Universidade do Minho
Escola de Psicologia

Rui Alexandre Nunes Costa

A multidimensional analysis of maternal separation impact: corticosteroids and oxytocin linkage

2010

Universidade do Minho
Escola de Psicologia

Rui Alexandre Nunes Costa

A multidimensional analysis of maternal separation impact: corticosteroids and oxytocin linkage

Master thesis Psychology
Area of knowledge in Clinical Psychology)

Trabalho efectuado sob a orientação do
Professor Doutor Nuno Jorge Carvalho Sousa
Professor Doutor Ana Raquel Marcelino Mesquita

2010

AGRADECIMENTOS

Foram mais de dois anos de investigação animal que, embora longos, se revelaram incrivelmente fascinantes. Foram muitas horas passadas num biotério, muitas horas em laboratórios no Instituto de Ciências da Vida e da Saúde, incalculáveis horas a ler artigos, livros e outras tantas à frente do computador. Mas, felizmente, não foi um trabalho solitário. Quero, neste pequeno espaço, agradecer a todos os que estiveram envolvidos, directa ou indirectamente, neste projecto que, além de académico, se tornou pessoal:

À Ana Raquel Mesquita por TUDO! Pela paciência imensa que teve ao longo destes últimos anos na orientação desta tese. À disponibilidade e conhecimentos incríveis que teve sempre que precisava de apoio laboratorial, estatístico, na produção literária, na revisão do inglês... Por tudo isso, e por tudo o resto, MUITO obrigado por ter tornado este projecto uma realidade encadernada e legível.

Ao professor Nuno Sousa, por ter aberto as portas do ICVS a um aluno de uma outra área científica e pelos preciosos comentários e orientação realizados ao longo de todo o projecto.

A todos os que trabalham no ICVS e que demonstraram uma disponibilidade e ajuda inacreditáveis, sobretudo nestes últimos meses nos laboratórios!

À professora Isabel Soares por me ter apresentado a grandiosa área da Vinculação e por me ter despertado o interesse na conjugação dessa imensa área com a investigação animal.

À professora Bárbara Figueiredo por me fazer reflectir sobre as implicações clínicas deste tipo de investigação. E, sobretudo, pela amizade e compreensão que demonstrou nos momentos mais complicados.

À Ana Domingues, pela paciência nos conselhos com o inglês.

À Margarida, pela tua precisosa ajuda e companhia nas longas tardes de trabalho no biotério!

À Silvana e Paula, pela vossa amizade neste último ano!

Ao Diogo. Por seres, indescritivelmente, a minha base segura!

Finalmente, aos meus pais e irmão dos quais me orgulho todos os dias pelo apoio e conselhos que me têm dado desde que iniciei o meu percurso académico!

A MULTIDIMENSIONAL ANALYSIS OF MATERNAL SEPARATION IMPACT: CORTICOSTEROIDS AND OXYTOCIN LINKAGE

ABSTRACT

Exposure to early life stress and emotional trauma appear to be critical for neurodevelopment, stress responsiveness, lifetime health, and behavioral programming. Importantly, the severity of these effects seems to be mediated by the glucocorticoid levels exposure and the specific development stage at which it occurs. Indeed, in the last decades elegant longitudinal research with humans informed about the impact of early life experiences on the adult general adaptation. However, the mechanisms underlying the stress programming are still unclear. Animal models have been developed to experimentally investigate the neural substracts of early chronic stress exposure. In the rat, maternal separation (MS) in early ages have been linked to endocrinal, neurochemical and behavioral disruptions in the rat. The present thesis aimed to investigate the long-term *imprinting* effects of early stress exposure into two developmental time windows ($MS_{2\text{-}15}$, $MS_{7\text{-}20}$), in terms of behavioral outcomes: spatial memory performance, anxiety behaviors, learn helplessness behavior and social behavior. Moreover, in order to clarify the cross-talk between corsticosteroid and oxytocinergic systems as possible mechanism underlying the proposed behavioral outcomes, we separately evaluated the long-term consequences of early life maternal separation in corticosteroid and oxytocinergic pathways.

Independently of the temporal windows in which the stressor occurred, MS rats showed increased adrenal glands and body's weight, which may reflect a disruption of the HPA axis, as corroborated by the higher levels of corticosteroids plasma found in the same animals. Behaviorally, $MS_{2\text{-}15}$ rats presented depressive-like behaviors and, indepently of the brain maturity, both MS groups showed hyperanxious behavior in the elevated plus maze test. Finally, early adverse experiences showed to influence the *mRNA OT-r expression* in specific brain areas linked to HPA axis regulation and to several cognitive- and social-emotional processes.

These findings could reflect the time-dependent imprinting effect of adverse experiences in childhood, since the impact of corticosteroids in the maturation of specific neuronal substract of behavior and neuroendocrine phenotypes were demonstrated.

Key Words: *Maternal Separation; Imprinting; Corticosteroids; Oxytocin*

UMA ANÁLISE MULTIDIMENSIONAL DO IMPACTO DA SEPARAÇÃO MATERNAL: RELAÇÃO ENTRE CORTICOESTERÓIDES E OXITOCINA

RESUMO

A exposição precoce a stress e a experiências traumáticas têm sido apontadas como críticas para o desenvolvimento neurocognitivo, da respostas ao stress, da saúde e da programação comportamental. Mais importante ainda, a severidade destes efeitos parece ser mediada pelos níveis de glucocorticóides e pelo período desenvolvimental em que as experiências ocorrem. De facto, nas últimas décadas estudos longitudinais realizados com humanos têm demonstrado o impacto das experiências precoces na adaptação global em idade adulta. No entanto, os mecanismos subjacentes à programação do stress ainda não estão totalmente clarificados. Neste sentido, têm-se desenvolvido modelos animais para identificar, experimentalmente, quais os substratos neurais subjacentes aos efeitos da exposição crónica ao stress. No rato, a separação materna (MS) em idades precoces tem sido associada a perturbações neurobioquímicas, neuroendócrinas e as perturbações de comportamento. A presente tese teve como objectivo avaliar, a nível comportamental, e em dois períodos sensitivos do desenvolvimento ($MS_{2\text{-}15}$, $MS_{7\text{-}20}$) os efeitos da programação a longo-prazo da exposição precoce a stress, nomeadamente: o desempenho da memória espacial, o comportamento ansioso, o comportamento depressivo e o comportamento social. Adicionalmente, e a fim de promover a compreensão sobre a relação entre os sistemas oxitocinérico e HPA como possível mecanismo subjacente aos *outcomes* comportamentais identificados, foram avaliadas separadamente as consequências a longo-prazo da separação maternal nas vias oxitocinérgica e na produção de corticoesteróides.

Independentemente dos períodos temporais em que ocorreu o stress, os ratos sujeitos à separação materna apresentaram um aumento ao nível do peso corporal e das das glândulas adrenais, o que pode reflectir uma ruptura do eixo HPA, como corroborado pelos maiores níveis plasmáticos de corticosteróides encontrados nos mesmos animais. Ao nível comportamental, os ratos $MS_{2\text{-}15}$ apresentaram comportamentos depressivos e, independente do nível de maturação cerebral, ambos os grupos experimentais apresentaram comportamentos ansiosos quando testados no Elevated Plus Maze. Finalmente, a experiência de stress em momentos precoces do desenvolvimento demonstrou influenciar a expressão do mRNA dos receptors de oxitocina em áreas específicas do cérebro associadas à regulação do eixo HPA e a vários processos cognitivos e sócio-emocionais.

Os presentes resultados permitem demonstrar que o impacto de experiências adversas na infância está dependente do período desenvolvimental em que estas ocorrem, tendo sido demonstrado o impacto dos corticosteroides na maturação de redes neuronais especificamente associadas a determinados fenótipos comportamentais e neuroendócrinos.

Palavras-chave: *Separação Materna; Programação Desenvolvimental; Corticoesteróides; Oxitocina*

TABLE OF CONTENTS

1. INTRODUCTION

The association between early life stress and emotional trauma with an increased risk to develop psychiatric disorders and medical problems have been widely demonstrated in non-experimental human studies (Heim & Nemeroff, 2001; Nemeroff, 2004). Specially, three types of early childhood stress linked to future physio- and psychopathology emerged: (i) prolonged separation from parents, (ii) abuse and neglect, and (iii) institutional experience.

The first associations between early *attachment-figure separation* and psychopathology, namely depression, emerged during the 1940's by Sptiz and colleagues observations of children during prolonged separation from their parents in hospitals or other institutional settings (Spitz, 1946). In the same perspective of Spitz, during 20 years J. Bowlby (1969) observed and described the hospitalized children's behavior in Tavistok clinic; trying to understand the implications of prolonged parental separation (a long period of temporarily inaccessibility) has in children's behavior. He found a specific type of behavior responses of children separated from their parents (intense protest, despair and detachment), introducing a new framework to look at child-parent relationship, what today is known as "Attachment Theory". He concluded that a healthy relationship between infant and parent is important for the quality of development of the children and has a profound impact in normal development in adulthood, namely in the anxiety phenotype expression (Bowlby, 1982). In fact, for the infant and young child, attachment relationship with caregivers is the major environmental factor that shapes the development of the brain during its period of maximal growth. In this relationship the baby has the possibility to respond to the stimulation provided by the caregiver, which enables proper development to occur, because it is during the first years of life that the basic pathways of the brain are becoming established. Disrupting this important relationship leads to immediate and long-term effects on the behavior of human infants (Rutter, 1979). Recent studies have shown that prolonged separations from parents early in life are associated with major depression later on (e.g. Chapman, Whitfield, Feliti, Dube, Edwards, & Anda, 2004; Young, Abelson, Curtis, & Nesse, 1997).

Psychological, physical or sexual abuse, neglect and parental separation are also to exert harmful effects on children development in the immediate moment, as long-term effects in physical and psychological functioning during adult life, increasing mortality and suicide risk, substance abuse, diabetic disease, obesity and cardiovascular disease (Bifulco, Brown, & Adler, 1991; Felitti, Vicent, Anda, Robert, & Nordenberg, 1998; Kendler, Kuhn, & Prescott, 2004; Nunes-Costa, Lamela, & Figueiredo, 2009). For instance, the longitudinal study by Egeland and Erickson (1987) shows the emotionally neglected children or with emotional unavailable mothers, to be associated with social withdrawn, inattentive, and cognitive underachievement in their elementary-school years (Erickson & Egeland, 2002). Among the evidence about adult consequences of childhood trauma and neglect, Kendler and colleagues (2000) in an epidemiological study found an increased risk of developing psychiatric disorders and substance abuse by adult women who reported childhood physical or sexual abuse. In another elegant study, Mullen, Martin, Anderson, Romans, and Herbison (1996), reported that New Zealand women citizens with history of emotional abuse in early ages were prone to develop adult psychopathology as

well as social deficits. Other studies also found that school-aged maltreated children have a higher chance of becoming more aggressive and social inhibited and to develop internalizing or externalizing symptomathology than non-maltreated children (Kim & Cicchetti, 2004). Accordingly, several studies found that families characterized by a lack of emotional support, or by parental overregulation or underregulation of children's behavior, are also associated with increased physical and mental health risks for children (for review Repetti, Taylor, & Seeman, 2002).

Current research has documented deleterious effects of *institutional rearing* on the development of young children. As recent data has shown, children with institutional experience have a higher probability to express a variety of medical problems (Albers, Johnson, Hostetter, Iverson, & Miller, 1997), delay on physical and brain normative growth (Benoit, Jocelyn, Moddemann, & Embree, 1996), and neurocognitive problems (e.g. Albers et al., 1997; Cermak & Daunhauer, 1997; Morison, Ames, & Chisholm, 1995; O'Connor, Rutter, Beckett, Keaveney, Kreppner, & the English and Romanian Adoptees Study Team, 2000). Social and behavioral problems are also reported in these studies even with young children adopted out of institutions. It is communally accepted that these children express a higher number of disturbed attachment behavior (O'Connor et al., 2000; Zeanah, Smyke, Koga, & Carlson, 2005) as well as inattention/hyperactivity (Roy, Rutter, & Pickles, 2004), anxiety, fearfulness or aggression (for review, see Frank, Klass, Earls, & Eisenberg, 1996). According to this data, there is significant empirical evidence for the importance of the caregiver's sensitive responsiveness and active engagement to children in distress moments (specially in social deprivation conditions) to allow an increase in the probability of the children's healthy development.

Despite the findings in retrospective research with humans suggesting that prolonged exposure to early adverse experiences contribute to the development of several physiological and psychiatric diseases, it is difficult to establish a direct causality between early life stress and psychopathology. Besides the methodological problems in controlling all possible causes for psychopathology (Kendler, Bulik, Silberg, Hettema, Myers, Prescott, 2000) or performing neurochemical analyses in humans, it is difficult to control and understand all the potentially traumatic life experiences as well as the environments in which these children develop throughout the rest of their lives (Hardt & Rutter, 2004; Kessler, 1997; van Praag, 2004). In spite of the relevance of longitudinal designs to assess the impact of early life stress, it is frequently cited as difficulties to overcome such as the financial support, time consumption and ethical restrictions. In order to answer these issues on this research field, during the last decades controlled laboratory animal models have been develop to experimentally test these hypothesis and to further understand mechanisms underling this relationship between maternal separation (early and chronic stress) and adult behavior (Plotsky & Meaney, 1993). Indeed, repeated separations of infant animals from their mothers or peers allude to major variations in behavior and physiological functioning when they are evaluated at adult age. Since the first studies with nonhuman primates (Suomi, Eisele, Grady, & Harlow, 1975) up to the most recent ones, empirical evidence suggested that stressful life events play a role in the development and maintenance of physical and psychiatric disorders in adulthood such as anxiety, social dysfunction,

aggression, altered ingestion and anhedonia (Margolin & Gordis, 2003). Studies with non-human primates also reveled neurochemical (Higley, Hasert, Suomi, & Linnoila, 1998; Le Marquand, Pihl, & Benkelfat, 1994), endocrine (Fahlke, Lorenz, Long, Champoux, Suomi, & Higley, 2000) and immune function deficits after long periods of maternal separation in childhood (Coe, Rosenberg, & Levine, 1988). As well as a non-human primate, the laboratory rat is a good alternative in experimental model to test the hypothesis of the impact of early postnatal adversities in adulthood functioning (Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001).

1.1. The Laboratory Rat Development and Maternal Separation Protocols

Much of the current knowledge about human biological functioning derives from animal research, and more specifically from the rat giving its similarity with humans in terms of genotype, physiology and brain functioning (Willis-Owen & Flint, 2006).

The effects of early life stress experiences in adulthood functioning have been investigated through a variety of experimental manipulations with rats in laboratory settings, mainly focused in the interference on mother-pup interaction. Among the environmental factors, the disruption of the mother–infant relationship is one of the strongest threats for the optimal development of the infants. When born, rat pups are unable to perform the most basic biological tasks, such as defecating, urinating or even regulate body temperature (Krinke, 2000). Maternal presence is necessary for pups' survival, providing primary source of warmth, nutrition, and licking, while it also regulates numerous physiological, behavioral and psychological processes (Francis & Meaney, 1999; Hofer, 2005; Liu, Diorio, Tannenbaum et al., 1997; Rosenfeld, Ekstrand, Olson, Suchecki, & Levine, 1993). To perform these tasks, the dam approaches litters and executes arched-back nursing and display licking/grooming behaviors, particularly on the head and the ano-genital region (Giodano, Siegel, & Rosenblatt, 1989). The time spent with the nest decreases significantly in the postnatal period (0-20 days after birth). At the beginning of the lactating period the mother spends between 80% and 85% of the time with the pups, decreasing for 30% around the weaning (PND 21) (Leon, 1979).

By two weeks of age the visual and auditory pathways are significantly developed, which together with the maturity of the motor system, allows them start exploring the surrounding environment and to reach complete autonomy by the end of the third post-natal week (Ostermeyer & Elwood, 1983). Approximately on PND 30-40, with hormonal and physiological changes, female rats reach puberty and the body weight stabilizes around 120g, almost half of the male animals.

Regarding the rat brain development, during prenatal and postnatal period intense processes of cell proliferation, migration, axonal outgrowth and dendritic maturation take place. From an evolutionary parallelism, it is possible to compare the immature brain of the rat at birth with the immaturity human brain during the last trimmest of pregnancy (Roman, 2004). While the rat's brain is not completely developed at birth and continues their development in the postnatal period, human brain is sufficiently mature at the same time. Besides the genomic programming of the central nervous system (CNS) development, environmental factors also play an

important role in these biological phenomena and are involved in the establishment of normal neural functions in the adult individual (Andersen, 2003). The longer the disruption of the interaction between mother and pups in the perinatal period (environmental factor), the higher is the probability to have implications in the normal developmental programming and mal-adaptive biological responses in adulthood. In this way, the Maternal Separation (MS) paradigm is commonly used to mimic childhood stress in caregiver neglect circumstances, and has been proved to have a widely immediate (Mesquita, Pêgo, Summavielle, Maciel, Almeida, & Sousa, 2007) and long-term (Sanchez, Ladd, & Plotsky, 2001) impact on behavioral and neurobiological functioning.

MS paradigm covers a range of methods in which litters are separated from their dam in the postnatal period, until weaning. This manipulation of dam-pup interaction has been done in several different ways by varying the frequency, the duration and the age at which the separation occurs and the level of social deprivation (pups could be separated either from their dams or from littermates); and the post deprivation environment (e.g. by rearing rats in isolation from others after protocol's deprivation period) (Ellenbroek & Cools, 2002). Taking into account the variety of manipulations described above, there is an attempt in the literature to discriminate three types of separation protocols: the maternal deprivation (MD) where there is a single 24h period of separation; the handling procedure, when rat pups are submitted to short periods of maternal separation while being stimulated by the experimenter (< 30 min/day); and maternal separation (MS), consisting on longer periods of separation between rats and their dams (3-12 h/day) for consecutive days (for review Gutman & Nemeroff, 2002). These simple manipulations appear to be critical in the behavioral and neurobiological outcomes of the pups in adult age. In fact, shorts periods of separation (handling), not necessarily with stroking, were found to have long-term effects on corticosteroid response, decreased emotional reactivity and better performances in attention and learning tasks (Levine, 2002). However, long periods of separation were proved to have opposite reactions in rat performances and neurobiological responses, found to increase behavioral and stress reactivity (e.g., Biagini, Pich, Carani, Marrama, & Agnati, 1998; Macrí, Mason, & Würbel, 2004; Mesquita et al., 2007), as we will discuss below.

Accordingly to the McKinney criteria (1977) the validity of these models of maternal separation is based in the fact of the existence of similarities in the etiological factors and pathophysiological mechanisms between rodents and humans, and in the response to therapeutic treatments.

1.2. Some Hypothesis Beyond Differences in Outcomes of Early Stressed Rats: Maternal Care, The Hypothalamus-Pituitary-Adrenal Axis Ontogeny and Peripheral Metabolic Signals

More than 50 years ago, Seymour Levine provided the first experimental evidence that rat stress response is modulated by early experiences (Levine, 1957; Levine, 1959). In fact, adult rats separated daily from their mothers for few minutes a day until weaning (handling procedure) showed reduced activity of the Hypothalamus-Pituitary-Adrenal (HPA) axis and a decrease in adrenal gland weights 24h after a single glucose injection (Levine, 2001). Indeed, handled rats submitted to environmental stress present lower corticosteroids (CS) increase and

high ability to return to basal levels, lasting this response until adulthood (Meaney, Aitken, Bodnoff, Iny, & Sapolsky, 1985). This data contrasts with that reported with MS procedures where repeated periods of separation with more than 3h are associated to deleterious effects on different biological systems (for review, see Francis & Meaney, 1999).

The quality and quantity of maternal behavior following reunion of dams and pups is proposed to be a suitable explanation for rodents' responses after periods of separation in early stages of development (for review, see Fish, Shahrohk, Bagot et al., 2004). Although it is not completely known how maternal behavior interferes with pups' HPA axis, some explanations focused on the role of feeding, distribution of nursing bouts and body temperature maintenance on the regulation of rodents' HPA axis has been explored (Macrí, Mason, & Würbel, 2004; Ruedi-Bettschen, Feldon, & Pryce, 2004; Suchecki, Rosenfeld, & Levine, 1993). The amount of maternal care and how these set of behaviors are performed is altered according to the time of separation. Prolonged periods of separation lead to longer recovery latencies in MS dams upon reunion, longer time to begin feeding and to exhibit licking/grooming and arched-back nursing care. In contrast, after brief separations (handling procedures) when returning to their mother's cage the pups are more licked and groomed than non-handled animals reducing emotionality and HPA axis responses and improving their adaptive stress response later in life (Liu, Caldji, Sharma, Plotsky, & Meaney, 2000). Francis and Meaney (1999) found that disrupted emotional responses reported in maternally separated rats were reversed when MS pups were cross-fostered by high-licking and -grooming adult females. Accordingly to this data, it is possible that the quantity and quality of maternal care are critical in the explanation of MS and handling procedures outcomes. Recently, molecular basis for this relationship between maternal care and offspring outcomes started to be explored. Diorio, Weaver and Meaney in a cDNA array study proved that the quality of maternal care impacts on hippocampal gene expression, namely for genes related to cellular metabolic activity (e.g. glucose transporter), glutamate receptor function and genes linked to the expression of growth factors (BDNF) (for review, see Diorio & Meaney, 2007). Additionally, recent data also suggests that the amount of licking and grooming provided by the dam alters the methylation pattern of the transcription factor NGFI-A, which activates glucocorticoid receptor gene expression in the hippocampus (Weaver, Cervoni, Champagne et al., 2004).

Because maternal care could not be *per se* the unique mediator in these responses (Macrě, Chiarotti, & Würbel, 2008), another way to understand differences between rodents responses in MS paradigm is to look at the corticosteroids (CS) role in organization, regulation and maturation of brain and peripheral tissues, as well as to the ontogeny of HPA axis. In the presence of physiological or psychological stressors, the HPA axis (also known as the LHPA axis due to its influence on the limbic system) is activated with the production of arginine-vasopressin (AVP) (Gunnar & Quevedo, 2007) and corticotrophin-releasing factor (CRF or CRH) on the medial parvocellular region of the parvoventricular nucleus of the hypothalamus. These hormones act synergistically, specially in chronic stress conditions (Pinnock & Herbert, 2001) and, before released from the median eminence nerve terminals into anterior pituitary (AP), have the capacity to inhibit the luteinizing hormone and,

consequently, sexual conduct. Once activated specific receptors on the corticotropic cells of the AP, CRH induces the synthesis of proopiomelanocortin (POMC), the precursor of several opioid molecules (β-lipotropin and β-endorphin) and adrenocorticotropic hormone (ACTH). After converted from POMC, ACTH enters the circulatory system by a set of capillaries surrounding the pituitary gland (reaching the maximum of release around 10-15min) and flows through this system until it reaches the cortex of adrenal glands. After ACTH stimulation these glands are responsible for the production of glucocorticoids (cortisol in humans; corticosterone in rodents - CS), and mineralocorticoids (aldosterone), reaching the maximum release only after 15-30 min (Sandi & Cales, 2000). Due to its lipophilic structure, CS crosses the blood-brain-barrier acting within the central nervous system (de Kloet, 2004). Glucocorticoids are pluripotent hormones acting in different tissues regulating many aspects of metabolism, growth and other immunological functions. Recently, glucocorticoids proved to have the ability to regulate gene expression in multiple brain structures (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007; Gunnar & Quevedo, 2007). Corticosteroids are also responsible in the maintenance of vascular tone and permeability and distribution of water in the body, to potentiate the effect of vasoconstriction (Beishuizen & Thijs, 2003).

This complex sequential of production of glucocorticoids occurs through a negative feedback process mediated by two receptors in the CNS (Beishuizen & Thijs, 2003; Stansbury & Gunnar, 1994): the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR receptors regulate the effects of stress as baseline blood pressure, maintaining the responsiveness of neurons to neurotransmitters and preserving the circadian rhythm of HPA axis (Sapolsky, 2000). On the other hand, the GR is activated only when large numbers of molecules of glucocorticoids are in the circulatory system (during extreme stress experiences or at the peak of its circadian production) (de Kloet, 2004). Indeed, the affinity cortisol/corsticosterone for MR is 10-fold higher than for GR (de Kloet et al., 1998). These receptors are located in areas involved in emotion, learning and memory. While the MR receptors can be found in the hippocampus, septum and in a small extension of the prefrontal cortex, amygdala, posterior pituitary and paraventricular nucleus of the hypothalamus, the GR can be detected all over the brain, focusing on hippocampus, hypothalamus, pituitary, amygdala formation, bed nucleus of the stria terminalis, nucleus accumbens, cerebral cortex (Han, Ozawa, Matsuda, Nishi, & Kawata, 2005; Ozawa, Ito, Ochiai, & Kawata, 1999), and medial dorsal nucleus of raphe (Härfstrand, Fuxe, Cintra et al., 1986).

For the regulation of this axis there are three types of negative feedback mechanisms known accordingly to the time required to inhibit the stress response. The rapid feedback, which occurs within minutes by inhibiting the CRF and ACTH, releases at the level of cell membranes of the PVN and the AP (Young & Vazquez, 1996). The intermediate feedback acts at the level of CRF synthesis and release, decreasing subsequently the ACTH levels. The delayed feedback is the only mechanism known to be able to inhibit the total ACTH stores. In this process the organism takes 1-2 hours after the presentation of the stressor until establishing the homeostasis. This process involves the reduction of pituitary ACTH by decreasing POMC mRNA levels. This transcriptional process implies changes on other HPA axis components such as hippocampal GR or the CRF levels (Cairns, Cairns, & Okret, 1993).

In rodents, these HPA axis feedback mechanisms undergo a process of maturation during development, which is one of the possible explanations for the differences between early life stress models. Preferentially, MS procedures occur in the period that has been identified as the stress hypo-responsive period (SHRP) comprising the first two weeks after birth and characterized by low levels of CS production. In the period lasting from birth to PND 4 CS basal levels are extremely high due to the inability of the rat's organism to process these hormones transmitted through the placenta from the mother during parturition. Since this point until the 2nd week of life, CS levels remain low due to the weak release of ACTH in consequence of CRF neurons immaturity, decreased pituitary peptide content and decreased sensitivity to CRF stimulus (Schoenfeld, Leathen, & Rabii 1980).

Manipulations comprising mother-pup separation <3h/day did not perturb this protective period and thereby did not cause any disruptions in CS production, neurotransmitter systems or behavior in the offspring. The neonates can respond to stressors during this SHRP (Walker et al., 1991), depending on the type of the stimuli and its frequency of presentation. In fact, neonatal animals respond centrally to specific stressors with elevation of ACTH levels such as bacterial endotoxin (Witek-Janusek, 1988), cold exposure or ether fumes (Walker, Scribner, Cascio, & Dallman, 1991). In MS procedures the stressor is presented chronically, following and shaping/programming the ontogeny of the receptors involved in the HPA feedback processes (e.g. Rosenfeld, Ekstrand, Olson, Sucheki, & Levine, 1993; Gunnar & Quevedo, 2007; and Mesquita, 2008 for review). MS leads to a downregulation of CRF-receptor density in the AP (Ladd et al., 1996), increase CRF mRNA expression in the paraventricular nucleus of the hypothalamus, and a significant decrease in MR and specially on GR receptors in the CNS, namely in the hippocampal formation, after chronic stress (Sutano, Rosenfeld, de Kloet, & Levine, 1996). In summary, the changes in receptors number and CRF mRNA expression leads to a inhibition of negative feedback mechanism of HPA axis what may explain the high basal and stress-induced CS levels.

Removing pups from the mother eliminates the pups' access to food, particularly breastfeeding. Leptin hormone, one of the well known peripheral metabolic signals, is largely expressed in maternal milk and signalizes arcuate nucleus of the hypothalamus in order to modulate the activity of POMC neurons and CRH expressing neurons through innervations to the PVN (Elias, Lee, Kelly, et al., 1998; Bouret, Draper, & Simerly, 2004). Lesions of the arcuate nucleus were found to increase HPA axis activity (van der Lely, Tschop, Heiman, & Ghigo, 2004). In this way, we can conclude that leptin inhibits the HPA axis activation, modulating in a long-term process the adrenal steroidogenesis through decrease of CRH and POMC production. Indeed, basal corticosteroid secretion is unaffected by leptin. This hypothesis is supported by the Rosenfeld (1993) research conclusions that feeding of the pups during the separation period prevents most of the peripheral responses to long periods of separation (Rosenfeld, Ekstrand, Olson, Suchecki, & Levine, 1993). Leptin is not the only peripheral metabolic that modulates glucocorticoid levels. Arcuate nucleus also has receptors for ghrelin (Cowley, Smith, Diano et al., 2003) and glucose (Wang et al., 2004). For example, glucose was found to regulate HPA axis function, even in the absence of corticosterone (Laugero, Bell, Bhatnagar, Soriano, & Dallman, 2001; Laugero, 2004). Although aspects of maternal care and HPA axis ontogeny undoubtedly regulate stress responses in the

neonate and later in life, the maternal separation impact on the HPA axis might also be triggered by deprivation of some metabolic factors like as ghrelin, glucose and leptin (Fig. 1).

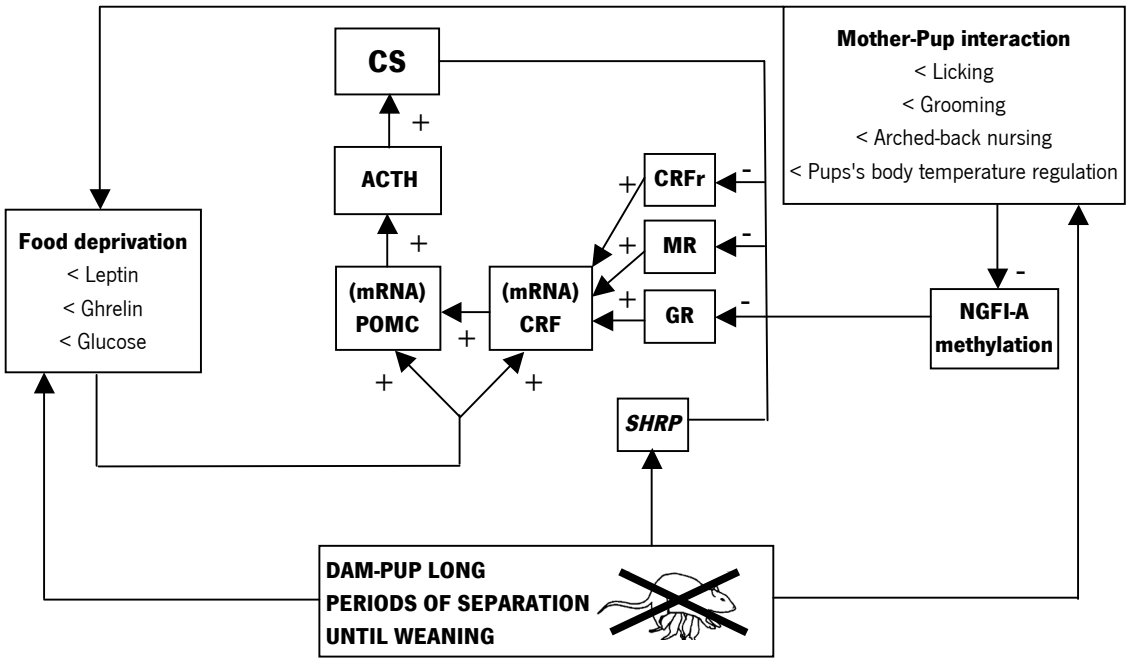

Figure 1 – Possible pathways through Maternal Separation paradigm affects HPA axis. Note. + (production) increases; - production decreases / down-regulation of.

1.3. Neurobiological Changes After a Period of Maternal Separation

It is unquestionable the fact that early social-emotional deprivation affects a variety of neurotransmitter systems in the rat brain (Kaufman, Plotsky, Nemeroff, & Charney, 2000 for profound review). Next, we briefly explore some neurochemical imbalances in some neurotransmitters pathways with well-known behavioral influences. According to this thesis' purpose, we will explore in greater detail the oxytocinergic pathway changes in rearing conditions.

Periodic maternal separations were proposed to alter adult brain serotonergic transporter and serotonergic 1A receptor levels and function in the forebrain regions (Vicentic, Francis, Moffett et al., 2006). Arborelius and colleagues (2004) also found changes in the sensitivity of the serotonin receptors and transporters (Arborelius, Hawks, Owens, Plotsky, & Nemeroff, 2004). Alterations in these serotonergic systems by early rearing deprivation conditions might increase vulnerability for behavioral disorders in adulthood, as showed by human studies (Gartside, Johnson, Leitch, Troakes, & Ingram, 2003).

Noradrenaline neurotransmitter has been proposed to mediate depression like-behaviors in humans and in other mammal species. The levels of this neurotransmitter in hypothalamus and hippocampus have been found to be markedly decreased in MS animals (Daniels, Pietersen, Carstens, & Stein, 2004). There was also significant differences between maternal separated and control pups in the levels of noradrenaline

neurotransmitter in the nucleus accumbens (Arborelius & Eklund, 2007), with stressed pups having lower values than controlled ones.

At birth, rat's dopamine system is not fully developed. Dopamine D1- and D2-receptor density increase during first weeks of life until the end of the first postnatal month when reaches adult levels (Johansson, Georgiev, & Fredholm, 1997). MS manipulation has also been suggested to alter D1-like receptor density (Brake, Zhang, Diorio, Meaney, & Gratton, 2004). There is also evidence that MS induces decrease dopamine transporter levels in some brain regions (e.g. nucleus accumbens and caudade putamen), increasing responses to stress and addictive behaviors in adulthood (Meaney, Brake, & Gratton, 2002).

Changes in GABA receptors, which mediate the majority of fast synaptic inhibition in adult brain, have been proposed as one potential mediator of endocrine and behavioral responses to stress. MS animals displayed reduced $GABA_A$ receptor and mRNA levels for the $\gamma 2$ subunit of this receptor in the locus coeruleus, the nucleus tractus solitarius, amygdala (Caldji, Francis, Sharma, Plotsky & Meaney, 2000; Jaworski, Francis, Brommer, Morgan, & Kuhar, 2005), and hippocampus formation (Hsu et al., 2003). GABAergic system has been considered as critical pathway for the anxiolytic and fear like-behaviors and free alcohol intake in adulthood.

In stress conditions, the endogenous opioid system is activated in order to reestablish organic homestasis, namely through reduction of pain sensation; it also displays an important role in brain reward pathways implicated in drug abuse (Lu, Shepard, Scott Hall, & Shaham, 2003). Ploj, Roman and Nylander (2003) discovered endogenous opioid system changes after MS with long-term alterations of dynorphin and enkephalin levels in the limbic structures, substantia nigra, pituitary lobe and periaqueductal gray.

Argenine-Vasopressin (AVP) and V_{1A} receptor seem to be susceptible to GC programming during early life. Immunoreactivity analyses in the paraventricular nucleus of the hypothalamus have shown a selectively increase of vasopressin levels in early maternal separated male rats (Veenema, Bredewold, & Neumann, 2007). In fact, an association of MS to higher vasopressin mRNA expression was also described not only in the same area, but also in the bed nucleus of the stria terminalis (Veenema & Neumann, 2008). Very recently, Lukas, Bredewold, Veenema, and Neumann (2010) extended the knowledge about the interaction between vasopressinergic and glucocorticoid's pathway, exploring the expression of $V_{1A}R$ in several brain areas of early stressed rats, namely in the piriform cortex, lateral septum, hypothalamus, dentate gyrus, arcuate nucleus and hippocampus. They also looked at social behaviors dependent of this neuropeptide in different developmental stages. Their conclusions suggest that $V_{1A}R$ bindings are likely associated with the maturation of aggressive behaviors and dependent of early life stress events (Lukas, Bredewold, Veenema, & Neumann, 2010).

1.4. Oxytocin: The Neuropeptide, Central Behavioral Effects and MS

The neurohypophysial hormone oxytocin (OT), a small nonapeptide, has a long established role at the moment of the birth, providing uterine contractions and milk ejection (Russel & Leng, 1998). However, as we will explore below, the role of the OT in mammals' behavior phenotype is much wider, having an important role in social

bonds formation and anxiolitic effects during stressful events. Physiologically OT operates through two separate systems according to their distinct anatomy, functionality and sites of release and action: the peripheral and the central oxytocinergic systems (Ring, Malberg, Potestio et al., 2006). In the present study we will only explore the central oxytocin system, where this neuropeptide acts as a neurotransmitter/neuromodulator and controls some central behavioral parameters.

OT is the neuropeptide with higher mRNA expression in the rat's hypothalamus (Gautvik, De Lecea, Gautvik et al., 1996). This hormone is synthesized in magnocellular neurons of the supraoptic (SON) and PVN of the hypothalamus and released into the circulation after extending down to the posterior pituitary. OT is ubiquitously distributed throughout the CNS by parvocellular neurons, having been detected in the hypothalamus, entorhinal cortex, medial and septal nuclei, mesencephalic gray nucleus, dorsal and ventral hippocampus, subiculum, amygdala, striatum, olfactory bulbs, raphe nuclei, locus coeruleus, the nucleus of the solitary tract, dorsal motor nucleus of the vagus nerve and sensory nuclei. OT fibers also end on the pineal gland and the cerebellum, with most of them continuing toward the dorsal horn of the spinal cord (e.g. Argiolas and Gessa, 1991). OT concentrations in the extracellular fluid of the SON were calculated to be 100-1000 higher than the basal OT concentrations in plasma (Landgraf, Neumann, Russel, & Pittman, 1992). Although peripheral OT, being non-steroid and water soluble, was theorized to not cross blood-brain-barrier (BBB), recent data points to the opposite direction: ~0.1% systemic OT was calculated to cross BBB (Jones & Robinson, 1982), allowing for interaction between the two oxytocinergic systems. For example, some researches have shown that peripheral injections of OT can depict effects seen with central administration of this peptide (e.g. Razzoli, Cushing, Carter, & Valsecchi, 2003).

Looking into the behavioral phenotype effects of OT, research demonstrated the key role of this neuropeptide in the development and maintenance of social cognition and social behavior. Much of what is known about central effects of OT has been derived from maternal behavior studies. Central injections of an antagonist or antiserum or lesions of the OT-producer brain areas in the hypothalamus, lead to inhibition of the onset (but not the maintenance) of *maternal behavior* (Insel & Harbaugh, 1989). Pedersen and Prange (1979) in their pioneer study demonstrated that injection of OT into the lateral ventricles of ovariectomized rats induces maternal care within 30 minutes. Important for dams-offspring bonding formation is the interaction of oxytocinergic pathway with steroids, such as nitric oxide (Okere, Higuchi, Kaba, Russel, Takahashi, & Murata, 1996), and infant olfactory cues (Yu, Kaba, Okutani, Takahashi, & Higuchi, 1996). The secretion of this peptide is hypothesized as being induced through physical contact with another animal (Carter, 1998) being a rewarding cue for the offspring in rodents dam-pups contact (Nelson & Panksepp, 1996). Indeed, OT is endogenously released in the pup during the infant-mother contact via somatosensory stimulation and by non-noxious stimuli such as warm temperature and odors/pheromones (Uvnäs-Moberg, 1998). Although not totally clarified, the OT seems to be also associated to maternal protective behavior, inhibiting aggression to pups (Giovenardi, Padoin, Cadore, & Lucion, 1998), but increasing aggression to identified intruder (Ferris, et al., 1992). Not only offspring bonding

formation is associated to OT; in fact, central and subcutaneous low doses administration of this peptide lead to *increase social contact/memory and preference for the familiar partner* in both males and females (Cho, DeVries, Williams & Carter, 1999), important for monogamy relationships.

In addition to the social behavior, OT centrally and peripherally administrated has been shown to induce *stereotypic behaviors* in the rat, such as repetitive grooming of the genital regions (van Wimersma Greidanus et al., 1990). Interestingly, elevated OT levels have been found in patients with obsessive-compulsive disorder (Leckman, Goodman, North et al., 1994). However, in human studies with autism disorder, a psychopathology characterized by a set of repetitive behaviors, OT administration is associated with a decrease in these behaviors (Hollander, Novotny, Hanratty, et al., 2003). This contradictory data suggests differences between behavioral phenotypes of obsessive-compulsive and autistic disorders or, even, unrevealed interactions of oxytocinergic pathway with other neurochemical pathways (e.g. dopamine and endorphins (Drago, Contarino, & Busa, 1999)).

Finally, concerning to central effects, OT exerts potent *anti-stress effects*, such as the inhibition of the rise in circulating glucocorticoid levels, decreases in blood pressure, and increases in insulin and cholecystokinin (Carter, 1998; Neumann, Krâmer, Toschi, & Ebner, 2000). OT's ability to reduce HPA axis activity may reside in the presence of both OT and CRF neurons and receptors in the PVN. Research with lactating women showed that this anti-stress effect also seems to occur in humans: a suppression of HPA activity has been observed if breast-feeding starts 30-60 min before exposure to a stressor (Heinrichs, Neumann, & Ehlert, 2002). Accordingly to these results, it is also well documented the *anxiolytic* and *antidepressive* proprieties of OT in animal models (McCarthy, McDonald, Brooks, & Goldman, 1997). For example, rat pups separation cries were inhibited by central administration of OT (Winslow & Insel, 1993). McCarthy (1995) suggests that much of the behavioral effects of OT may be associated to its anxiolytic effects. The ability of OT to reduce HPA axis activity may reduce the natural inhibition in social encounters and increase exploration behaviors.

In contrast to most biologically active compounds, only one OT receptor (OT-R) has been identified for both central and peripheral oxytocinergic systems (the same expressed in the uterus). However, the "anti-stress" effect of the OT (explored previously) cannot be reversed by OT antagonists, which strongly suggests the existence of other, unidentified, OT receptors (Uvnäs-Moberg, 1998b). Peripherally, OT receptors are found not only in the uterus and mammary tissue, but also in thymus and kidney, allowing the assumption of the OT effects in many body functions. Within the brain, OT receptors are abundantly present in limbic and autonomic areas, with large density variation within species and developmental stages. In rat brain, OT receptor mRNA was detected in some cortical areas, the olfactory system, the basal ganglia, the amygdala, the hippocampus, the thalamus, the ventromedial nucleus of the hypothalamus and brain stem (for review, see Gimpl & Fahrenholz, 2001). This distribution does not seem to be influenced by sex.

In rats, the gene for OT is transcribed in the gestational period, around the 18th day of intrauterine life, but it remains undetectable in the pituitary until 21st day of gestational period (Altstein & Gainer, 1988). OT general levels are low during gestation and its synthesis is only detected on the second day of postnatal life suggesting

that the gene encoding OT is regulated at the posttranscriptional level (Lipari et al., 2001). The OT-R also first appears in the postnatal period (Shapiro & Insel, 1989). Thus, it is possible that the production of OT and possibly its receptor as well, may be vulnerable to "hormonal imprinting" derived from postnatal experiences. However, empirical evidence for this assumption still remains scarce. In a very recent research, Lukas and collaborators (2010) examined the effects of MS on OT-R binding in forebrain regions of juvenile (5 weeks), adolescent (8 weeks), and adult (16 weeks) male rats submitted to 3h of MS between 1-14 postnatal days. They found lower binding OT-R in the agranular cortex at juvenile and adolescent age, and in the lateral septum and the caudate putamen at adult age after exposure to MS. In addiction, higher binding OT-R was found in the medial preoptic area (at adolescent age) and ventromedial hypothalamus (at adult age), also dependent of early stress experience. Shortly, the authors proved the existence of age-dependent changes in OT-R binding and the role of MS in the regulation of this receptor ontogeny (Lukas, Bredewold, Neumann, & Veenema, 2010). However, no behavioral tests were administrated for evaluation of MS-induced changes in OT dependent-behaviors.

1.5. Accessing Effects of Early Life Stress in Future Psychopathology in Rats: Anxiety, Depression, Memory and Social Interaction

It is well documented the organizational and regulatory effects of CS in the CNS and peripheral tissues, through the action on neurons and glia differentiation (Sousa & Almeida, 2002) and neurotransmitter expression (Lauder, 1983). As explored before, MS occurred in early stages of life have the capacity to induce significant changes in CS levels leading to behavior phenotypes. Different measurements have frequently been used to explore rats' emotional behavior after stress conditions.

Mesquita and collaborators (2007) found eye and ear opening anticipation in maternally separated newborns proving the CS role in the acceleration of some somatic milestones. However, in the same study the acquisition of some neurological reflexes, such as the postural reflex, air righting and surface righting reflexes, seemed to be delayed compared to non-separated pups (Mesquita et al., 2007).

As adults, maternally separated pups have shown increased emotionality/anxiety behaviors (reviewed in Ladd et al., 2000). In the Elevated Plus Maze (EPM), MS animals spend more time in the closed arms, an indicator of increased emotionality in these animals (Daniels, Pietersen, Carstens, & Stein, 2004; Kalinichev, Easterling, Plotsky, & Holtzman, 2002; Madruga, Xavier, Achaval, Sanvitto, & Lucion, 2005; Mesquita, 2008). Similar results were found in the Open Field test (OF), where adult MS animals spent more time in the periphery of the arena showing less exploratory behaviors (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000). In common, these two models are based on the conflict existing between the natural trend to explore a new environment (open arms in the EPM and the center of the arena and number of rearings in the OF) and its innate ability to explore unknown environments.

Depressive-like behaviors are commonly tested in the Forced Swimming Test. The immobility time spent by the rat is a good indicator for helplessness behavior. There is sufficient data to conclude the impact of this model in depressive-like behaviors, with neonatal separated pups demonstrating a significant increase in the immobility time compared to controls (Mesquita, 2008). These findings are corroborated by neurobiological changes in 5-HT system. Accordingly, the hippocampal contents of 5-HT and the raphe nucleus expression of 5-HTT mRNA were decreased in MS rats (Lee, Kim, Kim et al., 2007), young separated rats also showed increased serotonin turnover in the dorsal raphe nucleus (Mesquita et al., 2007). Pharmacology manipulations with Escitalopram have been proven to alleviate the depressive phenotype in adult rats maternally separated early in life (Khoury, Gruber, Mørk, & Mathé, 2006).

The majority of hippocampal granule neurons develops and maturates between postnatal days 1 and 21 (Amaral & Dent, 1981) and the peak of neurogenis overlaps the SHRP (Sapolsky & Meaney, 1986). Therefore, MS could modulate the normal maturation of hippocampal cells with memory and learning implications. Aisa et al. (2007; 2009) found that MS could induce significant decreases in markers of neuroplasticity in the hippocampal formation as well as the prevention of increases in NCAM expression, one plasticity marker that has been associated to some forms of stress effects on learning and memory. In the Morris Water Maze, a behavioral test for spatial memory assessment, MS increases the latency times in rats (Mesquita, 2008), showing the influence of early deprivation on spatial learning.

Among children, negative early life experiences such as maltreatment, have been associated to risk factor for aggression, violence and anti-social behaviors (Barnow & Freyberger, 2003; Fonagy, 2004). These findings were also found in primates and rodents, where increase in intermale aggression was detected in adult rats (Veenema, Blume, Niederle, Buwalda, & Neumann, 2006; Veenema, Bredewold, & Neumann, 2007) and also in juvenile pups (Veenema & Neumann, 2009) exposed to MS procedures.

As discussed above, abnormal social experiences during the neonatal period have been shown to alter the activity of neuropeptide systems, namely OT system. Furthermore, neurochemical synthesis and release changes of this neuropeptide may in turn mediate the regulation of social behaviors in adulthood (Ferris, 2005) such as partner preference formation, aggression, and maternal behavior (Bales and Carter, 2003). Therefore, early life stress (MS) may induce phenotype changes in social behaviors through oxytocinergic system.

1.6. Aims of the Study

The peak period of neurogenesis and denditric growth overlaps the SHRP in neonatal rats (Sapolsky & Meaney, 1986). Early life stress is a risk factor for the acquisition of (neuro)development milestones and future functioning if presented in this postnatal period in a chronically way. MS is a suitable animal model for understanding the potential mechanisms of environmental and developmental determinants of individual differences in stress response and future psychopathology (Holmes, le Guisquet, Vogel, Millstein, Leman, & Beizung, 2005). There is some consistent empirical evidence for the significant effects of MS in the behavior and

neuroendocrine phenotypes that last until adulthood. Even if CS role was commonly thought to have direct responsibilities in the abnormal phenotypes of early stressed pups, in fact these steroids probably affect the development of others neurochemical pathways influencing, in an indirect way, the abnormal reported behaviors of early MS pups. As many other interactions, CS have been proved to have disruptive functions on the OT production and release (Lukas, Bredewold, Neumann, & Veenema, 2010; Veenema et al., 2006; Veenema & Neumann, 2009).

In order to understand the mechanisms beyond MS impact on depressive-like behavior, anxious behavior, spatial memory performance and social behaviors, we explore the long-term impact of MS on the corticosteroid system, focusing the differential role of CS in two different time windows of development.

We expect that MS induce HPA axis disruption with increases of CS levels leading behavioral phenotypes changes in adulthood. However, CS are able to trigger changes in several neurotransmitters pathways, namely by region-specific changes in OTr mRNA expression which, in turn, might also contribute to variations in adult behaviors. The present study also tested the hypothesis that mRNA OT-r expression in some stress-sensitive brain areas could be affected by early life stress.

In summary, the present thesis aims to:

- Characterize the long-term consequences of stress exposure into two different developing periods, in terms of spatial memory performance, anxiety behaviors, depressive like-behaviors and social interactions.
- Evaluate long-term consequences of early life maternal deprivation in corticosteroid system.
- Evaluate the early life stress consequences in oxytocinergic central pathway at adult age.

2. MATERIAL AND METHODS

Animals

Wistar Han females (Charles River, Barcelona, Spain) during estrous were placed overnight with males in same cages under standard laboratory conditions (light/dark cycle 12/12 hours, 8:00 AM – 8:00PM, 22° C ambient temperature; food and water *ad libitum*, cage size 60 cm x 40 cm x 20 cm). Males were removed from the cage when a vaginal plug was confirmed; this day was designated as embryonic day 0 (E0) and the day of delivery as postnatal day 0 (PND 0). At the end of pregnancy (E22), all females were provided with nesting material and remain singly housed. Litters were delivered on gestation day 22; the size of each litter was adjusted to an average of 10 pups. The litters were randomly divided in 3 different groups: 2 maternal separation groups ($MS_{2\text{-}15}$, n=14 males; $MS_{7\text{-}20}$, n=20 males) and control group (Cont, n=11 males).

Separation procedure

Pups from $MS_{2\text{-}15}$ group were daily separated from their mothers between the 2nd and the 15th post natal days. Liters from each cage were separated from their dam for 360min (from 9.00 am to 3:00 pm) and kept together in the same plastic box, transferred to an adjacent room climatically controlled (37°C). After the period of separation the pups returned to their home cages in the colony room. The same procedure was applied to the other experimental group; however for the $MS_{7\text{-}20}$ animals, the period of separation occurred between the 7th and the 20th post-natal days. Control animals where left undisturbed with their dams until weaning,

Behavioral tests

After weaning (P21) all animals were housed in groups of two and left undisturbed they reach 5 months of age when they were behaviourally tested. All behavioral tasks were conducted sequentially and performed during the light phase of the light/dark cycle.

Open Field

At the age of 5 months, free exploratory behavior of 14 $MS_{2\text{-}15,}$ 20 $MS_{7\text{-}20,}$ and 11 Control rats, was assessed during 5 min in a white square open field (43.2 cm x 43.2 cm) surrounded with acrylic transparent walls (ENV – 515; MedAssociates, VT, USA). Room illumination was provided by a white bright light and the temperature was controlled. 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 of emotional reactivity were measured and recorded: (a) time spent in the central area (a measure of anxious-like behavior); (b) total distance travel (a measure of general locomotor activity) (c) number and duration of rears (a measure of general exploratory behavior). The apparatus was cleaned with 10% ethanol and wiped between sessions.

Elevated Plus Maze

The Elevated Plus Maze was used to examine the anxious-like behavior. The maze has been validated and used as a measure of anxiety (Hogg, 1996; Rodgers and Dalvi, 1997a, 1997b) and reflects a conflict between the rat's exploratory activity and its innate fear of height and exposed areas.

Animals were individually tested for 5 min in the experimental apparatus. This time interval was chosen due to decrease in avoidance and increase in fatigue behaviors after 5 to 10min of test (Pellow et al., 1985). The 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. In the beginning of the session the rats were placed in the centre of the maze and then allowed to explore either the open or closed arms of the maze. The time spent in the different arms of the maze was recorded using an infra-red beam system connected to a computer. The number of entries and time spent into the open arms were used as anxiety behavior indicators – more entries and higher

time spent in the open arms, less anxious behavior. The number of entries in the closed arms of the maze also served as a measure of the rat's locomotor activity. The maze was cleaned and wiped between every animal with 10% ethanol.

Forced Swimming Test

In order to test the depressive like-behavior the learned helplessness was analyzed using the Forced Swimming Test. As described by Porsolt et al. (1977), the rats were placed individually in acrylic cylinders filled with water (25°C) to a depth such the animals cannot reach the ground and had no solid support for their rear paws. The present test started with a 10 min pre-test session and, in the following day, animals were subjected to the same procedure for 5 min (test session). A video camera was used to record both of sessions. 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 water was clean between each trial. Video recordings were later scored by two investigators blind to the experimental conditions. Three different parameters were analyzed: a) Immobility -that was considered as rats floating passively, displaying only small movements to keep its nose above the surface; b) activity was considered when the animal swim to escape from the cylinder; c) latency to immobility consisted to the time at which the animal give up swimming for the first time).

Morris Water Maze

The Morris Water Maze (MWM) consisted of a black tank with 170 cm in diameter and 50 cm in deep, filled with water at 22°C, to a depth of 31 cm. A black platform (12 cm diameter, 30 cm high, invisible to rats) was placed in one of the 4 quadrants (that are divided by imaginary lines) below the surface of the water. The room was mild lighted and four extrinsic visual clues were glued in the room walls. Data related to the path swum and latency to reach platform were collected using video-tracking system fixed to the ceiling of the room (Viewpoint, Champagne au Mont d'Or, France).

A place learning task was performed in order to assess rats' ability to learn the position of the hidden platform. Animals were individually tested for 4 consecutive days (4 trials per day) and the platform was placed in the center of an arbitrary quadrant. Test session for all rats began with rodents being placed, 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 maximum duration of each trial was 120 sec and 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 dried before continuing the session. At the end of the four sessions, all rodents were dried with a heating lamp for 15min before placed at the home cage.

Social Interaction Task

The Social Interaction Test (SIT) was described by Field and Hyde (1978) as the first test using ethological sources of anxiety and a natural form of behavior as the dependent variables. Sensitive changes in anxiety

phenotype were found using this test (for a review, File & Seth, 2003). Thirty years later, SIT was extended to assess behaviors from different disorders (Tordjman et al., 2006), effects of pharmacological manipulations on behavioral phenotype (e.g. Bagdy, Graf, Anheuer, Modos, & Kantor, 2001; Bhattacharya & Mitra, 1992) or brain lesions and its implication on behavior (e.g. Duxon, Kennett, Lightowler, Blackburn, & Fone, 1997; Gonzalez, Andrews, & File, 1996). The original protocol's form (Field & Hyde, 1978) has been frequently modified according to the specificity of the research objectives, despite preserving the original objectives and measure parameters. In this way, we adapted Field and colleague protocol preserving its original bases.

The apparatus was constituted by an open arena of 100cm x 100cm x 40cm. The floor was from Morris Water Maze apparatus and sides were made of smooth black polyvinyl chloride (non-reflecting material). A video camera on the ceiling of the open arena was used to score the animals' behavior. Lighting in the room and temperature was the same as the light phase at colony room.

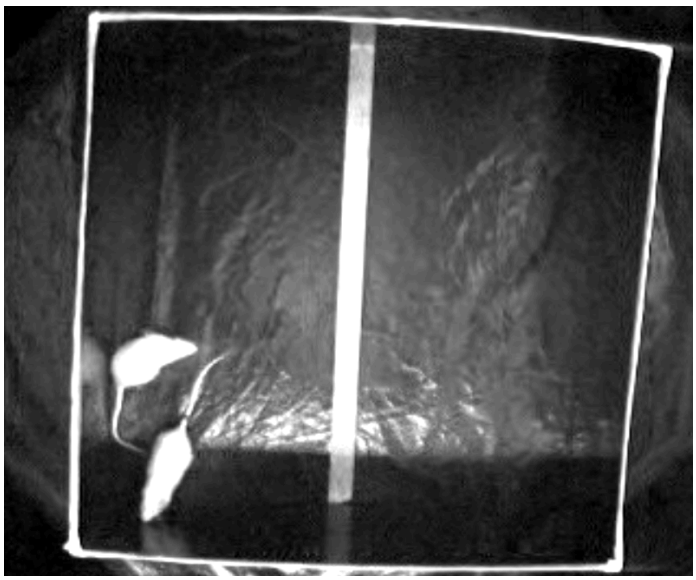

Figure 2 – The Social Interaction Task apparatus

In the present social interaction protocol, the rats were singly housed for 5 days pior the test. This was because social isolation reliably increases the time spent in social contact and the stress-like responses (e.g., heart rate and blood pressure) (Sharp, Zammit, Azar, & Lawson, 2002). Originally, this period of housing isolation was stipulate in 5 days, but according Niesink and van Ree (1982) 4-7 days of individual housing was sufficient period to produce a maximal peak of interaction during the test. In our protocol, animals were singly housed 5 days prior the test. The cages were located together in racks so that auditory and olfactory contacts were maintained.

Two days prior the test, the animals were familiarized with the test arena. For that purpose, each animal was allowed to explore the apparatus during two 7-min trials (one per day) Between each trial, the arena was cleaned with 10% ethanol and wiped. In the day of the test, each rat was tested for social interaction with an unknown test partner (from another litter, not from experimental or control groups) that not differs by more 80g in weight.

Each trial of social interaction test had 7-min duration and was video recorded for posterior analysis. The arena was cleaned and wiped between each trial. For the present experiment 6 rats from each MS groups and 6 rats from control group were tested. The rats were randomly selected.

Social behavior was analyzed in terms of *frequency* and *time* and have been classified in 4 different categories: **explorative behavior**, **non-aggressive** (healthy social interaction), **aggressive behavior** and **others** (see Table 1). Two investigators blind to the experimental condition analyzed the videotapes off-line.

Table 1
Rodent's behaviors assed in the Social Interaction Test

Exploration	Rearing	The state in which rodent' s body is vertically erected sniffing the air
	Movement without social interaction	Movements in arena, sniffing and inspecting the arena. This includes stereotyped walking along specific routes
Non-aggressive behaviors	Investigation	Sniffs at and investigates the other rat
	Nosing	Both rats meet nose to nose while stretching their body slightly
	Follow	The rat follows the other rat, with a distance of 20cm or less between the rats.
	Approach	The rat approach the other rat rapidly or changing the route exhibited till then.
	Grooming	Grooming between the rats
	Play behavior	Any type of chase, spar, pin or wrestle without any type of aggressive intention
Agressive behaviors	Lateral threat	The body is arched in a sideward posture towards the other rat
	Upright	The rat is standing on its hind legs and his facing the opponent
	Stand over and lie under	One rat is standing on top of the other rat, while the other is lying on its back beneath the first rat
	Clinch (kicking, boxing, wrestling, agressive grooming or biting	Active fighting between the rats
	Purse	The rat runs after the other rat during a fight
	Escape	The rat runs away from the other rat during a fight
Other type of behavior	Inactive	Sitting quietly
	Stationary stereotyped behavior	The rat is stationary and performs circular head movements o head weaving
	Movement stereotyped behavior	Body circling in the same position, digging, spontaneous activity, jumping... (note: do not include exploration of the arena in stereotype routes)
	Self-involved behaviors	The rat is stationary and is self-grooming, scratching, washing or involved in another type of self-injurious behaviors

Tissue preparation

All animals were sacrificed by rapid decapitation and blood samples were collected. The adrenal glands from all animals were also removed and weighted. The brain was removed and different areas were dissected using a microscope and following orientation marks provided by Paxinos and Watson (2005). Samples were snap-frozen by immersion in liquid nitrogen for posterior total RNA extraction.

Gene expression analysis

Total RNA was isolated from the left hippocampal formation, pre-frontal cortex, extended amygdaloid complex, and hypothalamus 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 (cycling parameters: 5min at 25°C, 60min at 42°C, and 5min at 85°C).

Using a standard reverse transcriptase-polymerase chain reaction (iQ5 real-time PCR Detection System) protocol (Bio-Rad, Hercules, CA, USA), the expression levels of OT-r were assessed for the above described areas of Cont group (n=5), $MS_{2\text{-}15}$ (n=5) and $MS_{7\text{-}20}$ (n=5) animals and duplicates were performed for each sample. The cycling parameters were 1 cycle of 95 °C for 15min, followed by 39 cycles of 94 °C for 15s, annealing temperature was 60°C for all genes analysed for 30s and 72 °C for 30s. Single acquisitions were done at the end of each annealing step. Primers: OT-r *fw*-5'-GTCAATGCGCCCAAGGAAGCTTC-3' and *rw*-5'-CTCTGGCTTGAGCTGCGACGG-3'

Endocrine evaluation

Radioimmunoassay was performed using the blood samples collected 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 CS concentration was determined using a γ counter. The minimum detectable dose is 7.7 ng/ml.

Statistical analysis

Data analysis were carried out using PASW 18.0 software (IBM® SPSS® Statistics) and statistical significance was accepted for p values ≤ 0.05. Whenever the homogeneity of variances or the normal distribution of the sample were not achieved, variables transformations were performed in the following sequence, till meet the criteria: Log_{10}, Square Root (SQRT) and Inverse (1/). Data are presented as mean ± SEM.

Biometric data (total body weight, thymus and adrenal glands weights) were statistically analyzed by one-way ANOVA ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont). Tukey post-hoc test was used to analyze univariate significances.

The Elevated Plus Maze task was analyzed using one-way ANOVA with exception for "entry open arms" variable where Kruskal-Wallis test was performed followed by Mann-Whiney tests with Bonferroni correction. In the same behavioral test, "time spent in the open arms" variable was transformed by Log_{10}.

In the Open Field, Kruskal-Wallis analysis was performed for "number of rearings in center" and "time in periphery" variables. The other Open Field variables were analyzed by one-way ANOVA tests ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont) in which the variables "time spent in the center" and "duration of rearings in center" were submitted to a LOG_{10} transformation, and "number of entries" was transformed by square root.

The Morris Water Maze task was analyzed using repeated measures ANOVA on the average results of the latency time of the four trials in each day. Additionally, we compared averages of latency times between groups for each day with one-way ANOVA ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont). Averages of Day 2 were transformed by LOG10 to meet sample normality criteria. ANOVA significant results were followed by Tukey post-hoc test.

Forced Swimming Test variables were all statistically analyzed by one-way ANOVA test ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont) followed by Tukey post-hoc test for inter-groups significances.

Social Interaction Test was analyzed for time spent in each set of behaviors ("Exploration", "Non-aggressive behaviors" and "Other type of behavior"). "Agressive behavior" variable was not analysed because there were no recordings of agressive behaviors between groups. The threee variables were analyzed by one-way ANOVA ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont) after LOG_{10} transformation of "Other type of behavior" variable.

Data regarding the CS assessment was analyzed using one-way ANOVA ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont). A LOG_{10} transformation of the data was performed for normality achievement before running inferential statistical test.

OT-r gene expression assay was analyzed with one-way ANOVA ($MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ x Cont) for the four areas assessed in real-time PCR. Tukey post-hoc test were performed whenever appropriate.

Finally, we calculated effect sizes (*d*) of the differences accordingly to Cohen's (1988) equation: $d = M_1 - M_2 / \sigma$ where $\sigma = \sqrt{[\sum(X - M)^2/N]}$ and X is the raw score, M is the mean, and N is the number of cases. All large effect sizes ($d \geq 0.6$) were reported, even in non-significant results ($p > 0.05$).

3. RESULTS

3.1. Biometric Data

Although no differences were found for adrenal glands weighs between groups (F (2,42)=7.41, p=0.15), statistically significant differences between groups for total body weight (F (2,42)=4.12, p=.02) and thymus weight (F (2,42)=2.02, p=0.002) were found (see Table 2). Tukey post-hoc tests reveled that $MS_{7\text{-}20}$ rats had heavier thymus comparatively to Control animals (p=0.001); and Control group weight less than $MS_{7\text{-}20}$ group (p=0.02).

Effect size analyzes revealed a large difference between Cont and $MS_{7\text{-}20}$ (d=1.04), $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ (d=0.66) groups concerning body weight. Large effect sizes were also found for thymus weights in the Cont-$MS_{2\text{-}15}$ (d=0.69), Cont-MS_{720} (d=1.38) and $MS_{2\text{-}15}$-$MS_{7\text{-}20}$ (d=0.76) group comparisons. Finally, we also observed a large effect size of the difference between Cont and $MS_{7\text{-}20}$ for adrenal glands weights (d=0.69).

Table 2
Biometric data analysis for Control group and Maternal Separation groups

	Cont (n=11) *M (SD)*	**$MS_{2\text{-}15}$ (n=14) *M (SD)***	**$MS_{7\text{-}20}$ (n=20) *M (SD)***	***F* (2,42)**	**Cont vs $MS_{2\text{-}15}$**	**Cont vs $MS_{7\text{-}20}$**	**$MS_{2\text{-}15}$ vs $MS_{7\text{-}20}$**
Body weigh	389.67 (61.49)	412.44 (40.77)	438.72 (38.96)	4.12	n.s.	*	n.s.
Thymus weigh	.338 (.074)	.291 (.051)	.253 (.049)	2.02	n.s.	**	n.s.
Adrenal Glands weigh	.041 (.008)	.043 (.006)	.047 (.009)	7.41	n.s.	n.s.	n.s.

Note: * $p \leq 0.05$; ** $p \leq 0.01$

3.2. Adult behavioral consequences of stress exposure in two different developing periods, in terms of spatial memory, anxiety, depressive like- and social interactions.

Open Field

In the OF, a test for exploratory and locomotor activities assessment, there were no statistical differences between experimental and control groups regarding the total distance travelled in the arena, F (2,42)=1.62, p=2.09 (Figure 4A), the number, F (4,42)=1.25, p=2.96, and the duration of rearings, F (2,42)=1.38, p=0.26 (Figure 4B), or time spent in the center of arena, F (2,42)=1.62, p=2.09 (Figure 4C). Beside the non-significant results in the total number of rearings between groups, the effect size of the difference between Control and MS2-15 rodents is d=0.67 (Cohen's d).

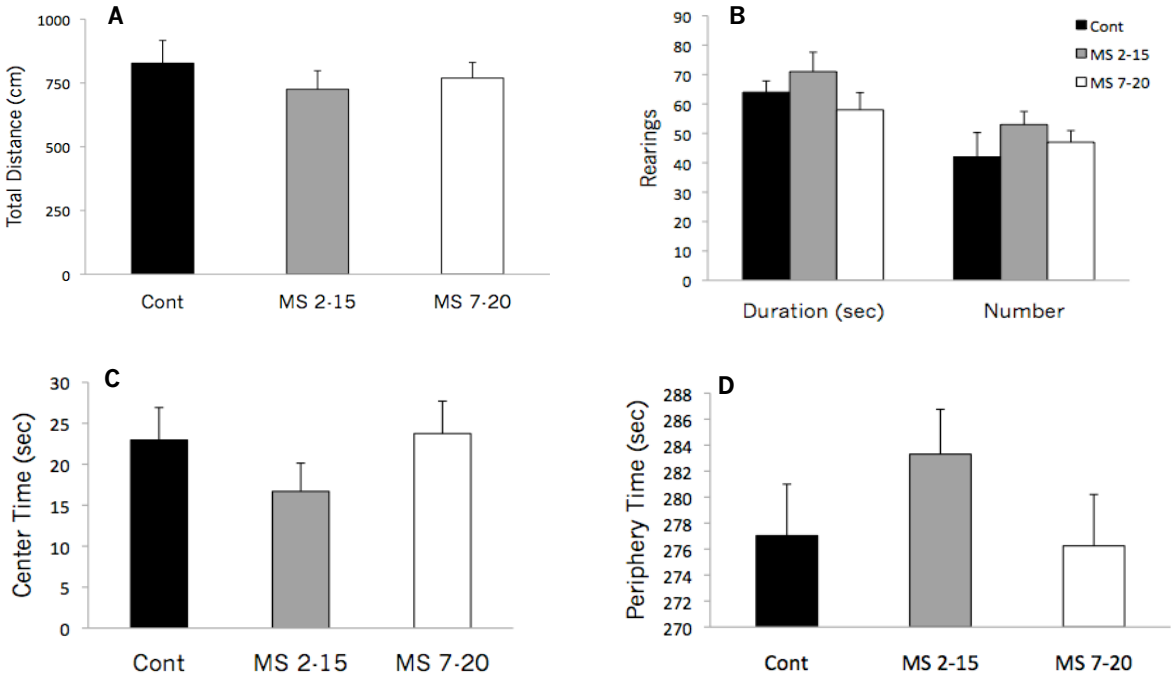

Figure 3 - Locomotor and exploratory activity assessed in the Open-Field. No significant differences were found in the total distance travelled in the arena (**A**) as well as for the number and duration (seconds) of vertical exploratory activity (rearings) (**B**) time spent in the center of the arena (**C**) or time spent in the periphery (**D**). All results are expressed as mean ± SEM. Large size effect was found between Cont and $MS_{2\text{-}15}$ groups in the number of vertical exploratory activity (d=0.67).

Elevated Plus Maze

Analysis of the EPM revealed a significant effect of MS in the number of open arms entries ($\chi^2(2) = 12.28$, p=0.002). Mann-Whiney tests with Bonferroni correction reveled that the number of entries in open arms is significantly higher for Control group than for $MS_{2\text{-}15}$,Z=3.1, p=0.002, and $MS_{7\text{-}20}$,Z=3.2, p=0.002, groups (Figure 3A). No significant differences were found between groups in the time spent in open arms, F (2,41)=0,39, p=0.68, and time spent in close arms, F (2,41)=1.24, p=0.30 (Figures 3B and 3C). Regarding the effect size analyzes, there were large differences between Cont group and MS groups ($MS_{2\text{-}15}$,d=1.73; $MS_{7\text{-}20}$,d=1.66) in the total number of open arms entries.

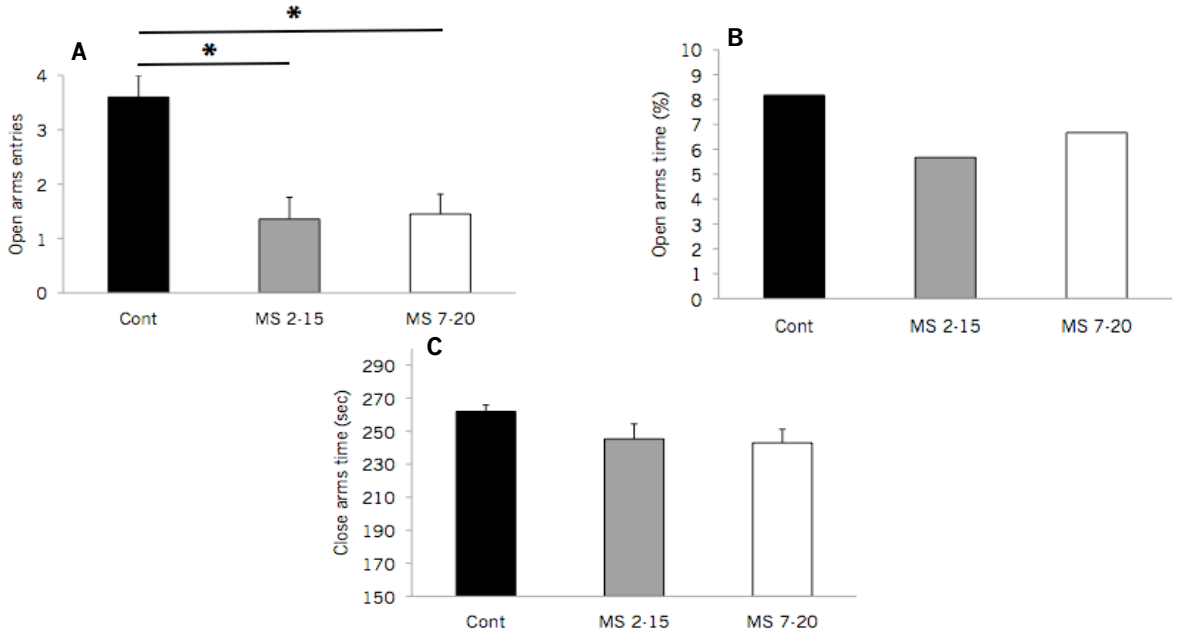

Figure 4 - Anxious-like behavior evaluation in the Elevated Plus Maze. Significant differences between Cont and experimental groups in the number of open arms entries (**A**).No satistically significance between groups in the percentage of time spent in the open arms over the total time (**B**), or time spent in closed arms (**C**) there were found. Results are expressed as mean ± SEM. * $p < 0.017$

Forced Swimming Test

Regarding the depressive like-behavior evaluated with the FST, a differential effect of earlier vs later maternal separation was observed in latency, F (2,41)=7.92, p<.001, activity, F (2,41)=4.41, p=0.02, and immobility, F (2,41)=4.41, p=0.02, times. Tukey post-hoc test reveled that $MS_{2\text{-}15}$ displayed a significant lower latency time compared to control rodents, p<.001; while, a marginally significant difference to $MS_{7\text{-}20}$, p=0.06 (Figure 5). Regarding the immobility and activity times, a significant decrease was observed in $MS_{2\text{-}12}$ compared to Cont, p=0.04, and $MS_{7\text{-}20}$, p=0.03. An inversed result with the same statistical significance was observed for the activity data.

Concerning effect sizes for latency times, there were large differences between Cont and $MS_{2\text{-}15}$ (d=1.71) and $MS_{7\text{-}20}$ (d=0.70), as well as between the two experimental groups (d=1.02). Large differences were also found between $MS_{2\text{-}15}$-Cont (d=0.95), and $MS_{2\text{-}15}$-$MS_{7\text{-}20}$ (d=1.13) for both activity and immobility times.

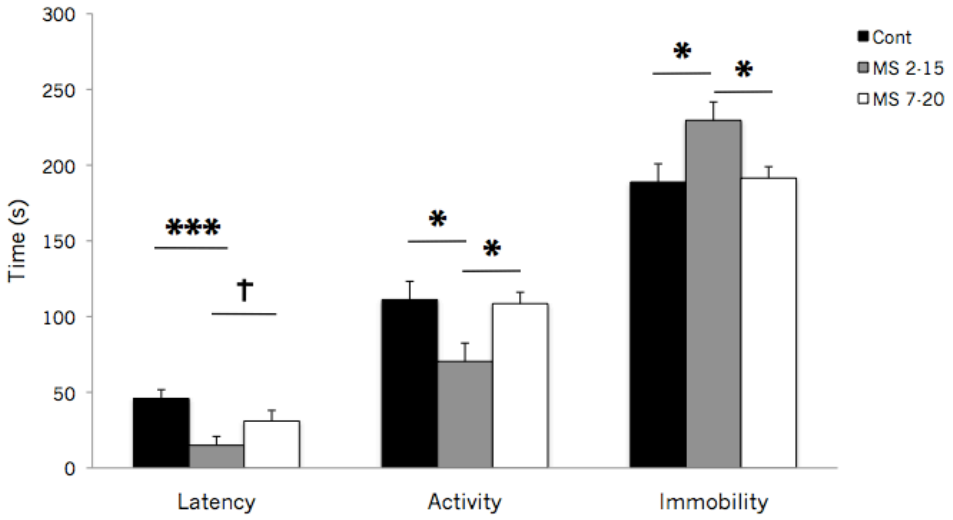

Figure 5 - Results from the Forced Swimming test. Depressive-like behavior was assessed using the latency, activity and immobility times. *** $p < .001$; * $p < 0.05$; † $p < 0.10$. All results are expressed as mean ± SEM.

Morris Water Maze

Analysis from repeated measures ANOVA failed to reveal a significant effect of MS on spatial memory performance. However, a marginally significant effect of maternal separation was observed in the third day, $F(2,42)=2.76$, $p=0.08$, when $MS_{2\text{-}15}$ group express higher latency times to find platform than Control group, $p=0.06$. For more details, see Figure 6.

There were also large differences between Control and $MS_{2\text{-}15}$ group ($d=0.92$), Control and $MS_{7\text{-}20}$ group ($d=0.86$) in the third day of the test; and an effect size of $d=0.92$ in the difference between Control and $MS_{7\text{-}20}$ groups in the fourth day of MWM.

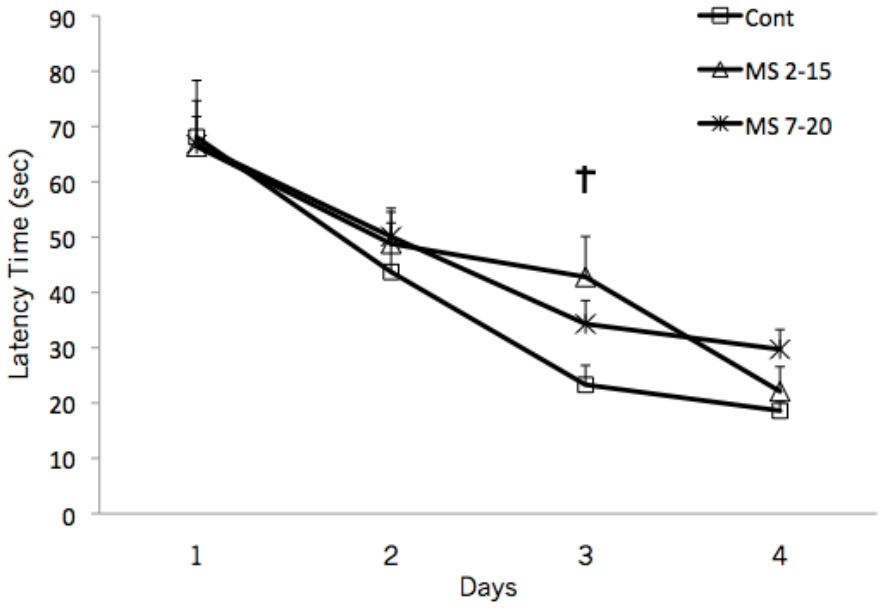

Figure 6 – Morris Water Maze performance. Spatial learning memory was assessed by the latency time swum to find a submerged platform. All results are expressed as mean of time of the 4 trials in 4 consecutive days ± SEM. † $p < 0.10$.

Social Interaction Test

There was no statistically evidence for the effect of maternal separation on the social behavior of adult rats assessed by SIT. No statistical differences were found between groups in the time spent with non-aggressive behaviors, F (2,15)=0.27, p=0.76 (Figure 7A), time exploring the arena, F (2,15)=2.22, p=0.14 (Figure 7B) or time spent on other type of behaviors such as, for example, self grooming, F (2,15)=0.73, p=0.50 (Figure 7C).

The effect size of the difference between Control and $MS_{2\text{-}15}$ groups in time spent exploring the arena is d=1.28; and d=0.69 for the difference between Control and $MS_{7\text{-}20}$ groups. Regarding to "other type of behaviors", the effect size of the difference between later maternal separated rats and control group is d=0.66.

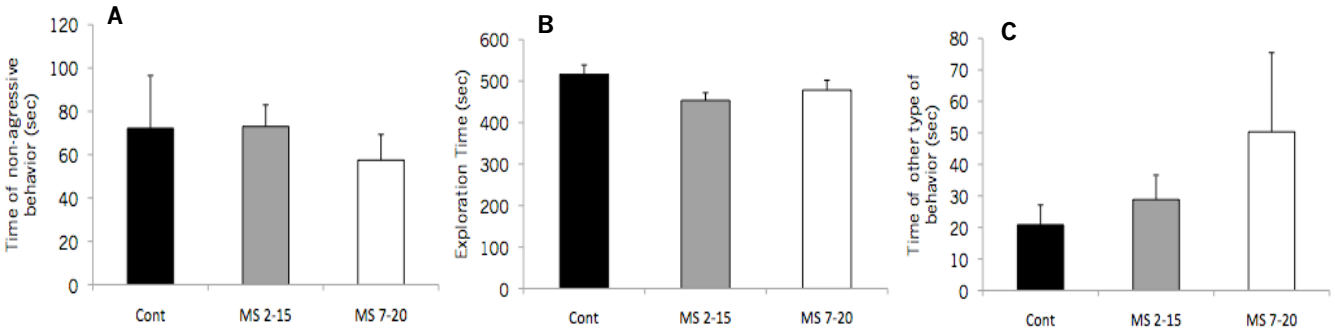

Figure 7 – Social Interaction Test results. All results are expressed as mean ± SEM for all set of behaviors. No statistically differences were found between groups.

3.3. Long-term consequences of early life maternal deprivation on corticosteroid system

In order to determine the impact of the early maternal deprivation in the corticosteroid system, we assessed the basal corticosterone levels at 5 months of age. Although one-way ANOVA analysis did not reveal significant differences in basal corticosterone levels between groups, F (2,30)=0.61, p=0.55 (Figure 8), a large effect size were found in the differences between control group and the two experimental groups (d=0.74 for $MS_{2\text{-}15}$ and d=0.57 for $MS_{7\text{-}20}$).

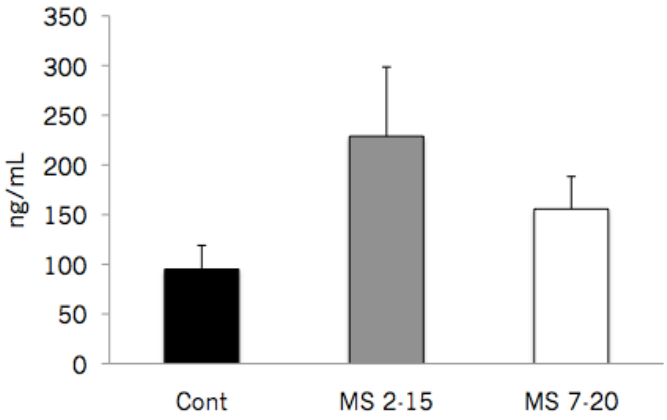

Figure 8 – Radioimmunoassay results expressed in ng/mL. All results are presented as mean ± SEM. No statistically differences were found between groups.

3.4. Long-term consequences of early life maternal deprivaion on oxytocinergic central pathway

One of the purpose of the present thesis was to understand how early life stress affects the oxytocinergic central system. In order to answer this issue, we assessed the OT-r expression in specific stress-sensitive areas: extended amygdala, prefrontal cortex, left hippocampus, and hypothalamus. No significant differences were found between groups in OT-r expression in amygdala, F (2,12)=1.56, p=0.25 (Figure 9A), prefrontal cortex, F (2,12)=1.63, p=0.24 (Figure 9B), and hypothalamus, F (2,12)=0.57, p=0.58 (Figure 9C). Regarding the OT-r expression in the hippocampus, marginally univariate differences were observed between groups, F (2,12)=3.09, p=0.08, with Cont animals expressing more receptors for oxytocin than $MS_{2\text{-}15}$ group, p=0.08, in this area (Figure 9D).

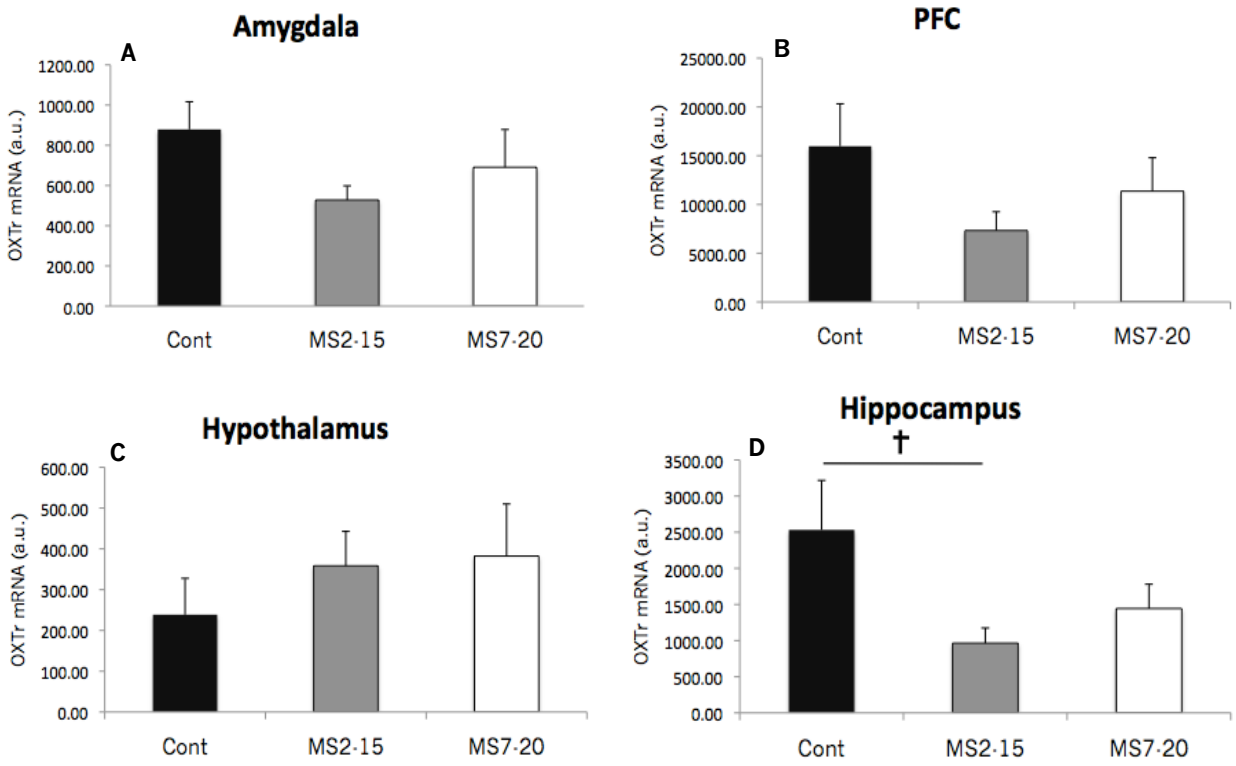

Figure 9 – Real-time PCR analysis of OT-r in the amygdala (**A**), prefrontal cortex (**B**), hypothalamus (**C**) and hippocampus (**D**) of Cont and MS animals. OT-r expression is normalized for housekeeping gene (HPRT) and are expressed as mean ± SEM. † $p < 0.10$.

Amygdala OT-r expression data reveled an expressive effect size in the difference between Cont and $MS_{2\text{-}15}$ groups (d=1.43). In the PFC results, the effect size of the difference between Cont and $MS_{2\text{-}15}$ rats is d=1.15; the effect size of the difference between the two MS groups is d=0.65. In the hypothalamus region, the effect size of the difference between Cont and $MS_{2\text{-}15}$ rats is d=0.62. Finally, regarding the left hippocampus region, the effect size of the difference between Cont and MS groups are d=1.38 and d=0.90 ($MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ respectively); additionally an effect size of d=0.76 was also found between $MS_{7\text{-}20}$ and $MS_{2\text{-}15}$ groups in this region.

Table 3

Maternal Separation effects on behavioral, neuroendocrinal and neurochemical phenotypes.

	Control *M ± SD*	$MS_{2\text{-}15}$ *M ± SD*	$MS_{7\text{-}20}$ *M ± SD*	Control x $MS_{2\text{-}15}$ *p* values	Cohen's *d*	Control x $MS_{7\text{-}20}$ *p* values	Cohen's *d*	$MS_{2\text{-}15}$ x $MS_{7\text{-}20}$ *p* values	Cohen's *d*
Biometric data									
Body weigh (g)	389.67 ± 61.49	412.44 ± 40.77	438.72 ± 38.96	n.s.	1.04	p < 0.05		n.s.	0.66
Thymus weigh (g)	0.334 ± 0.064	0.290 ± 0.051	0.253 ± 0.049	n.s.	0.69	p < 0.01	1.38	n.s.	0.76
Adrenal glands weigh (g)	0.041 ± 0.007	0.043 ± 0.006	0.047 ± 0.008	n.s.		n.s.	0.69	n.s.	
Behavioral data									
Elevated Plus Maze									
Open arms entries	3.6 ±.99	1.4 ± 1.50	1.5 ± 1.64	p < 0.01	1.73	p < 0.01	1.66	n.s.	
Open arms time (seg)	25 ± 12.71	17 ± 23.91	20 ± 21.04	n.s.		n.s.		n.s.	
Close arms time (seg)	262 ± 29.85	245 ± 33.98	243 ± 36.89	n.s.		n.s.		n.s.	
Open Field									
Rearings – duration (seg)	64.38 ± 19.38	71.02 ± 24.70	58.32 ± 21.30	n.s.		n.s.		n.s.	
Rearings - number	42.09 ± 12.66	52.71 ± 16.68	47.40 ± 18.51	n.s.	0.67	n.s.		n.s.	
Total distance (cm)	827.61 ± 296.03	725.32 ± 271.32	719.02 ± 245.98	n.s.		n.s.		n.s.	
Center time (seg)	22.96 ± 13.14	16.69 ± 12.90	23.75 ± 17.68	n.s.		n.s.		n.s.	
Periphery time (seg)	277.04 ± 13.14	283.31 ± 12.90	276.25 ± 17.68	n.s.		n.s.		n.s.	
Forced Swimming Test									
Latency (seg)	45.9 ± 23.0	15.0 ± 11.0	31.0 ± 19.3	p < .001	1.71	n.s.	0.70	p < 0.10	1.02
Activity (seg)	111.2 ± 50.1	70.4 ± 33.8	108.6 ± 33.7	p < 0.05	0.95	n.s.		p < 0.05	1.13
Immobility (seg)	188.8 ± 50.1	229.6 ± 33.8	191.5 ± 33.7	p < 0.05	0.95	n.s.		p < 0.05	1.13
Morris Water Maze									
Day 1 (seg)	68.1 ± 34.0	66.4 ± 30.9	66.8 ± 22.3	n.s.		n.s.		n.s.	
Day 2 (seg)	43.7 ± 29.4	48.8 ± 24.0	50.1 ± 19.8	n.s.		n.s.		n.s.	
Day 3 (seg)	23.3 ± 11.6	42.8 ± 27.4	34.3 ± 18.9	p < 0.10	0.92	n.s.	0.86	n.s.	
Day 4 (seg)	18.7 ± 5.7	22.1 ± 16.7	29.7 ± 16.0	n.s.		n.s.	0.92	n.s.	
Social Interaction Test									
Non-aggressive behavior (seg)	72.2 ± 59.7	73.0 ± 24.4	57.5 ± 29.0	n.s.		n.s.		n.s.	
Investigation (seg)	517.2 ± 53.9	453.7 ± 45.0	478.5 ± 58.3	n.s.	1.28	n.s.	0.69	n.s.	
Other behaviors (seg)	20.8 ± 15.4	28.8 ± 19.0	50.3 ± 61.5	n.s.		n.s.	0.66	n.s.	
Radioimmunoassay (ng/mL)	95.10 ± 79.42	228.87 ± 241.58	155.66 ± 103.52	n.s.	0.74	n.s.	0.65	n.s.	
mRNA OXTr expression (a.u.)									
Amygdala	878.83 ± 308.11	525.70 ± 158.59	690.55 ± 420.33	n.s.	1.43	n.s.		n.s.	
PFC	15968.42 ± 9740.81	7323.62 ± 4314.56	11356.41 ± 7675.91	n.s.	1.15	n.s.		n.s.	0.65
Hippocampus	2532.05 ± 1531.73	964.54 ± 469.49	1443.49 ± 752.79	p < 0.10	0.62	n.s.	0.90	n.s.	0.76
Hypothalamus	238.59 ± 199.34	358.37 ± 188.93	382.14 ± 285.82	n.s.	1.38	n.s.		n.s.	

Note. Only medium and large Effect Sizes (Cohen'*d*) are presented ($d \geq 0.6$).

4. DISCUSSION

The objectives of the study presented in this thesis were to examine the long-lasting *imprinting* effects of adverse early life experiences on the behavior phenotype, HPA axis, and oxytocinergic system in the rat. For this purpose Maternal Separation (MS) paradigm was used in two different "temporal windows" of rodent's development. Generally, when repeatedly separating rat pups from the dam, significant changes on behavior phenotypes and oxytocinergic system are detected. Furthermore, the present results demonstrate the effect of age of separation on the rat development, with early maternal separated rats showing worst behavioral outcomes and pronounced changes in OT-r expression in some brain areas. Taken together, our findings suggest that adverse early life experiences can have subtle effects on neurobiological development concomitant with behavioral expression. Additionally, the earlier these negative experiences occur, the more implications they have in the development of the neurobiological systems. Throughout this discussion, the results and the most significant clinical implications of our work will be explored.

Maternal separation and metabolic parameters

Early exposure to adverse experiences, associated to chronic stress, is known to contribute to the development of metabolic disorders such as diabetes (Lesage, Del-Favero, Leonhardt et al., 2004), cardiovascular disease (Felitti, Vincent, Anda, Robert, & Nordenberg, 1998; Louey & Thornburg, 2005) or body weight gain (Wright, Parkinson, & Drewett, 2006). Data from MS studies with rodents showed that early maternal separation experiences could promote hyperphagia and weight gain (Ryu, Yoo, Kang, Lee, & Jahng, 2009) and significant increases in heart rate throughout stress periods (Sanders, Anticevic, 2007). The present results also show the same relationship between early stress exposure and body weight gain, in which $MS_{7\text{-}20}$ rats significantly increase their weight when assessed in adulthood. Surprisingly, $MS_{2\text{-}15}$ animals did not significantly differ from control ones, although the large effect size in the difference between groups.

Thymus and adrenal glands weight could be important indicators of HPA axis functioning and CS impact. Although indirectly, our data support the idea that early life stress triggers negative consequences on immunological functioning, with MS rats expressing smaller thymus. On the other hand, the deregulation of HPA axis in MS rats could be detectable by the higher weights of MS adrenal glands, even though the differences between groups were not statistically different which leads to cautious when interpretating these data.

Maternal separation may trigger HPA axis deregulation

Although univariate analysis did not show statistical differences between groups on the plasma corticosterone levels, according to Cohen's *d* more than 70% of control group animals are below the CS basal average levels of $MS_{2\text{-}15}$ and $MS_{7\text{-}20}$ groups. We hypothesize that these nonstatistical differences may be explained by the small size of the sample. For this reason, our results should be analyzed with some caution. Since the Cohen's *d* values found in our research is in agreement with previous results in literature, we consider that our findings could provide some contribution to the field. We found a possible effect of period of separation on the HPA axis deregulation

when CS plasma levels were assessed. Early maternal separation group presents higher levels of circulating CS at adult age. Accordingly to our findings, previous studies found the same relationship between the development of the HPA axis and the early mother-infant bond disruption (Levine, Huchton, Wiener, & Rosenfeld, 1991; Mesquita, et al., 2007; Schmidt, Enthoven, Van Der Mark, Lenive, De Kloet, & Oitzl, 2003). One of the main features of maternal separation is the deprivation of the pup from a wide variety of environmental cues in the first days of life, namely food, body temperature regulation and maternal stimuli, many of which are essential for survival and non-pathological development. Furthermore, the ontogeny of different biological systems seems to be sensitive to the intensity of environmental stimuli and, more importantly, by the period in which stressor exposure occurs. Maternal separation procedures performed in the present study comprised two different "temporal windows" to assess more precisely the *imprinting* effects of different periods of stress exposure. Therefore, the relative state of "hypercorticalism" found in $MS_{2\text{-}15}$ group could be firstly explained by the chronically stressor exposure (repeated separations from dams for several days) and, secondly, by the stress programming and ontogeny effects on the receptors involved in the HPA feedback processes. In fact, the early period of separation takes place in the hypo-responsive period (SHRP) being hypothesized to have long-term implications on neural development, especially on the HPA axis maturation (Levine, 1994). During this period, comprising the first three postnatal weeks of life, the negative feedback mechanism of the HPA axis is not completely mature, exposing the organism to a susceptibility condition to the imprinting effects of stressor exposure (Pryce, 2008). In accordance to aforementioned data we also observed herein that $MS_{7\text{-}20}$ group also had higher levels of CS in plasma than control animals. Although not assessed in the present study, the differences in the CS plasma levels between groups allow us to speculate a possible downregulation of GR- and CRF-receptors density in important brain areas involved in the HPA axis negative feedback process. These possible neurochemical changes lead to HPA axis deregulation, expressed by higher production and release of CRF, ACTH and CS as documented in previous studies (e.g. Boyle, Brewer, Funatsu, et al., 2005; Kalinichev, Easterling, Plotsky et al., 2002; Nemeroff, 1996).

Early life stress exposure and exploration behavior

Regarding the question of early stress exposure interference on exploratory and basal locomotor behaviors, this study failed to reveal statistical evidence that MS interferes on the exploratory phenotype at adulthood. In fact, no statistical significant results were found in Open Field data analysis. Likewise, no differences between groups were observed in exploration of closed arms in Elevated Plus Maze (data not shown), as well as in the exploratory behaviors in Social Interaction Test (SIT), corroborating the assumption of no relationship between MS exposure and adult basal locomotor and exploratory behaviors' disruption. These results differ from previous reports, which demonstrated a reduction in exploratory locomotion and reduced exploration of the center of arena in MS rats compared to not stressed rats (Caldji, Diorio, & Meaney, 2000). However, it should be pointed out that the exploratory and basal locomotor behaviors are measures intrinsically related to fearfulness/anxiety phenotype.

When MS rats are assessd for these two set of behaviors, the findings on the literature are very inconsistent. Accordingly to some reports, MS increases fearfulness and reduces exploration (Wigger & Neumann, 1999; Shalev, Kafkafi, 2002). On the other hand, some researchers found MS to decrease fearfulness and increase exploratory behaviour (von Hoersten, Dimitrjevic, Markovic, & Jankovic, 1993). In spite of this incongruence, our data is in line with Kaneko, Riley and Ehlers (1994), which also failed to find differences between early separated and control groups in the Open Field test. However, these contradictory reports in the MS literature and our failure to observe significant differences may be, in part, due to differences in the methodology used. For example, light/dark schedules in this study contrast with other MS studies (e.g. Caldji et al., 2000; Huot, Thrivikraman, Meaney, & Plotsky, 2001); differences in the assessment methods could be found (e.g. Matthews, Dalley, Mathews, Tsai, & Robins, 1996); and our rats' strain (Wistar) contrast with other experiments (e.g. Kaneko, Riley, & Ehlers, 1994).

In summary, despite the large difference reveled by Cohen's *d* between $MS_{2\text{-}15}$ and control group in the SIT assessment, in general this test together with OF and EPM have been unable to demonstrate that MS experience disrupts exploratory and locomotor behaviors in adulthood.

Early life stress exposure and anxious phenotype

As adults, maternally separated pups have shown increased emotionality/anxiety in several test conditions (Kalinichev, Easterling, Plotsky, & Holtzman, 2002). In accordance with the literature, we confirmed that both MS groups displayed a hyperanxious phenotype when assessed in EPM. The EPM's principle is based on the inner conflict between exploration and avoidance of novel environments. We found that the number of entries in the open arms were dependent of early life adverse experience. Relatively to the neuroendocrinal mechanisms beyond such behavior, the state of hypercorticalism, also reported in our results, is known to influence anxiety (Pêgo, Morgado, Pinto, Cerqueira, Almeida, & Sousa, 2008). We hypothesize that the number of entries in the open arms could be understood as a measure of risk taking perception. In other words, it could be possible that the number of entries in open arms is inverse to the risk taking in the face of challenging events. In fact, human studies reveled that people with higher social anxiety levels presents approach-avoidance conflicts about a possible social involvement. These conflicts were more intense for social interactions but there are also evidence for non-social risk taking behaviors (Kashdan, Elhai, & Breen, 2008). It is possible that, in this study, MS rats express higher anxiety levels with the reduction of taking risk behaviors when exposed to challenging events (open arms in EPM).

No differences between groups were found for the time spent in open arms. Although not assessed, this data may be due to a possible freezing behavior of MS animals in the open arms. When MS rats enter in the open arms, freezing behavior (normally expressed by high anxious animals in new environments) could increase the time spent on this novel environment, approaching the controls' time values. In order to validate (or refute) our hypothesis, future studies exploring this $MS_{2\text{-}15}$ behavior at EPM open arms are therefore recommended.

Early life stress and depressive-like behavior

The FST revealed significant differences for latency to immobility, immobility and total activity times between $MS_{2\text{-}15}$ and Control groups, meaning that early separated rats displayed increase learned-helplessness, a core symptom of depression. Interestingly, the differences between MS groups are also significant, with $MS_{7\text{-}20}$ group expressing less depressive-like behavior. There are similarities between the depressive-like behaviors expressed by $MS_{2\text{-}15}$ in this study and those previously described in our laboratory by Mesquita (2008), but also by other authors (Khoury, Gruber, Mork, & Mathe, 2006). Interestingly, Marais, van Rensburg, van Zyl, Stein, and Daniels (2008) also found that MS increases the vulnerability of the rat to develop depressive-like behavior when subsequently exposed to other kind of chronic stress. There are some interesting explanations for this behavioral disruption (or MS imprinting effect). Changes on CS milieu, also observed in MS procedures, is recognized to precipitate depressive like-behaviors (Johnson, Fournier, & Kalynchuk, 2006). In fact, chronic exposure to stress leads to HPA axis changes with consequent neuronal remodeling in key brain regions associated with depressive-like behaviors, namely the hippocampus (Magarinos, McEwen, & Flugge, 1996) amygdala (Vyas, Bernal, & Chattarji, 2003) and medial pre-frontal cortex (Cook & Wellman, 2004). Furthermore, another explanations for depressive phenotype of maternally separated rats could be found taking in account the action of CS on different stress-sensitive neural substrates. For example, some recent data suggest that MS could produce depressive-like behaviors though activation of proinflammatory processes (for review, Hennessy, Schiml-Webb, & Deak, 2009; Kim et al., 2008). In addition, corticosteroid receptors are known to program the functional development of monoaminergic systems. MS experience-induced alterations of the CS system ontogeny have been related to the developmental programming of central catecholaminergic and serotoninergic activity which may have a permanent impact on the animals intellectual and socio-emotional competences (Braun, Lange, Metsger, & Poeggel, 2000; Matthews, Dalley, Matthews, Tsai, & Robins, 2001; Mesquita et al., 2007).

In human studies, there is a growing evidence demonstrating that exposure to traumatic events during childhood can increase later risk for mood disorders (Heim & Nemeroff 2001; Vythilingam, Heim, Newport, Miller, Anderson, Bronen, et al., 2002). Likewise our present data, suggest that early life represents a vulnerable period during which exposure to chronic stress can bias the development of neural systems mediating emotion towards depression later in life.

Early life stress and spatial memory

Our data failed to reveal an influence of early MS in spatial learning performance later in life. On Morris Water Maze (MWM); only at the day 3 $MS_{2\text{-}15}$ group presented a significant impaired performance compared to control group. Accordingly to literature, MS rats are liable to exhibit a stress hyperresponsive HPA axis in adulthood that could contribute to the cognitive deficits, namely learning and memory (Aisa, Tordera, Lasheras, Río, & Ramírez, 2007). In a recent study, Aisa, Elizalde, Tordera, Lasheras, Rio, and Ramírez (2009) found that MS experience reduces the increase of NCAM expression (one of the main plasticity markers with key roles in neural

development and in the synaptic plasticity in the adult brain) after MWM task. Cell proliferation was also decreased in the dentate gyrus of MS rats. In fact, this brain structure still goes through a stage of dynamic development during early postnatal life, being susceptible to external stressors (Jarrard, 1993) Although, some experimental data point that neurodegenerative and marked atrophy of apical dendrites effects of CS on hippocampus neurons could contribute to profound cognitive impairment after chronic stress exposure (Lu, Goula, Sousa, & Almeida, 2003). In fact, the present study has been unable to clearly demonstrate that MS exposure have imprinting effects on learning performance. However, when compared to control rats, MS rats performance shapes subtle memory impairment (c.f. MS group means). Further research should be done to investigate the neural chances of maternally separated rats.

MS have long lasting implications on oxytocinergic system

It was also our goal to explore the cross-talk between the stress response, triggered by early life stress, and the oxytocinergic system mainly known by its anxiolitic effects. According to this view we explored the expression of OT-r in critical brain sites. The present study showed possible brain region-specific changes in OT-r mRNA expression after early stress exposure. Effect size analyses reveled robust differences between control and $MS_{2\text{-}15}$ animals in OT-r mRNA expression in all four brain areas assessed. MS exposure leads to a robust decreased in the oxytocin receptors expression in amygdala, prefrontal cortex and in the left hippocampus. Most notably, for the $MS_{2\text{-}15}$ group a 2-fold decreased in OT-r mRNA expression was observed in the PFC and a 3-fold decreased in the left hippocampal formation. Even though the large effect sizes observed, the ANOVA (one way) analysis failed to reveal statistically significant for amygdala and prefrontal areas. Interestingly, in the hypothalamus the differences between groups showed an inversed tendency, with experimental groups expressing higher values in the RT-PCR for mRNA OT-r. The present results are in line with previous findings assessing OT-r binding capacity in 3h MS rat (Lukas, Bredehold, Neumann, & Veenema, 2010).

A wide range of literature has been exploring the anxiolytic action of OT, showing that the stress-induced central release of OT can ameliorate the stress-associated symptoms such as anxiety (Üvnas, Ahlenius, Hillegaart, & Alster, 1994). It is also well documented that OT can act as an antidepressant in stress conditions (Arletti & Bertoloni, 1987). In fact, synaptic associations between CRH- and OT neurons of the PVN have been demonstrated (Hisano, Kagotani, & Daikoky, 1992).

In response to acute stress exposure and consequent CS elevation, increased OT secretion was reported (Neumann, Johnstone, Hatzinger, Liebsch, Shipston, Russel, et al., 1998). With prolonged (hours to days) OT agonist stimulation there is a downregulation of OT receptors. Since oxytocin regulation is based on a positive feedback mechanism, OT-r dowregulation induced by the high concentrations of OT could be a possible way of oxytocinergic system to react to extreme conditions, preventing imminent collapse of the system. In this study we hypothesize that the hypercorticalism state of MS animals (high plasmatic CS levels) would lead to increased release of OT which, rises above a specific threshold, inducing OT-r downregulation. Limbic regions such as

hippocampus, amygdala, and prefrontal cortex are specific brain areas involved in negative feedback inhibition of the HPA axis, and possible targets for OT anxiolytic action. Together with data from Lukas and collaborators (2010), our data found a decrease of mRNA OT-r in these areas. Thus, one speculates that early life stress (MS) stimulates CS release and consequent OT levels increase, leading to a downreagulation of OT-r expression. Interestingly, in the hypothalamus (OT producing area) a higher mRNA OT-r expression was found in MS groups comparing with controls. Contrasting with other brain regions, the hypothalamus is one of the area with less OT binding sites detectable across life (for review Gimpl & Fahrenholz, 2001). Furthermore, if OT-r expression begins in intrauterine life for almost brain areas, the development of OT receptors in hypothalamus begins only in the first days of postnatal life (Yoshimuraa, Kirnurab, Watanabea, & Kiyama, 1996). For these reasons, it is possible that the OT-r in hypothalamus are less susceptible to the effects of MS.

The areas analyzed in this study are also intrinsically related to some social and other assed behaviors. Lesion studies with monkeys revealed the amygdala's role in the evaluation of potential dangers (Amaral, 2003). In the rat, this area is involved in the processing of olfactory cues (Ferguson, Aldag, Insel, & Young, 2001) and is the core regulator of fear behavior (Walker, Toufexis, & Davis 2003). Acting synergistically with CS levels, the possible long lasting changes on OT levels induced by MS experience may have changed the processing of olfactory cues in social contacts and fear/anxious behaviors in adulthood.

Some hypothalamic nuclei are biologically optimized to generate inter-male aggression (Toth, Fuzesi, Halasz, Tulogdi, & Haller, in press), while others are associated to maternal and paternal care (Bosch, Pförtsch, Beiderbeck, Landgraf, & Neumann, 2010) or regulation of partner preference formation (Young, Gobrogge, Liu, & Wang, in press). In this area, our data showed high levels of mRNA OT-r in MS groups compared to control one, and so OT levels are likely to be altered.

The prefrontal cortex is known to control/process motor programming, inhibition responses, temporal ordering, spatial orientation, social or affective behaviors, olfaction cues (for review Kolb, 1984). OT-r binding in this brain area may influence the processing of a many set of behaviors, such as social recognition, play-fighting and aggression, learning flexibility, working memory or high risk behaviors. We found that $MS_{2\text{-}15}$ group present the lower mRNA OT-r levels in this area that can result in cognitive and social impairments.

Recently, oxytocin was found to have a neuroprotective function against immature hippocampal cultures' hypoxia (Ceanga, Spataru, & Zagrean, 2010). Our results showed decreased mRNA OT-r in $MS_{2\text{-}15}$ group in the left hippocampus leading to suppose changing in OT levels in this same area. Its possible that MS experience has deleterious effects on the hippocampus neural networks not only by CS elevated levels, but also by changes on the OT inner-brain levels. In this line, some learning and memory phenotypes found in MS studies could also be explained by OT central pathway changes.

Further work is required to establish the relationship between OT change levels in specific brain neural pathways after chronic stress exposure.

Early life stress and social behavior

The assessment of mRNA OT-r in the described areas was also based on their possible implication in social behavior and social relationship establishment. In this particular study, using the SIT, MS failed to reveal significant changes in social behavior at adult age and no aggressive behaviors were observed. However, Cohens *d* values reveled an effect of MS (early and later) in the arena exploration (discussed elsewhere) and an increased number of self-grooming by $MS_{7\text{-}20}$ group.

There is increasing evidence from rodent studies that early conditions of deprivation and emotional disruption increase intermale aggression (Veenema & Neumann, 2009), inattentive and impulsive behavior (Braun, Kremz, Wetzel, Wagner, & Poeggel, 2003; Colorado, Shumake, Conejo, Gonzalez-Pardo, & Gonzalez-Lima, 2006) or self-involved behaviors (Hofer, 1976). Non-human primates exposed to prolonged periods of maternal deprivation also reveled the same marked behavioral changes, such as increased social dysfunction or aggression (for review, see Sanchez, Ladd, & Plotsky, 2001). The maternal separation procedure, used with non-human primates or rodents, has been widely used as a viable model to mimic the effects of inconsistent or inadequate early caretaking on human development. In fact, in humans, non-normative early life experiences (institutionalization experience, for example) have been associated with increased aggression and other forms of anti-social behaviors in adulthood (Dodge, Bates, & Pettit, 1990). However, in the present study maternal separation did not substantially alter adult social behavior as recently, Hulshof and collaborators (2010), in an extensive work proposed to explore MS impacts on a several behavioral and neurobiological phenotypes also shown. However, the authors also used a slightly different version of SIT, leading us to hypothesize that these incongruent data from different MS studies may be related to the kind of tests used to access social behavior in adulthood. Changes in adult social behavior phenotype might only become apparent under certain environmental and social conditions. Probably specific "social skills" of MS rodents could only become detectable under particular social contacts, and different methodologies could be useful to achieve this propose. For instance, we speculate that social interaction test could not be sufficient stressful to induce any aggression in MS rats or that other type of social interaction models is specifically optimized to induce this type of behavior, such as the Resident-intruder test (Mitchell & Redfern, 2005). Additionally, social recognition (social memory) parameters could be better observed by a social discrimination task (Macbeth, Edds, & Young III, 2009). Furthermore, numerous studies of oxytocin knockout mice have implicated OT and OT-r in the central mediation of social behaviors (Ferguson et al., 2000; Ferguson et al., 2001). Taking in account our mRNA OT-r data, it was expected that MS rats displayed changes in social behaviors. Therefore, although we have observed changes in mRNA OT-r levels in specific MS rats' brain regions, there were no significant shifts in social phenotype during SIT performance.

Gender questions should also be taken into account in the interpretation of our findings. As above-mentioned, our study aims a comprehensive integration of mRNA OT-r levels and social behavior data. Since OT takes a predominant role in female social behavior (Young, Winslow, Wang, Gingrich, Guo, Matzuk et al., 1997), the

regulation of male social behavior is majorly conferred by higher expression of central vasopressin (Goodson, Lindberg, & Johnson, 2004). Supported by these data, we conceivably hypothesized MS-induced changes in oxytocinergic system could impact more markedly in the social phenotypes of females rats. In future investigations it might be important to investigate female social-emotional phenotype and OT interaction after MS experience.

Clinical implications

Examining the ways in which early experiences with caregivers may impact on the regulation of the HPA axis is important for a better understanding of the relationship between early developmental environments and long-term adaptive behaviors. In humans, caregiver interactions shape children ability to cope with future stress events due to HPA axis' highly sensitivity to early adverse experiences. The Maternal Separation paradigm applied in the present study is a widely used model for the neurobiological and behavioral development assessment of animals exposed to early chronic stress. In support of the view that disturbances in early psychosocial relationships are triggering factors for future pathology, our findings showed that interfering with the mother-infant relationship early in life could affect a range of development spheres. Taken together, these results suggest the importance of looking to the early mother-infant relationship and, most importantly, to the quality of the environmental context where this development occurs, either in normative or other unexpected contexts (e.g. institutionalization experiences).

Additionally, we looked at CS-OT interrelationship as potential neuronal mechanism through which early life experiences can lead to adult psychopathology. The nonapeptide oxytocin is released into systemic circulation in situations of psychological interaction and during stressor exposure, acting like anxiolytic and antidepressant. Moreover, previous experimental researches (e.g. Geenen, Legros, Franchimont, Baudrihaye, Defresne, & Boniver, 1986) have been described OT receptors in the thymus supporting the concept of a close dialogue between the neuroendocrine and the immune systems, and increasing the evidence for the importance of OT on the control and developmental of important biological systems. Our findings provided a little more information about OT and corticosteroid systems crosstalk under chronic stress conditions. This OT's action at HPA axis control level and immunological system maturation could be a major challenge for future research in this field. In particular, it will be of interest explore deeper the OT specific aspects on the pharmacological actions of several antidepressants or, ultimately, the OT proprieties as independent pharmacological agent in the prevention or treatment of psychological disorders.

5. GENERAL CONCLUSIONS

Taken together the results in the present thesis show that early maternal separation overactivates the *HPA axis*, independently of the temporal window in which the separation occurs, leading to *hyperanxious phenotype* (for both periods of maternal separation performed) and *depressive-like behaviors* (only observed if MS occurred in a

period of high brain immaturity). Finally, early adverse experiences showed to influence the *mRNA OT-r expression* in specific brain areas linked to HPA axis regulation but that also contribute to several cognitive- and social-emotional processes.

REFERENCES

Aisa, B., Elizalde, N., Tordera, R., Lasheras, B., Del Rìo, J., & Ramìrez, M. (2009). Effects of neonatal stress on markers of synaptic plasticity in the hippocampus: implications for spatial memory. *Hippocampus, 19*(12), 1222-1231.

Aisa, B., Tordera, R., Lasheras, B., Del Rìo, J., & Ramirez, M. (2007). Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology*, *32*(3), 256-266.

Albers, L., Johnson, D., Hostetter, M., Iverson, S., & Miller, L. (1997). Health of children adopted from the former Soviet Union and Eastern Europe: Comparison with preadoptive medical records. *Jama, 278*(11), 922.

Altstein, M., & Gainer, H. (1988). Differential biosynthesis and posttranslational processing of vasopressin and oxytocin in rat brain during embryonic and postnatal development. *Journal of Neuroscience*, *8(*11), 3967.

Amaral, D. (2003). The amygdala, social behavior, and danger detection. *Annals of the New York Academy of Sciences, 1000*, 337-347.

Amaral, D., & Dent, J. (1981). Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *The Journal of Comparative Neurology*, *195*(1), 51-86.

Andersen, S. (2003). Trajectories of brain development: point of vulnerability or window of opportunity? *Neuroscience & Biobehavioral Reviews*, *27*(1-2), 3-18.

Arborelius, L., & Eklund, M. (2007). Both long and brief maternal separation produces persistent changes in tissue levels of brain monoamines in middle-aged female rats*. Neuroscience*, *145*(2), 738-750.

Arborelius, L., Hawks, B., Owens, M., Plotsky, P., & Nemeroff, C. (2004). Increased responsiveness of presumed 5-HT cells to citalopram in adult rats subjected to prolonged maternal separation relative to brief separation. *Psychopharmacology, 176*(3), 248-255.

Argiolas, A., & Gessa, G. (1991). Central functions of oxytocin. *Neuroscience & Biobehavioral Reviews*, *15*(2), 217-231.

Bagdy, G., Graf, M., Anheuer, Z., Modos, E., & Kantor, S. (2001). Anxiety-like effects induced by acute fluoxetine, sertraline or m-CPP treatment are reversed by pretreatment with the 5-HT2C receptor antagonist SB-242084 but not the 5-HT1A receptor antagonist WAY-100635. *The International Journal of Neuropsychopharmacology*, *4*(04), 399-408.

Bales, K., & Carter, C. (2003). Developmental exposure to oxytocin facilitates partner preferences in male prairie voles (Microtus ochrogaster). *Behavioral Neuroscience, 117*(4), 854-859.

Barnow, S., & Freyberger, H. (2003). The family environment in early life and aggressive behavior in adolescents and young adults. *Neurobiology of aggression: understanding and preventing violence*, *213*.

Beishuizen, A., & Thijs, L. (2003). Review: Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *Journal of endotoxin research, 9*(1), 3.

Benoit, T., Jocelyn, L., Moddemann, D., & Embree, J. (1996). Romanian adoption: The Manitoba experience. *Archives of Pediatrics and Adolescent Medicine, 150*(12), 1278.

Bhattacharya, S., Satyan, K., & Chakrabarti, A. (1997). Anxiogenic action of caffeine: An experimental study in rats. *Journal of Psychopharmacology, 11*(3), 219.

Biagini, G., Pich, E., Carani, C., Marrama, P., & Agnati, L. (1998). Postnatal maternal separation during the stress hyporesponsive period enhances the adrenocortical response to novelty in adult rats by affecting feedback regulation in the CA1 hippocampal field. *International journal of developmental neuroscience, 16*(3-4), 187-197.

Bifulco, A., Brown, G., & Adler, Z. (1991). Early sexual abuse and clinical depression in adult life. *The British Journal of Psychiatry, 159*(1), 115.

Bosch, O., Pförtsch, J., Beiderbeck, D., Landgraf, R., & Neumann, I. (2010). Maternal behaviour is associated with vasopressin release in the medial preoptic area and bed nucleus of the stria terminalis in the rat. *Journal of Neuroendocrinology, 22*(5), 420-429.

Bouret, S., Draper, S., & Simerly, R. (2004). Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science, 304*(5667), 108.

Bowlby, J. (1969). *Attachment and loss.* Vol. 1. Attachment: Hogarth London.

Bowlby, J. (1982). Attachment and loss: Retrospect and prospect. *American Journal of Orthopsychiatry, 52*(4), 664-678.

Boyle, M., Brewer, J., Funatsu, M., Wozniak, D., Tsien, J., Izumi, Y., et al. (2005). Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(2), 473.

Brake, W., Zhang, T., Diorio, J., Meaney, M., & Gratton, A. (2004). Influence of early postnatal rearing conditions on mesocorticolimbic dopamine and behavioural responses to psychostimulants and stressors in adult rats. *European Journal of Neuroscience, 19*(7), 1863-1874.

Braun, K., Kremz, P., Wetzel, W., Wagner, T., & Poeggel, G. (2003). Influence of parental deprivation on the behavioral development in Octodon degus: modulation by maternal vocalizations. *Developmental psychobiology*, *42*(3), 237-245.

Cairns, C., Cairns, W., & Okret, S. (1993). Inhibition of gene expression by steroid hormone receptors via a negative glucocorticoid response element: Evidence for the involvement of DNA-binding and agonistic effects of the antiglucocorticoid/antiprogestin RU486. *DNA and Cell Biology, 12*(8), 695-702.

Caldji, C., Diorio, J., & Meaney, M. (2000). Variations in maternal care in infancy regulate the development of stress reactivity. *Biological psychiatry, 48*(12), 1164-1174.

Blanchard, D., Hynd, A., Minke, K., Minemoto, T., & Blanchard, R. (2001). Human defensive behaviors to threat scenarios show parallels to fear-and anxiety-related defense patterns of non-human mammals. *Neuroscience & Biobehavioral Reviews, 25*(7-8), 761-770.

Carter, S. (1998). Neuroendocrine perspectives on social attachment and love. *Psychoneuroendocrinology*, *23*(8), 779-818.

Ceanga, M., Spataru, A., & Zagrean, A. (2010). Oxytocin is neuroprotective against oxygen-glucose deprivation and reoxygenation in immature hippocampal cultures. *Neuroscience Letters*.

Cermak, S., & Daunhauer, L. Sensory processing in the postinstitutionalized child. *The American journal of occupational therapy, 51*(7), 500.

Cerqueira, J., Mailliet, F., Almeida, O., Jay, T., & Sousa, N. (2007). The prefrontal cortex as a key target of the maladaptive response to stress. *Journal of Neuroscience, 27*(11), 2781.

Chapman, D., Whitfield, C., Felitti, V., Dube, S., Edwards, V., & Anda, R. (2004). Adverse childhood experiences and the risk of depressive disorders in adulthood. *Journal of affective disorders, 82*(2), 217-225.

Cho, M., Courtney DeVries, A., Williams, J., & Carter, C. (1999). The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (Microtus ochrogaster). *Behavioral Neuroscience, 113*, 1071-1079.

Coe, C., Rosenberg, L., & Levine, S. (1988). Prolonged effect of psychological disturbance on macrophage chemiluminescence in the squirrel monkey. *Brain, Behavior, and Immunity*, *2*(2), 151-160.

Colorado, R., Shumake, J., Conejo, N., Gonzalez-Pardo, H., & Gonzalez-Lima, F. (2006). Effects of maternal separation, early handling, and standard facility rearing on orienting and impulsive behavior of adolescent rats. *Behavioural processes, 71*(1), 51-58.

Cook, S., & Wellman, C. (2004). Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *Journal of neurobiology, 60*(2), 236-248.

Cowley, M., Smith, R., Diano, S., Tschöp, M., Pronchuk, N., Grove, K., et al. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron, 37*(4), 649-661.

Daniels, W., Pietersen, C., Carstens, M., & Stein, D. (2004). Maternal separation in rats leads to anxiety-like behavior and a blunted ACTH response and altered neurotransmitter levels in response to a subsequent stressor. *Metabolic brain disease, 19*(1), 3-14.

De Kloet, E. (2004). Hormones and the stressed brain. *Annals of the New York Academy of Sciences, 1018*, 1-15.

Diorio, J., & Meaney, M. (2007). Maternal programming of defensive responses through sustained effects on gene expression. *Journal of Psychiatry & Neuroscience, 32*(4), 275.

Dodge, K., Bates, J., & Pettit, G. (1990). Mechanisms in the cycle of violence. *Science, 250*(4988), 1678.

Drago, F., Contarino, A., & Bus , L. (1999). The expression of neuropeptide-induced excessive grooming behavior in dopamine D1 and D2 receptor-deficient mice. *European journal of pharmacology, 365*(2-3), 125-131.

Duxon, M., Kennett, G., Lightowler, S., Blackburn, T., & Fone, K. (1997). Activation of 5-HT2B receptors in the medial amygdala causes anxiolysis in the social interaction test in the rat. *Neuropharmacology, 36*(4-5), 601-608.

El Khoury, A., Gruber, S., Mørk, A., & Mathè, A. (2006). Adult life behavioral consequences of early maternal separation are alleviated by escitalopram treatment in a rat model of depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 30*(3), 535-540.

Elias, C., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R., Couceyro, P., et al. (1998). Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron, 21*(6), 1375-1385.

Ellenbroek, B., & Cools, A. (2002). Early maternal deprivation and prepulse inhibition: The role of the postdeprivation environment. *Pharmacology Biochemistry and Behavior, 73*(1), 177-184.

Erickson, M., & Egeland, B. (1987). A developmental view of the psychological consequences of maltreatment. *School Psychology Review, 16*(2), 156-168.

Erickson, M., & Egeland, B. (2002). Child neglect. *The APSAC handbook on child maltreatment*, *2*, 3-20.

Fahlke, C., Lorenz, J., Long, J., Champoux, M., Suomi, S., & Higley, J. (2000). Rearing Experiences and Stress Induced Plasma Cortisol as Early Risk Factors for Excessive Alcohol Consumption in Nonhuman Primates. *Alcoholism: Clinical and Experimental Research*, *24*(5), 644-650.

Felitti, M., Vincent, J., Anda, M., Robert, F., & Nordenberg, M. (1998). Relationship of Childhood Abuse and Household Dysfunction to Many of the Leading Causes of Death in Adults: The Adverse Childhood Experiences (ACE) Study. *American journal of preventive medicine, 14*(4), 245-258.

Ferguson, J., Aldag, J., Insel, T., & Young, L. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *Journal of Neuroscience, 21*(20), 8278.

Ferguson, J., Young, L., Hearn, E., Matzuk, M., Insel, T., & Winslow, J. (2000). Social amnesia in mice lacking the oxytocin gene. *Nature Genetics, 25*(3), 284-288.

Ferris, C. (2005). Vasopressin/oxytocin and aggression. Novartis Foundation Symposium. 268, 190–198 discussion 198–200, 242–153.

File, S., & Hyde, J. (1978). Can social interaction be used to measure anxiety? *British Journal of Pharmacology*, *62*(1), 19.

File, S., & Seth, P. (2003). A review of 25 years of the social interaction test. *European journal of pharmacology*, *463*(1-3), 35-53.

Fish, E., Shahrokh, D., Bagot, R., Caldji, C., Bredy, T., Szyf, M., et al. (2004). Epigenetic programming of stress responses through variations in maternal care. *Annals of the New York Academy of Sciences, 1036*(1), 167-180.

Fonagy, P. (2004). Early Life Trauma and the Psychogenesis and Prevention of Violence. *Annals of the New York Academy of Sciences*, *1036*(1), 181-200.

Francis, D., & Meaney, M. (1999). Maternal care and the development of stress responses. *Current Opinion in Neurobiology*, *9*(1), 128-134.

Frank, D., Klass, P., Earls, F., & Eisenberg, L. (1996). Infants and young children in orphanages: One view from pediatrics and child psychiatry. *Pediatrics, 97*(4), 569.

Gartside, S., Johnson, D., Leitch, M., Troakes, C., & Ingram, C. (2003). Early life adversity programs changes in central 5 HT neuronal function in adulthood. *European Journal of Neuroscience*, *17*(11), 2401-2408.

Gautvik, K., De Lecea, L., Gautvik, V., Danielson, P., Tranque, P., Dopazo, A., et al. (1996). Overview of the most prevalent hypothalamus-specific mRNAs, as identified by directional tag PCR subtraction. *Proceedings of the National Academy of Sciences of the United States of America, 93*(16), 8733.

Geenen, V., Legros, J., Franchimont, P., Baudrihaye, M., Defresne, M., & Boniver, J. (1986). The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. *Science, 232*(4749), 508.

Gimpl, G., & Fahrenholz, F. (2001). The oxytocin receptor system: Structure, function, and regulation. *Physiological Reviews, 81*(2), 629.

Giordano, A., Siegel, H., & Rosenblatt, J. (1989). Nuclear estrogen receptor binding in the preoptic area and hypothalamus of pregnancy-terminated rats: Correlation with the onset of maternal behavior. *Neuroendocrinology, 50*(3), 248-258.

Giovenardi, M., Padoin, M., Cadore, L., & Lucion, A. (1998). Hypothalamic paraventricular nucleus modulates maternal aggression in rats: effects of ibotenic acid lesion and oxytocin antisense. *Physiology & Behavior, 63*(3), 351-359.

Gonzalez, L., Andrews, N., & File, S. (1996). 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. *Brain Research, 732*(1-2), 145-153.

Goodson, J., Lindberg, L., & Johnson, P. (2004). Effects of central vasotocin and mesotocin manipulations on social behavior in male and female zebra finches. *Hormones and behavior*, *45*(2), 136-143.

Gunnar, M., & Quevedo, K. (2007). The neurobiology of stress and development. *Annual review of psychology, 58*, 145.

Gunnar, M., & Quevedo, K. (2007). Early care experiences and HPA axis regulation in children: A mechanism for later trauma vulnerability. *Progress in Brain Research, 167*, 137-149.

Gutman, D., & Nemeroff, C. (2002). Neurobiology of early life stress: rodent studies.

Härfstrand, A., Fuxe, K., Cintra, A., Agnati, L., Zini, I., Wikström, A., et al. (1986). Glucocorticoid receptor immunoreactivity in monoaminergic neurons of rat brain. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(24), 9779.

Han, F., Ozawa, H., Matsuda, K., Nishi, M., & Kawata, M. (2005). Colocalization of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus and hypothalamus. *Neuroscience Research*, *51*(4), 371-381.

Hardt, J., & Rutter, M. (2004). Validity of adult retrospective reports of adverse childhood experiences: Review of the evidence. *Journal of Child Psychology and Psychiatry, 45*(2), 260-273.

Heim, C., & Nemeroff, C. (2001). The role of childhood trauma in the neurobiology of mood and anxiety disorders: Preclinical and clinical studies. *Biological psychiatry, 49*(12), 1023-1039.

Heinrichs, M., Neumann, I., & Ehlert, U. (2002). Lactation and stress: Protective effects of breast-feeding in humans. *Stress: The International Journal on the Biology of Stress, 5*(3), 195-203.

Hennessy, M., Schiml-Webb, P., & Deak, T. (2009). Separation, Sickness, and Depression. *Current Directions in Psychological Science, 18*(4), 227.

Higley, J., Hasert, M., Suomi, S., & Linnoila, M. (1998). The serotonin reuptake inhibitor sertraline reduces excessive alcohol consumption in nonhuman primates: Effect of stress. *Neuropsychopharmacology, 18*(6), 431-443.

Hofer, M. (2005). The psychobiology of early attachment. *Clinical Neuroscience Research, 4*(5-6), 291-300.

Hogg, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology Biochemistry and Behavior*, *54*(1), 21-30.

Hollander, E., Novotny, S., Hanratty, M., Yaffe, R., DeCaria, C., Aronowitz, B., et al. (2003). Oxytocin infusion reduces repetitive behaviors in adults with autistic and Asperger's disorders. *Neuropsychopharmacology*, *28*(1), 193-198.

Holmes, A., le Guisquet, A., Vogel, E., Millstein, R., Leman, S., & Belzung, C. (2005). Early life genetic, epigenetic and environmental factors shaping emotionality in rodents. *Neuroscience & Biobehavioral Reviews, 29*(8), 1335-1346.

Hulshof, H., Novati, A., Sgoifo, A., Luiten, P., Boer, J., & Meerlo, P. (2010). Maternal separation decreases adult hippocampal cell proliferation and impairs cognitive performance but has little effect on stress sensitivity and anxiety in adult Wistar rats. *Behavioural brain research.*

Huot, R., Thrivikraman, K., Meaney, M., & Plotsky, P. (2001). Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology, 158*(4), 366-373.

Insel, T., & Harbaugh, C. (1989). Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. *Physiology & Behavior, 45*(5), 1033-1041.

Jarrard, L. (1993). On the role of the hippocampus in learning and memory in the rat. *Behavioral and neural biology*, *60*(1), 9-26.

Jaworski, J., Francis, D., Brommer, C., Morgan, E., & Kuhar, M. (2005). Effects of early maternal separation on ethanol intake, GABA receptors and metabolizing enzymes in adult rats. *Psychopharmacology, 181*(1), 8-15.

Johansson, B., Georgiev, V., & Fredholm, B. (1997). Distribution and postnatal ontogeny of adenosine A2A receptors in rat brain: comparison with dopamine receptors. *Neuroscience, 80*(4), 1187-1207.

Johnson, S., Fournier, N., & Kalynchuk, L. (2006). Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. *Behavioural brain research, 168*(2), 280-288.

Jones, P., & Robinson, I. (1982). Differential clearance of neurophysin and neurohypophysial peptides from the cerebrospinal fluid in conscious guinea pigs. *Neuroendocrinology, 34*(4), 297-302.

Kalinichev, M., Easterling, K., Plotsky, P., & Holtzman, S. (2002). Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. *Pharmacology Biochemistry and Behavior, 73*(1), 131-140.

Kaneko, W., Riley, E., & Ehlers, C. (1994). Behavioral and electrophysiological effects of early repeated maternal separation. *Depression, 2*(1), 43-53.

Kashdan, T., Elhai, J., & Breen, W. (2008). Social anxiety and disinhibition: An analysis of curiosity and social rank appraisals, approach-avoidance conflicts, and disruptive risk-taking behavior. *Journal of anxiety disorders, 22*(6), 925-939.

Kaufman, J., Plotsky, P., Nemeroff, C., & Charney, D. (2000). Effects of early adverse experiences on brain structure and function: clinical implications. *Biological psychiatry, 48*(8), 778-790.

Kendler, K., Bulik, C., Silberg, J., Hettema, J., Myers, J., & Prescott, C. (2000). Childhood sexual abuse and adult psychiatric and substance use disorders in women: An epidemiological and cotwin control analysis. *Archives of General Psychiatry, 57*(10), 953.

Kendler, K., Kuhn, J., & Prescott, C. (2004). Childhood sexual abuse, stressful life events and risk for major depression in women. *Psychological Medicine, 34*(08), 1475-1482.

Kessler, R. (1997). The effects of stressful life events on depression. *Annual review of psychology, 48,* 191-214.

Kim, J., & Cicchetti, D. (2004). A longitudinal study of child maltreatment, motherñchild relationship quality and maladjustment: The role of self-esteem and social competence. *Journal of Abnormal Child Psychology, 32*(4), 341-354.

Kolb, B. (1984). Functions of the frontal cortex of the rat: a comparative review. *Brain Research Reviews, 8*(1), 65-98.

Krinke, G. (2000). *The laboratory rat.* Academic Press.

Ladd, C., Owens, M., & Nemeroff, C. (1996). Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. *Endocrinology, 137*(4), 1212.

Landgraf, R., Neumann, I., Russell, J., & Pittman, Q. (1992). Push pull perfusion and microdialysis studies of central oxytocin and vasopressin release in freely moving rats during pregnancy, parturition, and lactation. *Annals of the New York Academy of Sciences, 652*(1), 326-339.

Lauder, J. (1983). Hormonal and humoral influences on brain development. *Psychoneuroendocrinology, 8*(2), 121-155.

Laugero, K. (2004). Reinterpretation of basal glucocorticoid feedback: Implications to behavioral and metabolic disease. *Vitamins & Hormones, 69*, 1-29.

Laugero, K., Bell, M., Bhatnagar, S., Soriano, L., & Dallman, M. (2001). Sucrose ingestion normalizes central expression of corticotropin-releasing-factor messenger ribonucleic acid and energy balance in adrenalectomized rats: A glucocorticoid-metabolic-brain axis? *Endocrinology, 142*(7), 2796.

Leckman, J., Goodman, W., North, W., Chappell, P., Price, L., Pauls, D., et al. (1994). The role of central oxytocin in obsessive compulsive disorder and related normal behavior. *Psychoneuroendocrinology, 19*(8), 723-749.

Lee, J., Kim, H., Kim, J., Ryu, V., Kim, B., Kang, D., et al. (2007). Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. *Neuroscience research, 58*(1), 32-39.

LeMarquand, D., Pihl, R., & Benkelfat, C. (1994). Serotonin and alcohol intake, abuse, and dependence: findings of animal studies. *Biological psychiatry, 36*(6), 395-421.

Leon, M. (1979). Mother-young reunions. *Progress in psychobiology and physiological psychology, 8*, 301-334.

Lesage, J., Del-Favero, F., Leonhardt, M., Louvart, H., Maccari, S., Vieau, D., et al. (2004). Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. *Journal of Endocrinology, 181*(2), 291.

Levine, S. (1957). Infantile experience and resistance to physiological stress. Science, 126(3270), 405.

Levine, S. (1959). The effects of differential infantile stimulation of emotionality at weaning. *Canadian Journal of Psychology/Revue canadienne de psychologie, 13*(4), 243.

Levine, S. (1994). The Ontogeny of the Hypothalamic Pituitary Adrenal Axis. The Influence of Maternal Factorsa. *Annals of the New York Academy of Sciences, 746*(1), 275-288.

Levine, S. (2001). Primary social relationships influence the development of the hypothalamic-pituitary-adrenal axis in the rat. *Physiology & Behavior, 73*(3), 255-260.

Levine, S., Huchton, D., Wiener, S., & Rosenfeld, P. (1991). Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. *Developmental psychobiology, 24*(8), 547-558.

Liu, D., Caldji, C., Sharma, S., Plotsky, P., & Meaney, M. (2000). Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. *Journal of Neuroendocrinology, 12*(1), 5-12.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., et al. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science, 277*(5332), 1659.

Louey, S., & Thornburg, K. (2005). The prenatal environment and later cardiovascular disease. *Early human development, 81*(9), 745-751.

Lu, J., Goula, D., Sousa, N., Almeida, O.F.X. (2003). Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic *N*-methyl-D-aspartate receptors. *Neuroscience, 121*, 123-131.

Lu, L., Shepard, J., Scott Hall, F., & Shaham, Y. (2003). Effect of environmental stressors on opiate and psychostimulant reinforcement, reinstatement and discrimination in rats: A review. *Neuroscience & Biobehavioral Reviews, 27*(5), 457-491.

Lukas, M., Bredewold, R., Neumann, I., & Veenema, A. (2010). Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. *Neuropharmacology, 58*(1), 78-87.

Macbeth, A., Edds, J., & Young, W. (2009). Housing conditions and stimulus females: a robust social discrimination task for studying male rodent social recognition. *Nature Protocols, 4*(11), 1574-1581.

Macrì , S., Chiarotti, F., & Würbel, H. (2008). Maternal separation and maternal care act independently on the development of HPA responses in male rats. *Behavioural brain research, 191*(2), 227-234.

MacrÌ, S., Mason, G., & Würbel, H. (2004). Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. *European Journal of Neuroscience, 20*(4), 1017-1024.

Magarinos, A., McEwen, B., Flugge, G., & Fuchs, E. (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *Journal of Neuroscience, 16*(10), 3534.

Marais, L., van Rensburg, S., van Zyl, J., Stein, D., & Daniels, W. (2008). Maternal separation of rat pups increases the risk of developing depressive-like behavior after subsequent chronic stress by altering corticosterone and neurotrophin levels in the hippocampus. *Neuroscience research, 61*(1), 106-112.

Marmendal, M. (2005). *Maternal separation in the rat: long-term effects of early life events on emotionality, drug response and neurobiology*. Doctoral dissertation, Department of Psychology, Göteborg University, Sweden.

Matthews, K., Dalley, J., Matthews, C., Tsai, T., & Robbins, T. (2001). Periodic maternal separation of neonatal rats produces region-and gender-specific effects on biogenic amine content in postmortem adult brain. *Synapse, 40*(1), 1-10.

Matthews, K., Hall, F., Wilkinson, L., & Robbins, T. (1996). Retarded acquisition and reduced expression of conditioned locomotor activity in adult rats following repeated early maternal separation: effects of prefeeding, d-amphetamine, dopamine antagonists and clonidine. *Psychopharmacology, 126*(1), 75-84.

McCarthy, M. (1995). Estrogen modulation of oxytocin and its relation to behavior. *Advances in experimental medicine and biology, 395*, 235.

McCarthy, M., McDonald, C., Brooks, P., & Goldman, D. (1997). An anxiolytic action of oxytocin is enhanced by estrogen in the mouse. *Physiology & Behavior, 60*(5), 1209-1215.

McKinney, W. (1977). *Animal behavioral/biological models relevant to depressive and affective disorders in humans. Depression in childhood: Diagnosis, treatment, and conceptual models* (JG Schulterbrandt & A. Raskin, eds.). *New York: Raven,* 107-122.

Meaney, M., Aitken, D., Bodnoff, S., Iny, L., & Sapolsky, R. (1985). The effects of postnatal handling on the development of the glucocorticoid receptor systems and stress recovery in the rat. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 9*(5-6), 731-734.

Meaney, M., Brake, W., & Gratton, A. (2002). Environmental regulation of the development of mesolimbic dopamine systems: a neurobiological mechanism for vulnerability to drug abuse? *Psychoneuroendocrinology, 27*(1-2), 127-138.

Mesquita, A. R. (2008). *An integrative biological assessment of the programming effects of glucocorticoids upon the developing brain : insights on their relevance for future psychiatric pathologies*. Doctoral dissertation, University of Minho, Portugal.

Mesquita, A., Pego, J., Summavielle, T., Maciel, P., Almeida, O., & Sousa, N. (2007). Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience, 147*(4), 1022-1033.

Mitchell, P., & Redfern, P. (2005). Animal models of depressive illness: the importance of chronic drug treatment. *Current pharmaceutical design, 11*(2), 171-203.

Morison, S., Ames, E., & Chisholm, K. (1995). The development of children adopted for romanian orphanages. *Merrill-Palmer Quarterly, 41*(4), 411-430.

Mullen, P., Martin, J., Anderson, J., Romans, S., & Herbison, G. (1996). The long-term impact of the physical, emotional, and sexual abuse of children: a community study. *Child Abuse & Neglect, 20*(1), 7-21.

Nelson, E., & Panksepp, J. (1996). Oxytocin mediates acquisition of maternally associated odor preferences in preweanling rat pups. *Behavioral Neuroscience, 110*(3), 583-592.

Nemeroff, C. (1996). The corticotropin-releasing factor (CRF) hypothesis of depression: new findings and new directions. *Molecular psychiatry, 1*(4), 336.

Nemeroff, C. (2004). Early-life adversity, CRF dysregulation, and vulnerability to mood and anxiety disorders. *Psychopharmacology bulletin, 38*(1), 14.

Neumann, I., Johnstone, H., Hatzinger, M., Liebsch, G., Shipston, M., Russell, J., et al. (1998). Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. *The Journal of physiology, 508*(1), 289-300.

Neumann, I., Krâmer, S., Toschi, N., & Ebner, K. (2000). Brain oxytocin inhibits the (re) activity of the hypothalamo-pituitary-adrenal axis in male rats: involvement of hypothalamic and limbic brain regions. *Regulatory peptides, 96*(1-2), 31-38.

Niesink, R., & Van Ree, J. (1982). Short-term isolation increases social interactions of male rats: a parametric analysis. *Physiology & Behavior, 29*(5), 819-825.

Nunes-Costa, R., Lamela, D., & Figueiredo, B. (2009). Psychosocial adjustment and physical health in children of divorce. *Jornal de Pediatria, 85,* 385-396.

O'Connor, T., Rutter, M., Beckett, C., Keaveney, L., & Kreppner, J., The English and Romanian Adoptees Study Team (2000). The effects of global severe privation on cognitive competence: Extension and longitudinal follow-up. *Child Development, 71*(2), 376-390.

Okere, C., Higuchi, T., Kaba, H., Russell, J., Takahashi, S., & Murata, T. (1996). Nitric oxide prolongs parturition and inhibits maternal behavior in rats. *Neuroreport, 7(*10), 1695.

Ostermeyer, M., & Elwood, R. (1983). Pup recognition in mus musculus: Parental discrimination between their own and alien young. *Developmental psychobiology, 16*(2), 75-82.

Ozawa, H., Ito, T., Ochiai, I., & Kawata, M. (1999). Cellular localization and distribution of glucocorticoid receptor immunoreactivity and the expression of glucocorticoid receptor messenger RNA in rat pituitary gland. *Cell and tissue research, 295*(2), 207-214.

Paxinos, G., & Watson, C. (2005). *The rat brain in stereotaxic coordinates: The new coronal set-161 diagrams*. San Diego: Academic Press.

Pedersen, C., & Prange, A. (1979). Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. *Proceedings of the National Academy of Sciences of the United States of America, 76*(12), 6661.

Pego, J., Morgado, P., Pinto, L., Cerqueira, J., Almeida, O., & Sousa, N. (2008). Dissociation of the morphological correlates of stress induced anxiety and fear. *European Journal of Neuroscience, 27*(6), 1503-1516.

Pellow, S., Chopin, P., File, S., & Briley, M. (1985). Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of neuroscience methods, 14*(3), 149-167.

Pinnock, S., & Herbert, J. (2001). Corticosterone differentially modulates expression of corticotropin releasing factor and arginine vasopressin mRNA in the hypothalamic paraventricular nucleus following either acute or repeated restraint stress. *European Journal of Neuroscience, 13*(3), 576-584.

Ploj, K., Roman, E., & Nylander, I. (2003). Long-term effects of maternal separation on ethanol intake and brain opioid and dopamine receptors in male Wistar rats. *Neuroscience, 121*(3), 787-799.

Plotsky, P., & Meaney, M. (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Molecular Brain Research, 18*(3), 195-200.

Pryce, C. (2008). Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: Inter-species and intra-species differences. *Brain Research Reviews, 57*(2), 596-605.

Ruedi-Bettschen, D., Feldon, J., & Pryce, C. (2004). The impaired coping induced by early deprivation is reversed by chronic fluoxetine treatment in adult Fischer rats. *Behavioural pharmacology, 15*(5-6), 413.

Razzoli, M., Cushing, B., Carter, C., & Valsecchi, P. (2003). Hormonal regulation of agonistic and affiliative behavior in female Mongolian gerbils (Meriones unguiculatus). *Hormones and behavior, 43*(5), 549-553.

Repetti, R., Taylor, S., & Seeman, T. (2002). Risky families: Family social environments and the mental and physical health of offspring. *Psychological Bulletin, 128*(2), 330-366.

Ring, R., Malberg, J., Potestio, L., Ping, J., Boikess, S., Luo, B., et al. (2006). Anxiolytic-like activity of oxytocin in male mice: Behavioral and autonomic evidence, therapeutic implications. *Psychopharmacology, 185*(2), 218-225.

Rodgers, R., Cao, B., Dalvi, A., & Holmes, A. (1997). Animal models of anxiety: An ethological perspective. *Brazilian Journal of Medical and Biological Research, 30,* 289-304.

Rodgers, R., & Dalvi, A. (1997). Anxiety, defence and the elevated plus-maze. *Neuroscience & Biobehavioral Reviews, 21*(6), 801-810.

Roman, E. (2004). *Maternal Separation in Rats: An Experimental Model for Long-Term Effects of Early-Life Experiences on Neurochemistry, Voluntary Ethanol Intake and Exploration and Risk Assessment Behavior.* Doctoral dissertation. Faculty of Pharmacy, Uppsala University, Sweeden.

Rosenfeld, P., Ekstrand, J., Olson, E., Suchecki, D., & Levine, S. (1993). Maternal regulation of adrenocortical activity in the infant rat: effects of feeding. *Developmental psychobiology, 26*(5), 261-277.

Roy, P., Rutter, M., & Pickles, A. (2004). Institutional care: Associations between overactivity and lack of selectivity in social relationships. *Journal of Child Psychology and Psychiatry, 45*(4), 866-873.

Russell, J., & Leng, G. (1998). Sex, parturition and motherhood without oxytocin? *Journal of Endocrinology, 157*(3), 343.

Rutter, M. (1979). Maternal deprivation, 1972-1978: New findings, new concepts, new approaches. *Child Development, 50*(2), 283-305.

Ryu, V., Yoo, S., Kang, D., Lee, J., & Jahng, J. (2009). Post-weaning isolation promotes food intake and body weight gain in rats that experienced neonatal maternal separation. *Brain Research, 1295,* 127-134.

Sanchez, M., Ladd, C., & Plotsky, P. (2001). Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Development and Psychopathology, 13*(03), 419-449.

Sanders, B., & Anticevic, A. (2007). Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats. *Behavioural brain research, 183*(1), 25-30.

Sandi, C., & Calès, J. (2000). *Estrès: Consecuencias psicológicas, fisiológicas y clínicas.* Madrid: Sanz y Torres.

Sapolsky, R. (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Archives of General Psychiatry, 57*(10), 925.

Sapolsky, R., & Meaney, M. (1986). Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Research Reviews, 11*(1), 65-76.

Schmidt, M., Enthoven, L., Van Der Mark, M., Levine, S., De Kloet, E., & Oitzl, M. (2003). The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. *International journal of developmental neuroscience, 21*(3), 125-132.

Schoenfeld, N., Leathem, J., & Rabii, J. (1980). Maturation of adrenal stress responsiveness in the rat. *Neuroendocrinology, 31*(2), 101-105.

Shalev, U., & Kafkafi, N. (2002). Repeated maternal separation does not alter sucrose-reinforced and open-field behaviors. *Pharmacology Biochemistry and Behavior, 73*(1), 115-122.

Shapiro, L., & Insel, T. (1989). Ontogeny of oxytocin receptors in rat forebrain: A quantitative study. *Synapse, 4*(3), 259-266.

Sharp, J., Zammit, T., Azar, T., & Lawson, D. (2002). Stress-like responses to common procedures in male rats housed alone or with other rats. *Journal of the American Association for Laboratory Animal Science, 41*(4), 8-14.

Sousa, N., & Almeida, O. (2002). Corticosteroids: sculptors of the hippocampal formation. *Reviews in the neurosciences, 13(*1), 59.

Spitz, R. (1946). Hospitalism: A follow-up report. *The psychoanalytic study of the child, 2,* 113.

Stansbury, K., & Gunnar, M. (1994). Adrenocortical activity and emotion regulation. *Monographs of the Society for Research in Child Development, 59*(2), 108-134.

Suchecki, D., Rosenfeld, P., & Levine, S. (1993). Maternal regulation of the hypothalamic-pituitary-adrenal axis in the infant rat: The roles of feeding and stroking. *Developmental Brain Research, 75*(2), 185-192.

Suomi, S., Eisele, C., Grady, S., & Harlow, H. (1975). Depressive behavior in adult monkeys following separation from family environment. *Journal of abnormal psychology, 84*(5), 576-578.

Sutanto, W., Rosenfeld, P., de Kloet, E., & Levine, S. (1996). Long-term effects of neonatal maternal deprivation and ACTH on hippocampal mineralocorticoid and glucocorticoid receptors. *Developmental Brain Research, 92*(2), 156-163.

Toth, M., Fuzesi, T., Halasz, J., Tulogdi, A., & Haller, J. (in press). Neural inputs of the hypothalamic, aggression area. *Behavioural brain research.*

Uvnäs-Moberg, K. (1998). Oxytocin may mediate the benefits of positive social interaction and emotions. *Psychoneuroendocrinology, 23*(8), 819-835.

Uvnäs-Moberg, K. (1998b). Antistress pattern induced by oxytocin. *News in physiological sciences, 13*(22).

Uvnäs-Moberg, K., Ahlenius, S., Hillegaart, V., & Alster, P. (1994). High doses of oxytocin cause sedation and low doses cause an anxiolytic-like effect in male rats. *Pharmacology Biochemistry and Behavior, 49*(1), 101-106.

van der Lely, A., Tschop, M., Heiman, M., & Ghigo, E. (2004). Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrine Reviews, 25*(3), 426.

Van Praag, H. (2004). Can stress cause depression? *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 28*(5), 891-907.

Van Wimersma Greidanus, T., Kroodsma, J., Pot, M., Stevens, M., & Maigret, C. (1990). Neurohypophyseal hormones and excessive grooming behaviour. *European journal of pharmacology, 187*(1), 1-8.

Veenema, A., Blume, A., Niederle, D., Buwalda, B., & Neumann, I. (2006). Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. *European Journal of Neuroscience, 24*(6), 1711-1720.

Veenema, A., Bredewold, R., & Neumann, I. (2007). Opposite effects of maternal separation on intermale and maternal aggression in C57BL/6 mice: Link to hypothalamic vasopressin and oxytocin immunoreactivity. *Psychoneuroendocrinology, 32*(5), 437-450.

Veenema, A., & Neumann, I. (2007). Neurobiological mechanisms of aggression and stress coping: a comparative study in mouse and rat selection lines. *Brain, Behavior and Evolution, 70*(4), 274-285.

Veenema, A., & Neumann, I. (2008). Central vasopressin and oxytocin release: Regulation of complex social behaviours. *Progress in Brain Research, 170,* 261-276.

Veenema, A., & Neumann, I. (2009). Maternal separation enhances offensive play-fighting, basal corticosterone and hypothalamic vasopressin mRNA expression in juvenile male rats. *Psychoneuroendocrinology, 34*(3), 463-467.

Vicentic, A., Francis, D., Moffett, M., Lakatos, A., Rogge, G., Hubert, G., et al. (2006). Maternal separation alters serotonergic transporter densities and serotonergic 1A receptors in rat brain. *Neuroscience, 140*(1), 355-365.

von Hoersten, S., Dimitrijevic, M., Markovic, B., & Jankovic, B. (1993). Effect of early experience on behavior and immune response in the rat. *Physiology & Behavior, 54*(5), 931-940.

Vyas, A., Bernal, S., & Chattarji, S. (2003). Effects of chronic stress on dendritic arborization in the central and extended amygdala. *Brain Research, 965*(1-2), 290-294.

Vythilingam, M., Heim, C., Newport, J., Miller, A., Anderson, E., Bronen, R., et al. (2002). Childhood trauma associated with smaller hippocampal volume in women with major depression. *American Journal of Psychiatry, 159*(12), 2072.

Walter, C., Scribner, K., Cascio, C., & Dallman, M. (1991). The pituitary-adrenocortical system of neonatal rats is responsive to stress throughout development in a time-dependent and stressor-specific fashion. *Endocrinology, 128*(3), 1385.

Walker, D., Toufexis, D., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European journal of pharmacology, 463*(1-3), 199-216.

Weaver, I., Cervoni, N., Champagne, F., D'Alessio, A., Sharma, S., Seckl, J., et al. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience, 7*(8), 847-854.

Wigger, A., & Neumann, I. (1999). Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. *Physiology & Behavior, 66*(2), 293-302.

Willis-Owen, S., & Flint, J. (2006). The genetic basis of emotional behaviour in mice. *European Journal of Human Genetics, 14*(6), 721-728.

Winslow, J., & Insel, T. (1993). Effects of central vasopressin administration to infant rats. *European journal of pharmacology, 233*(1), 101-107.

Witek-Janusek, L. (1988). Pituitary-adrenal response to bacterial endotoxin in developing rats. *American Journal of Physiology- Endocrinology And Metabolism, 255(*4), E525.

Wright, C., Parkinson, K., & Drewett, R. (2006). How does maternal and child feeding behavior relate to weight gain and failure to thrive? Data from a prospective birth cohort. *Pediatrics, 117*(4), 1262.

Yoshimura, R., Kimura, T., Watanabe, D., & Kiyama, H. (1996). Differential expression of oxytocin receptor mRNA in the developing rat brain. *Neuroscience research, 24*(3), 291-304.

Young, E., Abelson, J., Curtis, G., & Nesse, R. (1997). Childhood adversity and vulnerability to mood and anxiety disorders. *Depression and Anxiety, 5*(2), 66-72.

Young, E., & Vazquez, D. (1996). Hypercortisolemia, hippocampal glucocorticoid receptors, and fast feedback. *Molecular psychiatry, 1*(2), 149.

Young, K., Gobrogge, K., Liu, Y., & Wang, Z. (in press). The neurobiology of pair bonding: insights from a socially monogamous rodent. *Frontiers in Neuroendocrinology*.

Young, L., Winslow, J., Wang, Z., Gingrich, B., Guo, Q., Matzuk, M., et al. (1997). Gene Targeting Approaches to Neuroendocrinology: Oxytocin, Maternal Behavior, and Affiliation. *Hormones and behavior, 31*(3), 221-231.

Yu, G., Kaba, H., Okutani, F., Takahashi, S., & Higuchi, T. (1996). The olfactory bulb: a critical site of action for oxytocin in the induction of maternal behaviour in the rat. *Neuroscience, 72*(4), 1083-1088.

Zeanah, C., Smyke, A., Koga, S., & Carlson, E. (2005). Attachment in institutionalized and community children in Romania. *Child Development, 76*(5), 1015-1028.