brain stem nuclei and the hippocampus (Russell *et al.*, 2003). In fact, in support of the hypothesis that early dysregulation of the vestibular region can compromise hippocampal-dependent tasks later in adulthood, we observed that MS animals display impairments in spatial reference memory in the Morris water maze task later in life (Chapter 3).

5.3 Long-term behavioural implications and principal mediators

Several lines of evidence have proved that early life stress triggers a programming effect in several brain regions that are under maturation. However, it is important to discuss, at this point, which are the mediators of these actions. A first consequence of disrupting maternal-pup interaction was an increased activation of the HPA axis. Indeed, the impact of MS in the control of the HPA axis is preserved throughout life; this effect occurs even without exposure to stressful conditions and seems to be sustained in time (as revealed by the atrophy of the thymus observed in both MS groups). Such state of hypercorticalism is known to influence several behaviours, including anxiety (Pego *et al.*, 2008) and memory (Sousa *et al.*, 2000). In accordance, we confirmed that both MS groups displayed a hyperanxious phenotype as well as impaired performance in the spatial memory task. Whereas anxiety and spatial memory were independent from the period in which MS had occurred, performance in the forced-swimming test and open-field revealed a higher susceptibility of $MS_{2\text{-}15}$ animals for "depressive-like" behaviour and decreased exploratory activity. Quite interestingly, the performance in these behavioural paradigms is highly dependent on motor performance and, thus, it is tempting to speculate that the exposure to stressful conditions in the first week of life, is likely to impact on the development of brain circuits related to motor performance, which further highlights the relevance of our demonstration of delayed acquisition of neuronal milestones in the $MS_{2\text{-}15}$ animals (Mesquita *et al.*, 2007).

More speculative, but not mutually exclusive, is the hypothesis that the differential expression of CRF in the amygdala might be implicated in these distinct performance of MS groups. Indeed, $MS_{2\text{-}15}$ animals display increased CRF levels in the extended amygdala when compared to $MS_{7\text{-}20}$. Moreover, the pattern of changes observed in the levels of this neurotransmitter in the amygdala in MS groups is paralleled by similar changes in the expression of synapsin I, a synaptic protein

involved in vesicle trafficking/docking in the synapse, thus suggesting that there is an altered neuronal activity in this brain region as a result of the different temporal exposure to MS. Importantly, some studies correlate the increased activation of the amygdala with alterations in emotional behavior. Indeed, functional neuroimaging studies in human show that hyperactivity of the amygdala is positively correlated with the severity of depression (Dannlowski *et al.*, 2007) as well as with the increase vulnerability to the disease in the offspring of depressed parents (Monk *et al.*, 2008).

5.4 Immunological correlates

In parallel with these actions on behaviour and endocrine control, we have also found a remarkable effect of stress exposure during the perinatal period upon specific immune cell populations. While this observation has been described by others (Lubach *et al.*, 1995), we have shown, for the first time, that there is a specific period of vulnerability to this effect. In fact, the consequences of the same stressor, with the same intensity, but applied five days later in life failed to produced any effect in immune cells (Chapter 3). This distinct response illustrates that the impact of stress is diverse when it occurs at different stages of maturation/differentiation of the immune system. In fact, the reduction in the number of splenic T cells can be viewed as a consequence of altered maturation of these cells during thymic T-cell selection that is coincident with the period of stress exposure.

Irrespective of the underlying mechanism, the altered cellular profile observed in the early stressed animals is likely to affect the normal immune response, leading to increased susceptibility to infectious or allergic diseases. Interestingly, in this particular topic, data derived from clinical studies has provided some of the most relevant evidence to support this hypothesis (Wright, 2007). Asthma and other allergic diseases, for instance, have been associated with deficient caregiving early in life (Wright *et al.*, 2004). Although these disorders are not directly associated with a reduction in the number of cells, it has been shown that CS are able to influence the T cells response toward a TH2 phenotype (Wiley *et al.*, 2004).

The impact of an altered immune response and psychopathology has received the attention of several researchers (Coe & Laudenslager, 2007; Irwin, 2008). One of the most consistent neuro-immune links is the observation of increased levels of pro-inflammatory cytokines in the serum of

depressed patients. Even though we could not find consistent differences in the serum levels of pro-inflammatory cytokines in our animal model, there was enhanced expression of these cytokines in regions known to be implicated in depressive behaviours such as the PFC (Dunkin *et al.*, 2000) and amygdala (Monk *et al.*, 2008). Interestingly, the increased levels of IL-1β and TNF-α were only present in animals which displayed a depressive-like phenotype in the forced-swimming test. Such evidence, together with other reports showing that functional allelic variants of the genes for IL-1β and TNF-α increase the risk for depression and are associated with reduced responsiveness to antidepressant therapy (Jun *et al.*, 2003; Yu *et al.*, 2003) engage cytokines as important biomarkers of depression.

5.5 The role of cytokines

Recent studies suggest the mechanisms by which cytokines can interfere with mood (Muller & Schwarz, 2007). In fact, these pro-inflamatory cytokines seem to increase serotonin turnover (a fact also confirmed by us) and tryptophan degradation leading to the generation of toxic compounds such as quinolinic acid (NMDA agonist) and kynurenic acid (NMDA antagonist) through the kynurenin pathway (Muller & Schwarz, 2007). Interestingly, we already have evidence that $MS_{2\text{-}15}$ display an increased rate of serotonin degradation, at least in the vestibular area at PND21 (Chapter2) as well as decreased levels of 5-HT in the PFC in adult $MS_{2\text{-}15}$ animals (previous results from our laboratory).

If, indeed, cytokines play a role in the etiology of psychiatric disorders, such as depression, it becomes of relevance to evaluate this mediation through multiple perspectives. Although much has being described regarding the influence of pro-inflammatory cytokines in the CNS as modulators of specific behaviours, the contribution of anti-inflammatory molecules has been under-appreciated. This fact is rather surprising, as it is known that these “limbs” (pro- and anti-inflammatory) of the immune system are not completely independent and might influence, or even antagonize, each other. Therefore, we thought of interest, to explore the role of one of the most well described anti-inflammatory cytokines, IL-10, for which neuronal and neuroendocrine cells in rodent and human CNS display receptors (Gallo *et al.*, 1994; Hughes *et al.*, 1994; Rady *et al.*, 1995).

Using transgenic mice models for IL-10, our findings in Chapter 4 call for a re-appraisal of the ‘‘cytokine hypothesis of depression” (Smith, 1991), as it was demonstrated that pro-inflammatory cytokines are not the only ones implicated in psychopathology. Interestingly, the influence of IL-10 in human disorders has been already explored in the context of autoimmune and inflammatory diseases (as there are important associations with polymorphisms in the IL-10 family gene clusters) (van Boxel-Dezaire *et al.*, 1999). Of notice, there is also a high prevalence of depression in patients suffering from autoimmune and inflammatory disorders (Pucak *et al.*, 2007). Based on our observations, we speculate a possible role of IL-10 on the development of depressive phenotype observed in disorders such as multiple sclerosis: in one hand, it is known the high prevalence of depression in these patients; while on the other, it was shown the ability of INF-β, a widely use treatment in this disease, to increase IL-10 levels (Rep *et al.*, 1999), in parallel with an improvement in mood.

Another relevant fact was the gender effect observed. There is clinical evidence showing that women have a stronger immune response than men, which not only leads to a higher capacity to suppress inflammation, but also to a high prevalence of autoimmune disorders (Ansar Ahmed *et al.*, 1985). Moreover, in cases of estrogens increment (as it occurs during pregnancy) a clinical remission in the course of disorders such as multiple sclerosis and rheumatoid arthritis are observed, followed by an exacerbation of the symptoms in the post-partum period (Ostensen & Husby, 1983; Confavreux *et al.*, 1998). The fluctuation in estrogens levels also influence mood in women; indeed, hypo-estrogenic states are associated with vulnerability to depression (Osterlund *et al.*, 2005). These evidence suggest that estrogens can also mediate the link between mood and immunological conditions. In accordance, our data in transgenic mice models clearly showed a gender specific impact of IL-10 variations in the depressive-like behaviour; indeed, females were more susceptible to the absence and overexpression of this anti-inflammatory cytokine than males, suggesting a possible interaction between estrogens and IL-10.

Another line of evidence seems to support this hypothesis. Combined treatment (of T cell receptor (TCR) protein and estrogens) for the experimental model of multiple sclerosis (EAE) has shown to improve the course of the disease with a concomitant increase in IL-10 levels (Offner *et al.*, 2000). Accordingly, our results suggest that in animals that do not express IL-10, the possible protective effects induced by estrogens are lost since IL-10$^{-/-}$ female mice displayed increased

signs of depressive-like behavior when compared with male animals. In contrast, the higher levels of IL-10 present in PMT10 animals were probably reinforced by estrogens, bolstering their anti-depressive effects in female mice.

5.6 Epigenetic mechanisms

Despite all the evidence referred before, the mechanisms through which early life experiences can lead to adult psychopathology are still not understood. However, several studies have proposed changes in gene expression as one of the molecular mechanisms underlying the persistent behavioural changes observed in psychiatric disorders. Interestingly, disorders such as depression usually require chronic treatment to have a therapeutical effect. Changes in the gene control, without altering the germinative line, are possibly contributing to the maladaptations in specific structures of the brain. Most of the recent studies in the field have highlighted the role of specific modifications in chromatin structure, that can mediate the up or down-regulation of gene expression. This notion of altered regulation of the expression of specific genes without changes in the DNA structure gave rise to the concept of *epigenetics*. In fact, chromatin, which includes DNA, histones and other non-histone proteins within the cell nucleus can undergo several enzymatic changes that inhibit or facilitate gene transcription, according to its structure. In a simplistic way, chromatin can exist in a condensed state meaning that it is inactive or in an open state which translates activity. The structure of the chromatin is dependent on enzymatic reactions that occur in specific histones surrounding the DNA structure. Usually acetylation is associated with active chromatin and consequent high gene expression, while methylation, for instance, usually leads to inactive chromatin or to repression of specific genes.

Animal studies have already demonstrated that maternal behaviour is able to induce stable alterations in the epigenome leading to long-lasting consequences in adult animals (Weaver *et al.*, 2004). In referred study, it was shown an epigenetic modification in the GR gene, crucial in the HPA axis activity that is also mediated by maternal-pup interaction. In a series of elegant studies, this group has shown that the methylation status of the promoter of the GR exon 17 changes in response to the nature of maternal care (high versus low licking/grooming mothers). The authors propose that these epigenetic modifications of the GR gene are the underlying mechanism for the effect of maternal behavior on offspring GR expression in the hippocampus, HPA axis activity, and subsequently behaviour (Weaver *et al.*, 2004).

In the immune system, epigenetic modifications also have a critical role, for instance, in the T cell fate for Th1 or Th2 differentiation (Sanders, 2006). Specifically in the context of programming effects, others have described that LPS can directly bind to histones and regulate acetylation thus providing a mechanism for LPS to affect directly the epigenome (Musikacharoen *et al.*, 2003).

In our animal models it is also possible that MS, as an early stressful event, would lead to epigenomic programming. Besides the effects on GR in selected brain regions that have been already demonstrated by others, we also speculate about the role of early life environment in the immune function. In fact, having into account the data presented herein, it is possible that the *imprinting* effects of MS were also programming the immune system that can then, in parallel with epigenetic modifications in neural substrates, contribute to emotional disabilities later on as well as to increase vulnerability to infections or even allergies. Further studies are required to confirm this hypothesis

5.7 Conclusions

The present work has demonstrated that:

- MS impacts on neurodevelopment, disrupting serotoninergic neurotransmission in critical brainstem regions;

- MS permanently activates the HPA axis system leading to a hyperanxious phenotype and impaired spatial memory, independently of the temporal window in which the separation occurs;

- The "depressive-like behaviour" and the impairment in exploratory behaviour are only observed if MS occurs in a period of high brain immaturity;

- The changes in the immune cells at the periphery are also observed if the maternal separation occurs from PND2-15;

- The interplay between the immune system and the emotional behaviour is complex and the anti-inflammatory cytokine IL-10 is shown to play a role in mood regulation.

In summary, these studies provide new insights into the complex interactions that early life disrupting events can trigger in the immune and neuroendocrine systems and their relevance for subsequent psychopathology. Of notice, are the similarities of processes occurring in both systems, that are linked by many phenomenon and (if we think on T cells and neurons, for instance) share crucial mechanisms such as “synaptic transmission” and “memory”. At the end of this thesis, I begin to realize that *understanding one system will probably contribute to unveil the other’s mystery*.

5.8 References

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Chapter 6

Future perspectives

Future perspectives

The data obtained with this work demonstrated the impact of early life stressful events on future psychopathology, highlighting critical developmental periods and immunological mediators for different phenotypic outcomes. However new questions arise from these observations that need further investigation.

Future work should be performed in order to clarify the mechanisms by which the intracellular crosstalk between the immune and neuroendocrine systems leads to the observed behaviours. Molecular, neurochemical and pharmacological approaches will be important to identify the pathways, but also intracellular cascades involved in the *imprinting* effects observed in these studies:

These goals can be achieved using the following strategies:

1) Pharmacological intervention with CRF_{R1} antagonist would clarify the role of this neurotransmitter as a mediator in the neuro-immune interactions explored in the MS model.

2) In this study we used synapsin-I as a marker of synaptogenesis, which showed differential region expression. To further clarify the impact of MS on the synaptic formation and transmission, other markers (or proteins involved in this process) as well as tracers combined with electronic microscopy would be useful tools.

3) In order to explore possible epigenetic mechanisms as consequence of MS, it would be of interest to explore some nucleossomal alterations, namely acetylation or methylation modifications in specific promoter regions of target genes. Due to the central role of CS receptors, both in the CNS, but also in the immune system, they will be likely candidates to those studies. However, the regulation of the expression of other hormones involved in the control of the HPA axis, such as CRF and ACTH would not be underestimated.