Universidade do Minho
Escola de Ciências

Marlene Sofia Belinho Nogueira

The impact of early life experiences on oxytocinergic system modulation: implications on social behavior development

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The impact of early life experiences on oxytocinergic system modulation: implications on social behavior development

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação da
Doutora Ana Raquel Mesquita
Doutora Ana Magalhães
Doutora Paula Sampaio

Fevereiro de 2016

Anexo 3

DECLARAÇÃO

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Viver no mundo sem ter a consciência
do significado desse mesmo mundo
é como deambular por uma enorme
biblioteca sem tocar nos livros.

The Secret Teachings of All Ages

Abstract

For all mammals, the relationship with the mother is the first social bond that it is crucial for the maintenance of the homeostasis. This bond along with the maintenance of the proximity between the dam and litter, and the stimulation of the maternal care has a particular role on the establishment of the infant's stress coping style. It has been described that the mother-pup interaction has the intervention of many systems, namely the oxytocinergic. Both oxytocin (OXT) hormone and its receptor (OXTR) are widely distributed across the central nervous system and have clear impact on brain structures' development and maturation. Maternal separation (MS) (for long and repeated periods) is a well-known and established model of early life stress induction that has been demonstrated as a severe modulator of the mother-pup interaction and capable to induce long-last impairments at behavior and neuroendocrine level that remain to adulthood. However, less is known about the consequences of short repeated periods of MS on adolescent social behavior and their implications on the oxytocinergic system maturation.

To explore this issue the present study aimed to: 1) Evaluate the impacts of short daily MS, during two different periods early in life, on the social behavior of adolescent rats. 2) Investigate the ability of environmental enrichment (EE) to protect from deleterious effects of maternal separation on the adolescent social behavior. The stimulation provided via EE applied early in life impacts both on brain and behavior and may be beneficial for the behavior development; 3) Determinate the expression profile of OXT and OXTR, and correlate it with the effects of MS.

Wistar rats' litters were daily maternally separated for 2 hours at postnatal days (PND) 2-6 or 10-14, during which half of the litter was in EE and the other half in a standard environment. To assess the modulation of different components of social behavior during adolescence it was performed social recognition and interaction tests with familiar and unfamiliar subjects. It was also determined the expression profile of oxytocin and its receptor by RT-PCR in the same developmental period. Results showed that even subtle MS induced impairments in social behavior and oxytocinergic system. Rats exposed to MS from 2-6 PND did not expressed social affiliation/motivation neither preference for social novelty (impairment of social memory). The EE during this period of MS protected adolescent rat from this social memory impairment. Additionally, MS during 10-14 PND altered social interaction (mainly by increasing affiliative behavior) with the familiar peer but not with the unfamiliar one, whereas MS during 2-6 PND had more modest effects on social interaction in both cases. These behavioral data are positively and negatively correlated with an increased on the expression of oxytocin's receptor expression on prefrontal cortex. Essentially, these data provide new evidence that early short periods of MS are able to shape adolescent social behaviors that are differentially sensitive to MS across ontogeny and modulate the oxytocinergic system, with some of those impairments being recovered by the EE.

Resumo

Em toda a classe de mamíferos, a relação com a mãe é a primeira ligação social que é crucial para a manutenção da homeostasia. Este laço afectivo, para além de promover a proximidade física entre a portadora de cuidados e a sua ninhada, estimula o comportamento maternal, tendo um papel particular no estabelecimento de estratégias de adaptação da descendência. A interação entre mãe e cria tem a intervenção de diversos sistemas, nomeadamente o oxitocinérgico. Quer a oxitocina (OXT), quer o seu receptor (OXTR) estão plenamente distribuídos ao longo do sistema nervoso central e possuem um claro impacto no desenvolvimento e maturação de estruturas cerebrais. Os primeiros dias pós natais são um período de elevada plasticidade, especialmente para o sistema de stress, sendo isso, particularmente sensíveis a stressores. A separação maternal (SM) (por períodos longos e repetitivos) é um bem conhecido e estabelecido modelo de indução de stress precoce que tem demonstrado ser não só um modulador severo da interação mãe-cria, assim como capaz de induzir danos a nível comportamental e neuroendócrino, que permanecem até idade adulta. No entanto, pouco é conhecido sobre as consequências de separações maternais curtas e repetitivas no comportamento adolescente, assim como as suas implicações na maturação do sistema oxitocinérgico.

De forma a explorar este assunto, o presente estudo tem como objectivos: 1) Avaliar o impacto de curtas SM diárias durante dois períodos diferentes em idade precoce, no comportamento de ratos adolescentes. 2) Investigar a capacidade do ambiente enriquecido (EE) proteger dos efeitos tóxicos da SM no comportamento social adolescente. A estimulação providenciada via EE aplicada precocemente tem impacto tanto no cérebro como no comportamento, podendo ser benéfica para o desenvolvimento do comportamento. 3) Determinar o perfil de expressão da OXT e do OXTR correlacionando com os efeitos da SM.

Ninhadas de ratos *Wistar* foram separados diariamente por 2 horas entre os dias 2-6 e 10-14 pós natais, durante os quais metade da ninhada estava em EE e a outra metade em condições padrão. De forma a avaliar os diferentes componentes do comportamento social durante a adolescência foram executados testes de reconhecimento e interação social com sujeitos familiares e não familiares. Foi também determinado o perfil de expressão da oxitocina e do seu receptor por RT-PCR no mesmo período de desenvolvimento. Os resultados demonstram que até SM mais curtas induzem alterações no comportamento social e sistema oxitocinérgico. Os ratos expostos a SM entre o período 2-6 não expressaram nem motivação/afiliação social nem preferência pela novidade social (dano da memória social). O EE durante este período de SM protegeu o rato adolescente deste dano da memória social. Adicionalmente, a SM durante o período 10-14 alterou a interação social (principalmente o comportamento

afiliativo) com o par familiar mas não com o não familiar, enquanto que no período 2-6 teve efeitos mais modestos na interação social em ambos os casos. Estes dados comportamentais estão positiva e negativamente correlacionados com um aumento da expressão do OTR no córtex pré frontal.

Essencialmente, este estudo providencia novas evidências de que curtos perídos de SM podem modular o comportamento social adolescente, que são diferencialmente sensíveis pela descendência, modulando também o sistema oxitocinérgico, com alguns destes danos a serem revertidos pelo EE.

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Abbreviation Index

ACTH	Adrenocorticotropic Hormone
cDNA	Complementary DNA
CNS	Central Nervous System
CRF	Corticotrophin – Releasing Factor
CRH	Corticotrophin – Releasing Hormone
C_t	Cycle Threshold
E	Embryonic Day
EE	Enriched Environments
FMRI	Functional Magnetic Resonance Imaging
HPA	Hypothalamic – Pituitary – Adrenal
IL	Interleukin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G Protein Coupled Receptors
mRNA	Messenger RNA
MPOA	Medial Pre Optic Area
MS	Maternal Separation
OXT	Oxytocin
OXTR	Oxytocin Receptor
OXT – IR	Oxytocin Immunoreactive Neurons
PCR	Polymerase Chain Reaction
PFC	Pre Frontal Cortex
PND	Postnatal Day
PVN	Paraventricular Nucleus
RT – PCR	Real – Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SON	Supraoptic Nucleus
SHRP	Stress Hypo Responsive Period
VTA	Ventral Tegmental Area

Introduction

1.1 First social interaction in mammals: the mother-pup bond

In mammals and rodents in particular, the mother is vital for homeostatic regulation and constitutes the first social bond in the life of offspring. Rats are a special model to study this specific social bond, because unlike other mammals that are promiscuously maternal, female rats only present interest in pups shortly before parturition (Insel, 2000). The first social interaction of a pup has the intervention of the dam and it is the result of specific interactions between them. This bond helps to maintain proximity, stimulates maternal care (Bowlby, 1969) and has a particular role on the establishment of the infant's stress coping style (Lucassen et al., 2015). Indeed, the quality and quantity of maternal care was demonstrated to be very important for both humans and rodents considering it modulation of the expression of glucocorticoids and corticotrophin – releasing hormone (CRH) genes, which are involved in the appropriate stress response (Korosi & Baram, 2009).

During the weaning period, mammalian infants display motivation to seek out maternal contact and care producing affiliative behaviors and attachment bonds with their caregivers (Hofer et al., 1989). In turn, the dam adequately respond to litter needs, providing essential thermal, somatosensory, olfactory, visual and auditory stimulation, as well as maternal milk, for an extended period of postnatal development (Pryce, 1996). The maternal behavior reinforces infants' innate bonding behavior that ensures their physical development and consequent adequate growth increasing the offspring's probability of survival (Insel & Young, 2001).

Milk transfer is one of the primary functions of the mother-pup relationship. In fact, breastfeeding is frequently described as a unique form of social contact that promotes a strong affiliative bond (Febo et al., 2005) and has been shown to reduce activity and induce calmness on pups (Blass & Fitzgerald, 1988). Furthermore, the glucocorticoids present on caregiver's milk have been connected with an enhance of the negative feedback on the hypothalamic – pituitary – adrenal (HPA) system (Ciruili et al., 1992). Interestingly, Kikusui and his colleagues proved that the dam has an important role on the regulation of the pups' corticosteroid levels. Indeed, they observed high corticosteroid levels of pups, which where weaned at postnatal day 14 for 48 hours, return to baseline levels within 2 hours after pups return to the nest and reset interaction with the mother (Kikusui et al., 2009). The decrease of corticosteroid levels seems to be a side effect of the mother-pup bonding which has also been described as a social buffer that prevents infants from experiencing acute stress at least during weaning.

The mother-pup interaction depends on several elements to establish the bond depending on the olfactory learning, in which pups learn how to identify the mother through tactile stimulation from the dam and the dam's odor, and it has the intervention of many hormones. The olfactory learning mechanism enables pups, that are born deaf and blind, to direct its behavior towards the mother but also for the mother to direct contact and licking (Moriceau et al., 2010; Moriceau et al., 2009; Reis et al., 2014). A crucial hormone for the mother-pup bond (Feldman et al., 2010; Nelson & Panksepp, 1998) as well as other social behaviors (Insel & Young, 2001; Insel, 2010; Lieberwirth & Wang, 2014) is oxytocin (OXT). This hormone has been proved to facilitate the onset of the maternal behavior (Nelson & Panksepp, 1998). In fact, studies of Pedersen and his colleagues reported a full profile of maternal behavior when administered OXT to virgin female rats (Pedersen et al., 1982). Besides, when female rats were administered with OXT antiserum (van Leengoed et al., 1987), injected with selective OXT antagonist into ventral tegmental area (VTA), medial pre optic area (MPOA) and the olfactory bulb (Pedersen et al., 1994; Yu et al., 1996) or presented lesions on paraventricular nucleus (PVN) (Insel & Harbaugh, 1989) the maternal behavior was impaired. This suggest that the oxytocinergic system present in brain areas involved in the regulation of olfactory discriminations, emotions and reward, is extremely important to the expression of the maternal behavior and subsequently that it may strengthen mother-pup bond formation, which is also supported by studies with functional magnetic resonance imaging (FMRI) that shown that during breastfeeding, the release of OXT activated brain regions previously associated with the mother-pup bond (Febo et al., 2005). Literature suggests activation of hypothalamic OXT neurons immediately after birth, however its release has not been examined (Mogi et al., 2011). The expression the OXT receptors has been described as different in infants in different brain areas when compared to adults. In particular, the cingulate cortex is one of these altered brain sites and has been described in humans as an important area for emotional and emphatic responses (Compton, 2003; Singer et al., 2006). Additionally, OXT and OXT receptor knockouts were described as emitting less ultrasonic vocalizations induced by isolation when compared to wild types. This might be result of a reduced sensitization of the knockout rats to social isolation, as maternal separation (MS) than wild type pups (Mogi et al., 2011; Takayanagi et al., 2005; Winslow et al., 2000), resulting in a lower exploration of the maternal contact and care. Notably, it has been also revealed that early-weaned rats display decreased maternal behavior in adulthood towards pups, which subsequently is transmitted to the next generation non-genetically (Mogi et al., 2011). On the contrary, oxytocin receptor (OXTR) overexpression has been associated with dysmenorrhea as demonstrated by

studies of Sun-Wei Guo (2013) (Guo et al., 2013), and increased expression has also being linked to enhanced alloparental responsiveness and accelerated partner preference formation as adults (Keebaugh & Young, 2011). Besides, studies from Lozic (2014) demonstrated for the first time that the overexpression of OXTR in the PVN was associated with the cardiovascular short-term variability and autonomic control of the circulation. In their research they found that rats overexpressing OXTR in this specific brain area had enhanced sensitivity of the baroreceptor reflex, which is meant to maintain blood pressure at constant levels and it buffered the stress induced baroreceptor variability response mediated by an increased outflow to blood vessels and stimulation of respiration (Lozić et al., 2014). This buffer effect might be mediated by the production of OXT by the magnocellular neurons and it supports other studies that suggest that OXT activates an anxiolytic response (Windle et al., 1997; Windle et al., 2004).

Together this data suggests that the OXT neural system might be also related to infant bonding and regulated by the presence of the mother (Mogi2011). Besides the mother infant bond might be an adaptive system that ensures infant's sociality development.

1.2 Oxytocinergic system

1.2.1 Oxytocin

Oxytocinergic system has served as a suitable example for discovering important molecular and cellular mechanisms of neuropeptide synthesis, precursor processing, and cellular trafficking. Sir Henry Dale first discovered it in 1906 during an experiment with extracts from human posterior pituitary that contracted the uterus of a pregnant cat (Viero et al., 2010). Since then, it has been explored in thousands of studies, being the first peptide hormone to be sequenced and synthesized by Vincent du Vigneaud in 1953, onwards having multiple interests and employments (du Vigneaud, et al., 1953).

OXT is a neurohypophyseal peptide synthesized in the hypothalamus and released into the blood stream via axon terminals in the posterior pituitary or neurohypophysis (Gainer, 1998). As all neurohypophyseal hormones it has a disulfide bridge between Cys residues 1 and 6, with hormonal effects and constituted of a 6 amino acid cyclic and a 3 amino acid C-terminal parts as seen in Figure 1. From an evolutionary point of view it is extraordinary that OXT homologs are still present in

invertebrates species (Knobloch & Grinevich, 2014) and that it is produced OXT or OXT-like hormones in all vertebrates (Hoyle, 1999). These are nonapeptides that differ from each other by the substitution of aminoacids in the polypeptide chain (Barberis et al., 1998).

Currently, several studies establish it's important role in a wide spectrum of central and peripheral effects (Gimpl et al., 2001; Lee et al., 2009; Macdonald & Feifel, 2014; Ross & Young, 2009; Skopek et al., 2012; Uvnäs-Moberg, 1998) further it best known effects on parturition and lactation (Blanks & Thornton, 2003; Wakerley & Lincoln, 1973). Its effects come from the modulation of neuroendocrine responses (Stoop, 2012) to central mediation of social complex behaviors (Aoki et al., 2014) including attachment behaviors related to reproduction and care of the offspring (Caldwell & Iii, 2006; Neumann, 2008), but it also has an important role as a neurotransmitter in the brain (Insel & Young, 2001).

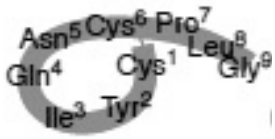

Figure 1 - Schematic model of the OXT's amino acid structure (Retrieved from Zingg et al., 1995)

Gene structure: anatomical synthesis and distribution

The OXT gene is expressed mostly in the PVN and supraoptic nucleus (SON) (see figure 2) of the hypothalamus and it is mainly released (by exocytosis) in response to multiple physiological stimuli (Gainer, 1998; Ivell & Richter, 1984; Viero et al., 2010). Actually, there were identified in the PVN two populations connected to the production of OXT: magnocellular neurons with projections that terminate in the neurohyphophysis and parvocelullar neurons with projections to specific areas of the central nervous system (CNS) (Ivell & Burbach, 1991).

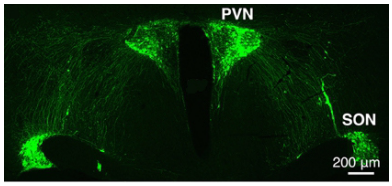

Figure 2 – Oxytocin neurons in PVN and SON in rat brain revealed by green fluorescence-protein (Retrieved from Knobloch & Grinevich, 2014).

In all species, OXT genes are located on the same chromosomal locus but are transcribed in opposite directions (Hoyle, 1999) as a result from the duplication of a common ancestral gene, which was followed by inversion of one of the genes. The OXT gene is found on chromosome 3 in rats (Caldwell & Iii, 2006; Ivell & Richter, 1984) and in chromosome 20p13 in humans (Dutil et al., 2001; Rao et al., 1992). Both genes consist in three exons: the first encodes a translocator signal peptide and the first nine amino acids of neurophysin, an important carrier peptide; the second exon encodes the central part of neurophysin (which primary function is the correct targeting, packaging, and storage of OXT) while the third encodes the COOH-terminal region of neurophysin (Marini et al., 1993; Sausville et al., 1985) (see figure 3). After transcription and synthesis as an inactive precursor, the OXT prepeptide is subject to cleavage and other post transcriptional modifications being conveyed axonally to the posterior pituitary where it can be released by appropriate stimulation (Gimpl et al., 2001; Ivell & Richter, 1984).

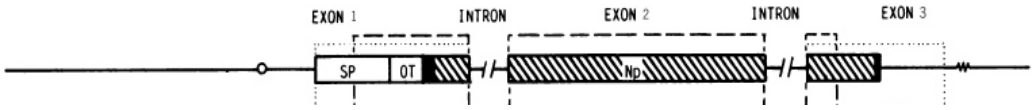

Figure 3 - Representation of the structural organization of the OXT gene (Retrieved from Ivell & Richter, 1984)

1.2.2 Oxytocin receptor

Receptor Structure

The OXTR is a cell surface membrane protein of 43 kDa and it consists in 389 amino acids (Kimura et al., 1992). It is a classic member of the rhodopsin-type (class I) GPCR (G protein coupled receptors) family with seven transmembrane domains. This receptor display the structural hallmarks characteristic of most GPCRs: glycosylation on Asn residues present in the extracellular domains, a disulfide bridge between two highly conserved Cys residues in the second and third extracellular domains, and two relatively well-conserved Cys residues within the C-terminal receptor domain, which have been shown to be palmitoylated in other GPCRs (Barberis et al., 1998) (see Figure 4). The conserved residues among the GPCRs appear to be involved in a common mechanism for activation and signal transduction to the G protein. The switching from the inactive to the active conformation is

associated with a change in the relative orientation of transmembrane domains 3 and 6, (Bockaert & Pin, 1999). It has two (mouse, rat) or three (human, pig, sheep, rhesus monkey, bovine) potential N-glycosylation sites (N-X-S/T consensus motif) in its extracellular NH2-terminal domain, however it does not seem to be essential for proper expression and has no effect on the functional properties of the receptor (Kimura et al., 1997).

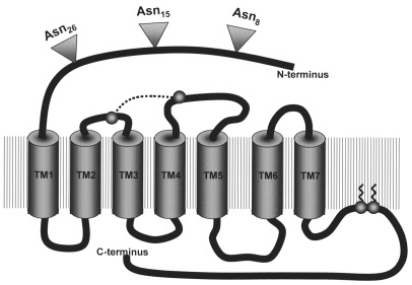

Figure 4 – Structure and organization of the OXTR indicating the three potential sites for N-glycosylation in the extracellular N-terminus. Cysteines are shown as filled circles (Retrieved from Ivell et al., 2001).

Gene Structure

The OXTR gene is present in single copy in the human genome in the chromosome 3p25–3p26.2, contains 3 introns and 4 exons (Inoue et al., 1994; Michelini et al., 1995; Simmons et al., 1995) and encodes approximately 389 amino acids. The rat OXTR gene is present in chromosome 4 (Caldwell & Iii, 2006), contains 3 exons and it is 93% identical to the human OXTR sequence (Kubota et al., 1996; Rozen et al., 1995).

Exons 1 and 2 correspond to the 5-prime noncoding region followed by exons 3 and 4 that encode the amino acid sequence of the OXT receptor. In fact, exon 4 contains the sequence encoding the seventh transmembrane domain, the COOH terminus, and the entire 3-noncoding region, including the polyadenylation signals (see Figure 6) (Kimura et al., 1992).

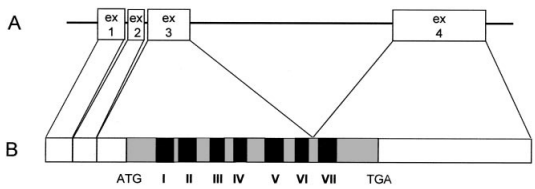

Figure 5 – Structure of human OXTR: OXTR gene (A) and OXTR cDNA (B) (retrieved from Gimpl et al., 2001)

Localization of OXT and OXTR within the brain and gene expression through development

In the brain, OXT gene is essentially expressed in magnocellular neurons in the hypothalamic PVN, which terminate in neurohypophysis and SON that have terminations in other parts of the CNS. Despite this, OXT fibers and OXT neurons have been widely described in the CNS in rat (for more details see Buijs et al., 1985). The distribution and number of OXT binding sites changes in critical periods of development (Olazábal & Alsina-Llanes, 2015; Tribollet et al., 1992; Yoshimura et al., 1996). The OXTRs are abundantly distributed in several brain areas including the limbic system and the hypothalamus (Ostrowski, 1998; Yoshimura et al., 1993) (see table 1 for more details) although only a fraction of the OXTRs are constantly present through development. Independently of the sex, two critical periods were recognized, in which the expression differs from infant to older ages: the 3rd postnatal week, right after the weaning period, and puberty (Tribollet et al., 1992). In fact, during the early postnatal life, areas containing high densities of OXT-binding sites like the *cingulate, retrosplenial cortex* and *substancia gelatinosa* posteriorly drop, or are strongly reduced, between postnatal days 10 and 21. On the opposite, some OXT-binding sites only appear at the time of puberty, between postnatal days 40 and 45, at areas like the olfactory tubercle, hypothalamic ventromedial nucleus, bed nucleus of the stria terminalis and the central amygdaloid nucleus (for more details see table 1). This can be explained based on studies by Tribollet et al. (1992) and Yoshimura, (1996) in which they demonstrated that the developmental profile of OXTRs is classified as constantly or transiently expressed through development depending on the brain area and age of the animal. The transient expression corresponds to restricted intervals in which there is a detectable OXTR expression while the constant expression consists in a continuous expression until the adult age of the animal. Additionally, it is extremely important to have in concern the different circulation and release of OXT during the early development versus the adult, due to incomplete maturation of important structures. Actually, the OXTR mRNA appears for the first time at E (embryonic day) 13, representing the earliest expression in the CNS. Then, a stronger signal is detected at E 15, which is maintained throughout development being slightly reduced in adults (Yoshimura et al., 1996). Nevertheless, the ligand, OXT hormone, is only detected at time of birth (Buijs, 1992). The discrepancy in the timing of appearance between the OXTR and OXT might be due to the early expression of the receptor, which anticipates the release of OXT to express their function through development (Yoshimura et al., 1996).

Regulation of OXTR

As many members of the GPCR superfamily, OXTR expression undergoes through dramatic and cell-specific up and down regulation. The tissue-specific regulation of OXTR expression enables circulating OXT to switch its target organs. Besides, it appears to have both inhibitory and stimulatory influences acting upon a constitutive pattern of basal expression (Ivell et al., 2001).

There is plenty evidence suggesting that gonadal steroids can regulate various aspects of the OXT system (Insel et al., 1993; Patchev et al., 1993; Tribollet et al., 1990). In uterus as well as hypothalamus, the regulation seems to be indirectly correlated with gonadal steroids probably involving intermediate transcription factors or cofactors (Ivell & Walther, 1999). In rats, increased expression of OXT mRNA in certain areas of the hypothalamus are coincident with the onset of puberty and vary across the estrous cycle; when both of these events are associated with increased concentrations of circulating estrogens. Apart from regulating the transcription of OXT, gonadal steroids also affect serum and pituitary levels of OXT, axonal and dendritic release of OXT, as well as the electrical activity of OXT neurons (Dhakar et al., 2013; Insel et al., 1993).

It has also been proposed that human OXT receptor gene is associated with transcriptional gene suppression (Yoshimura et al., 1996) by a genomic element in the third intron of it's sequence as it was hypermethylated in nonexpressing tissues and hypomethylated in myometrium when the OXT receptor gene was up regulated (Mizumoto et al., 1997).

Besides, though the 50-flanking regions of the rat and human OXTR contain several potential interleukin (IL). ILs were thought to represent potential regulators of OXT receptor expression and recent findings indicate that the human OXTR promoter is negatively regulated by IL-1b and IL-6, and that both cytokines decrease messenger RNA (mRNA) of OXTR accumulation in myometrial cells (Schmid et al., 2001).

Nevertheless and unlike others GPCRs, the OXTR undergoes desensitization by persistent agonist stimulation (Evans et al., 1997). This is, when these receptors are stimulated for a long period, they desensitize, being posteriorly phosphorylated and internalized. This process is very fast (within seconds or minutes) and can happen by numerous processes operating at transcriptional, translational, and protein levels (Evans et al., 1997; Gimpl et al., 2001).

Table 1 - Distribution and Relative Densities of OXT-Binding Sites in the Adult (PND 90) and the Infant (PND 10) in the rat CNS (retrieved from Tribollet et al., 1992)

	Adult	Infant
Cortical Areas		
Dorsal peduncular cortex	+++	++
Insular cortex	+	+
Perirhinal cortex	+	+
Cingulate cortex	0	++++
Retrosplenial cortex	0	++++
Olfactory System		
Anteroventral olfactory nucleus	++	++
Basal Ganglia		
Caudoputamen	+	+++
Islands of Calleja	++++	0
Ventral pallidum cell groups	++++	0
Limbic System		
Bed nucleus of the stria terminalis	+++	++
Lateral septal nucleus, intermediate	+	+
Central amygdaloid nucleus	+++	++
Medial amygdaloid nucleus	+	+
Basolateral amygdaloid nucleus	+	+
Dorsal subiculum	+	++++
Ventral subiculum	++++	+
CA1	+	+
Amygdalohippocampal area	+	+
Parasubiculum	++	++
Presubiculum	++	++
Thalamus		
Anteroventral thalamic nucleus	0	+
Periventricular thalamic nucleus	+	++
Hypothalamus		
Ventromedial nucleus	++++	0
Medial tuberal nucleus	++	++
Supramammillary nucleus	+	+
Lateral mammillary nucleus	+	+++
Medial mammillary nucleus	0	+++
Brainstem		
Dorsal motor nucleus of the vagus nerve	+	+
Inferior olive	+	+
Substantia gelatinosa of the trigeminal nucleus	+	++++
Spinal Cord		
Substantia gelatinosa	+	++++

[a] Just detectable (+) to highest density observed (++++). No binding, (0). PN90, postnatal day 90.

1.2.3 Oxytocinergic system and social behavior

Social behavior is the establishment of a pair bond between two animals and it is critical for social organization (DeVries et al., 1996). To perform any type of social interaction it is required the communication between conspecifics, in which it is used species-specific social and behavioral cues to to "understand" and "read" the intentions of the other (Veenema & Neumann, 2008). The development of social familiarity in rodents depends predominantly on olfactory or pheromonal cues, which define the capacity to recognize a conspecific, to determine the adequate response and formation of the "social memory" of individuals when within a social group (Neumann, 2008; Sanchez-Andrade & Kendrick, 2009; Winslow & Insel, 2004).

Adolescence is a gradual period (at about 40 days of age) in which the animals transit from childhood to adulthood characterized by certain characteristic behaviors, including increases in peer-directed social interactions and elevations in novelty-seeking and risk-taking behaviors (Spear, 2000). Social interactions and affiliation with peers take on particular importance during this time, and juveniles usually have more quality time of playful behaviors (Michael J. Meaney & Stewart, 1981; Panksepp et al., 2007; Spear, 2000). In fact, studies in animals that were deprived of play during the juvenile period indicate that social play is critical for normal social, motor, cognitive, and emotional (Argue & McCarthy, 2015; Graham, Burghardt, & Wiens, 2010; Pellis, Pellis, & Bell, 2010; Spinka, Newberry, & Bekoff, 2001; Van Den Berg et al., 1999).

Early social experience, stress or stress hormones have been described as important modifiers of the formation of the "social memory", so they can modulate social behavior as well as the partner preference (DeVries et al., 1996; Young & Wang, 2004). OXT has been described has having a key role on regulating the formation of social memories and social recognition for a lot of species (Lee et al., 2009; Popik et al., 1992) as well as on mediating the benefits of positive social interaction and emotions (Uvnäs-Moberg, 1998). Indeed, the recognition of a familiar partner and subsequent pair bonding are essential to social organization. Therefore understanding the interaction between the OXT and social bonding has been and continues to be a source of stimulating studies in neuroendocrinology (DeVries et al., 1996). Several studies link OXT to pair bonding, which is mainly characterized by social preference (the subject spends more time in close proximity) of a familiar partner instead of a stranger one, being also referred in other studies as partner preference (Getz et al., 1981; Young & Wang, 2004). Interestingly, OXT-knock out male mice failed to recognize familiar conspecifics that have

previously encountered and social memory deficits were also found in mice lacking OXT or OXTR (DeVries et al., 1996; Winslow & Insel, 2002). Additionally, some pharmacological studies demonstrated that intracerebroventricular injections of small doses of OXT on medial amygdala could rescue the social recognition deficit (Ferguson et al., 2001). Nevertheless, some other brain areas as pre frontal cortex (PFC), nucleus accumbens and ventral pallidum have been also referred as essential to the formation of this pair bond using the reward brain system (Wise, 2002).

Furthermore, there are additional OXT effects related to rodent social behaviors, namely maternal care, aggression, affiliative behaviors, pair bonding, social recognition, cognition and sexual behaviors (Arakawa et al., 2015; Calcagnoli et al., 2015; Lukas et al., 2010; Miller & Caldwell, 2015; Ross et al., 2009; Uvnäs-Moberg, 1998; Young & Wang, 2004). Besides, there are some evidence towards similar behavioral effects on humans and indirect evidence supports also the OXT anxiolytic and anti stress effects (Carter et al., 2001; Carter & Altemus, 1997; Kosfeld et al., 2005), (for more details see table 2).

Table 2 – Behavioral effects of OXT (Retrieved from (Lee et al., 2009)

Behavioral classes	Behaviors	Effects of Oxt in rodents	Effects of Oxt in humans
Social behaviors			
Social memory	Social recognition	–⇑ odor processing in olfactory bulb –⇑ social memory –⇓ social recognition in Oxtr KO mice – abnormal Bruce effect in female Oxt KO mice	–⇓ amygdalar activation to social stimuli –⇑ memory for faces
Affiliation	Sexual behavior	–⇑ erections (with T) and ejaculation frequency in males –⇑ receptivity (with E) in females	–⇑ arousal in men and women –⇑ uterine contractions at parturition
	Paternal behavior	–⇓ parental behavior with concomitant Avp/Oxt antagonism –⇓ adult paternal behavior with Oxt antagonist on PND1	no known effect
	Maternal behavior	– ⇑ Oxtr throughout the brain with onset of maternal behavior – necessary for lactation – induces full repertoire of maternal behaviors (in presence of E) – ⇓ pup retrieval and pup survival in Oxtr KO	no known effect
Aggression	Female aggression Male aggression	–⇑ Oxt levels in CeA correlated with aggression – may have organizational effect during prenatal period	no known effect –⇑ plasma Oxt levels in males with conduct disorder
Non-social behaviors			
Learning and memory	Non-spatial memory	–⇓ memory in passive avoidance tasks	–⇓ episodic memory in men and women –⇓ verbal recall of certain categories of words
	Spatial memory	–⇑ memory when injected into hippocampus – ⇓ memory when injected into NBM	no known effect
Anxiety and depression	Anxiety	–⇓ anxiety following Oxt administration –⇑ anxiety in some Oxtr KO mice; sexually dimorphic	–⇓ amygdalar response to threatening stimuli –⇓ anxiety to social stressors
	Depression	–⇑ active/coping behaviors with i.p. Oxt administration	– ⇓ plasma Oxt associated with major depression

1.3 Maternal Separation Paradigm

The MS is a well-established and powerful model of early life stress, applied in different research areas that can be used to characterize alterations on behavior and biological development.

Events occurring in early life are powerful modulators and can produce profound and long lasting changes in the development of different biological systems. Distinctive behavioral changes include impaired maternal care (Lovic et al., 2001), decreased maternal aggression in rats (Boccia & Pedersen, 2001), increased inter male aggression (Veenema et al., 2006), increased dominant juvenile play-fighting behaviors (Veenema & Neumann, 2009), impaired social recognition (Lukas et al., 2011) and play behavior (Arnold & Siviy, 2002; Zimmerberg & Sageser, 2011)) plus some evidences suggests impaired spatial learning and memory ability (Huang et al., 2002). It can also affect growth, metabolism, reproduction and inflammatory/immune responses (Chrousos & Gold, 1992; Darnaudéry & Maccari, 2008; Mesquita et al., 2007; O'Mahony et al., 2009; O'Malley, Dinan, & Cryan, 2011; Roque et al., 2014), increase susceptibility to addictions (Moffett et al., 2007; O'Mahony et al., 2009b; Ploj et al., 2003) and lead to psychiatric disorders (Charmandari et al., 2005; Chrousos & Gold, 1992; Chrousos, 2009). In particular, this stress induction model impacts the CNS that regulates the stress responsiveness (Hennessy et al., 2009; Lukaset al., 2010; Nishi et al., 2014; Zhang, & Zhao, 2013; Zhang et al., 2012) altering the trajectory and programming the response to stress (Darnaudéry & Maccari, 2008).

The optimal regulation of stress response is essential to adaptation, health and maintenance of homeostasis (De Kloet & Derijk, 2004; Ulrich-Lai & Herman, 2009). When the homeostasis is disturbed, the organism rapidly assemble a response in order to restore it (De Kloet et al., 1998). The physiological response to stress involves the activation of "classical" neuroendocrine mechanisms, from an efficient and highly conserved set of interlocking homeostatic systems, and aims to maintain physiologic integrity even in the most challenging situations (Ulrich-Lai & Herman, 2009). In order to restore the physiological and behavioral homeostasis, the autonomic nervous system and the HPA system are activated culminating with activation of stress centers in the brain (Cerqueira et al., 2007; De Kloet et al., 1998; Herman et al., 2003). There are two forms in which the stress response occurs. Initially, in an immediate response of a "proactive" mode, the corticotrophin – releasing factor (CRF) is liberated from the hypothalamic PVN in response to the stimuli. CRF is a very important molecule to organize the response to stressors and when it is released into hypophysial portal vessels it accesses the anterior pituitary gland. The binding of CRF to its receptor induces the release of adrenocorticotropic hormone (ACTH) into the systemic circulation (Charmandari et al., 2005; Smith & Vale, 2006). This immediate reaction is compensated by the parasympathetic nervous system activity

and in the endocrine dominion, ACTH targets it main focus, the adrenal glands, where it leads to an elevation of circulating levels of glucocorticoids (De Kloet et al., 2005; Herman et al., 2003; Kellendonk et al., 2002; Smith & Vale, 2006). Next, a slower approach takes place, the "reactive" mode, which facilitates the adaptation and helps re-establishing homeostasis (De Kloet et al., 2005). The corticosteroids feedback helps to terminate the stress-induced HPA activation by negative feedback regulation (Kellendonk et al., 2002) (Figure 6).

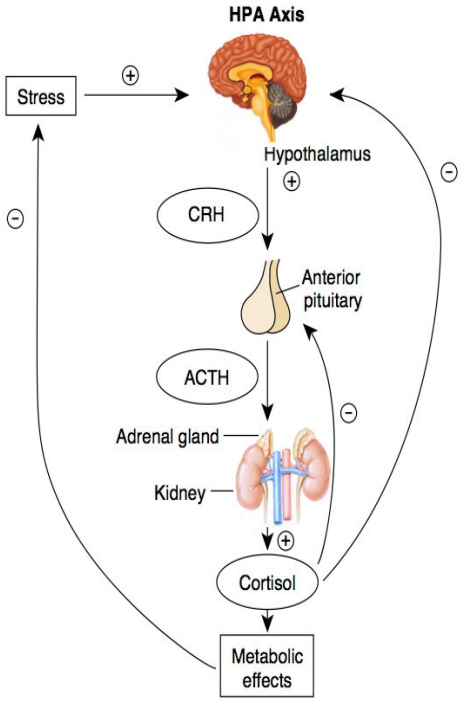

Figure 6 – The influence of stress in the HPA axis and how its metabolic effects ends regulating the stress response (Adapted from ThePaleoMom, 2014).

In rodents, during the first two weeks of life there is a marked reduction of corticosterone secretion, which ensures low and stable levels of glucocorticoids (Levine, 2001; Sapolsky & Meaney, 1986). This is very important because it acts as a protective mechanism during this postnatal developmental window, designated as the stress hypo responsive period (SHRP) (De Kloet et al., 1998; Sapolsky & Meaney, 1986). Furthermore, it was demonstrate that the exposure to high levels of glucocorticoids, during the neonatal period leads to irreversible reduction of the brain weight (Huang et al., 1999), influences cerebellar development showing a total decrease in DNA content in the brain (Velazquez & Romano, 1987), impaired neuronal myelination (Dunlop et al., 1997), widespread

reduction of dendritic spines (Antonow-Schlorke et al., 2003) and inhibition neural precursor cell proliferation in brain areas that show postnatal neurogenesis (Scheepens et al., 2003).

1.3.1 Maternal Separation Protocols

The diversity of MS protocols is vast since they can vary in length and according to different developmental periods. MS protocol can occur in single or repeated periods of separation with sessions of few minutes to several hours but the majority of them occur during the SHRP (Schmidt et al., 2011). Additionally, for MS protocol, not only the periodicity of the separation is very important but also the amount of time that the pups remain separated from the dam. Literature suggests that a minimum of 2 hours is necessary to induce an immediate effect on HPA responsiveness (Kuhn & Schanberg, 1998; Pihoker, 1993) and more than 8 hours to produce an effect that is measurable numerous days after the manipulation (Rosenfeld et al., 1992). Many MS protocols are often used as a model for early life adversity mimicking maternal neglect where the pups are deprived from maternal care for several hours (Bulbul et al., 2012; Carlyle et al., 2012; Roque et al., 2014; Slotten et al., 2006). Nevertheless, there are MS paradigms that do not mimic maternal neglect. Instead, there is a physical separation between animals for lower time periods comparing to the MS protocols described above. In his studies, Levine manipulated the litter for approximately 15 min, where the dam was separated from pups during this period revealing several differences between non handled vs. handled animals (Levine, 1957). In one hand, early stress experience result in greater infant's capability to adapt to psychological and physiological stress in adulthood and so presenting better stress coping. On the other, more recent studies have shown that this short periods of MS has a stimulating effect of maternal care, providing higher levels to pups (Pryce et al., 2001). However, this is not very consistent with literature since there are studies that use repeated daily separations, even for shorter periods, that describe deleterious effects on pups development (Li et al., 2003; Rana et al., 2015; Roman et al., 2005). Additionally, longer separation periods are portrayed as capable to reduce the amount of maternal care for the pups and thereby modeling emotional as well as physical neglect (Schmidt et al., 2011) which may contribute to psychopathology in later life (Pryce & Feldon, 2003). Besides, pups that were maternally separated for long periods exhibit more anxious behaviors (Boccia & Pedersen, 2001), exaggerated behavioral and neuroendocrine responses to stress (Kalinichev et al., 2002) and display less maternal care in adulthood (Lovic et al., 2001). Indeed, a study from Mesquita and colleagues

(2007) suggest that MS acts on the serotoninergic system, and transiently impairs the acquisition of innate reflexes, which might contribute to the long-lasting effects of MS on emotionality and cognition (Mesquita et al., 2007).

1.3.2 Impact of MS on social behaviors

Early stressful events like the MS result in a withdrawal of regulatory processes that form the complex pattern of physiology and behavior responses (Hofer1994). The effects of the MS on social behaviors have been source of interest for more than 50 years for many mammallian species due to the alteration of the context of interactions between mother and offspring in the postnatal social environment (Hofer, 1973; Kaufman & Rosenblum, 1969). This early life stress protocol usually acts on critical time periods for the nervous system, like the SHRP during which the brain undergoes functional organization, neuronal proliferation, migration and differentiation, gliogenesis and myelination (Rice et al., 2000).

During the separation period the two-week-old rat pups show an immediate response consisting of repeated, high intensity vocalizations (in the ultrasonic range), accompanied by agitated searching behavior and high levels of self-grooming (Hofer & Shair, 1978). The body temperature declines, the HPA axis is activated and glucocorticoid secretion increases (Kuhn & Schanberg, 1998; Pihoker, 1993). Most of these manifestations are rapidly ameliorated when they return into contact with the dam or a familiar littermate (Hofer, 1994), or, even diminish as the pups gets older (Noirot, 1972).

Right after the MS period, rats demonstrated to respond to a novel environment with heightened levels of locomotion and rising behavior (Hofer, 1973), displayed more self-grooming, defecation, urination and a delayed sleep onset in comparison to their mothered littermates. Similar studies showed that rats were MS (Roman et al., 2005). Other studies have demonstrated to have neurobiological consequences in areas that significantly overlap with the neural circuitry involved in complex social behaviour, such as social recognition. There have been also studied the potential impact that MS may have on the long-term development of the neurobiology and behaviour involved in social interaction in rodents and higher primates (Lukas et al., 2011; Sabatini et al., 2007; Varlinskaya, 2008; Veenema, 2012; Zimmerberg & Sageser, 2011) further altered social preference patterns (Wang et al., 2015). It was also revealed an association between the duration of separation and attachment

disorder behaviors (O'Connor & Rutter, 2015).

The MS protocol disrupts the mother-pup bond by decreasing mother-pups contact and maternal care, changing temporal patterns. As described above, social proximity, olfactory learning, access to milk, and physical contact between animals are adversely affected (Reis et al., 2014). This disruption has also been proved to affect future pup's HPA development and therefore future responses to stress (Liu et al., 1997). Other studies showed that maternally separated pups present maternal care deficits in adulthood (Lovic et al., 2001). Because OXT has been described to modulate the HPA axis, which has been proved to be altered on social behaviors, we can hypothesize that this is an extremely important hormone that has a particular role on behavior development.

1.3.3 Impact of MS on oxytocinergic system

As discussed above, MS can affect different biological systems. Indeed, MS induces alterations on the HPA axis, which is a major stress response system, and results in a hyperresponsiveness to stress from the animal in adulthood. Indeed, maternally separated rats tend to display more social avoidance and behavioral inhibition during social encounters (Cavigelli et al., 2007; Núñez et al., 1996; Roelofs et al., 2009). The oxytocinergic system has been described as one that it is disturbed by this stress-induction model (Kong et al., 2015; Lukas et al., 2010). In fact, OXT has been associated as a major HPA axis modulator (Neumann, 2002; Windle et al., 2004; Zheng et al., 2010), and also implicated in the expression of behavioral responses to stress (Ferguson et al., 2001; Ferguson et al., 2002; Lukas et al., 2010; Neumann, 2008). Indeed, brain OXT has a major role in the regulation of complex physiological stress responses including the stress-induced neuronal activation (Windle et al., 2004). Besides, the early exposure to relevant stimuli is known to permanently alter the responsiveness of the hormone receptor (Miller & Caldwell, 2015), so alterations by MS are probably noticeable at an OXTR distribution and expression levels.

Neurochemical data has been suggesting a key role of OXT in the development and maintenance of social cognition and social behavior. In fact, there are some studies in which OXT and OXTR knock out mice that were maternally separated that revealed decreased motivation to be in contact with the dam affecting their attraction towards the mother (Winslow & Insel, 2002). Nevertheless, at a molecular level it was found that presynaptic OXT was not essential to normal neuroanatomical distribution of OXTR. In accordance with this, there is studies in male rats from Lukas

and colleagues (2010) demonstrated that exposure to early life stress, using MS model, interferes with the normal development of OXTR binding in some specific forebrain regions (decreased levels in the agranular cortex, lateral septum and increased levels in the ventromedial hypothalamus) suggesting that environmental manipulations can, indeed, shape the developmental expression of OXTR through the brain. Also, the maternally separated rats displayed a decrease of OXTR binding throughout age, resulting in a lower OXTR binding density at adult age when compared with control rats (Lukas et al., 2010). Also, studies from Kong et al. (2015) in monogamous rodents revealed that repeated separation from pups affected the number of oxytocin immunoreactive neurons (OXT-IR) in PVN (Kong et al., 2015). Actually, long repeated periods of separation revealed an upregulation of the OXT-IR, while brief repeated periods of separation increased the central number of OXT-IR. The same was previous found in studies with rat dams after two weeks of MS from pups (Boccia & Pedersen, 2001).

Knowing that MS is a stress induction model that introduces several animal behavior and molecular impairments and that animal's are particular sensitive to the surrounding environment, it becomes extremely important the understanding of how enriched environments (EE) provide extra comfort might be capable of attenuating some of those impairments.

1.4 Reverting MS impact: environmental enrichment

From all the different factors that influence social interaction, the environmental condition in which the animals are housed have been described to induce changes on it (Francis et al., 2002). The postnatal handling has been pointed out as a functional recover of the effects of prenatal stress through compensation on long-term impairment of the glucocorticoid feedback and induction of anxiety and even in reversing the effect of MS on the HPA function (Francis et al., 2002; Maccari et al., 1995; Vallée et al., 1997). In fact, there are studies that support that environmental handling is almost the opposite of MS when it comes to its effects (Caldji et al., 2010; Darlene D Francis et al., 2002).

Environment enrichment (EE) can be divided in two forms: physical and social. Social enrichment consists in housing animals in groups wherever possible, to promote social interaction. The physical includes bedding enhanced with natural materials (paper parchment), plastic tunnels, wooden objects to gnaw, ropes, swings, running wheels, balls, ramps, ladders and other appropriately sized animal toys (Simpson & Kelly, 2011).

Actually, it is believed that EE can improve animals' well-being, brain weight, and it is used to demonstrate learning and brain plasticity in response to different environments (Baumans, 2005). There are also multiple studies in which we can observe that there is significant differences when it comes to EEs, specially in social interactions: increase on social play and social rest (Morley-Fletcher et al., 2003) and a decrease on social exploration (Magalhaes et al., 2007; Magalhães et al., 2006) (for more alterations see table 3).

Table 3 – Consequences of EEs in housed rats (adapted from Simpson & Kelly, 2011)

Attribute	Effects of enriched housing vs Standard Conditions	
Body weight	↓ (males)	(Moncek et al., 2004)
	– (females)	(Harati et al., 2011)
General behavior	↑ habituation, ↓ activity	(Brenes et al., 2008; 2009)
	↑ grooming, ↓ rearing	
Anxiety	**Elevated Plus Maze:**	
	↑ open arm entries	(Pena et al., 2006)
	↑ open arm movement	(Peña et al., 2009)
	↑ percent open arm entries and time	(Galani et al., 2007)
	Social Interaction:	
	↑ social play, ↑ social rest and social exploration after prenatal stress	(Morley-Fletcher et al., 2003)
	Pre-weaning EE ↓ social exploration, social behaviour and play	(Magalhaes et al., 2007; Magalhães et al., 2006)
Depressive- like Behaviours	**Forced Swim Test:**	
	↑ swimming, climbing, diving, ↓ immobility	(Brenes et al., 2008; 2009)
Learning and Memory	**Morris Water Maze:**	
	↓ escape latencies	(Leggio et al., 2005; Zhong et al., 2009)
	Novel Object Recognition:	(Bruel-Jungerman et al., 2005; Pamplona et al., 2009)
	↑ novel object preference	(Hoffmann et al., 2009; Leggio et al., 2005)
	Radial Arm Maze:	
	↓ errors and time in arms	

The effects of EE in behavioural tests: EE increases (↑), decreases (↓) or has no effect (-) on behaviours.

1.5 Aims of the study

At early ages all interactions and experiences are determinant for acquiring competences and adapt to environmental challenges. Early life stress events have been shown to play a critical role on social behaviors and act as severe modulators of the oxytocinergic system having long lasting implications throughout adulthood.

The MS model has been typically performed during the SHRP with establishment of "temporal windows" of vulnerability to the stress programming effects. Contrary to the most MS protocols, our MS paradigm involved short separations and was used as a non-negligent model in which physical MS is necessary. Instead of the typical MS group we decided to have two different periods (within the SHRP): from 2nd to 6th PND and 10th to 14th PND, corresponding approximately in humans to the period, in which children initiate the nursery at age of five month and at age of 3 years, creating a bridge between rodents and humans.

The current knowledge in this field has yet many open questions, some of which will be addressed in this thesis.

The study developed in the scope of this thesis aims to:

- Evaluate the impact of short daily MS, during two time points periods of early life, on the modulation of different components of social behavior of adolescent rats;
- Investigate the effect of early interventions that can be designed to overcome or reduce the effects of these environmental insults and challenges.
- Determine the expression profile of OXT and OXTR during adolescence, and correlate it with the behavioral impacts of MS.

Materials and Methods

2.1 Animals

Wistar Han females (*Rattus norvegicus*) (Charles River, Barcelona, Spain) during estrous were placed overnight with males in same cage under controlled environmental conditions (ambient temperature 22°C, 60% humidity, 12 h light/12 h dark cycle, lights on at 00:00 a.m.) with free access to food and water.

Males were removed from the cage when a vaginal plug was confirmed; this day was designated as embryonic day 0 (E0). Pregnant Wistar females were left undisturbed during pregnancy. At the end of pregnancy (E22), all females were provided with nesting material and remained singly housed. Litters were delivered on gestation day 22. At birth (considered the postnatal day (PND) 0), pups were culled to 8 pups per litter, normalized in terms of gender and placed in a new cage. The experiments carried out were approved by local ethics committee and by the Portuguese Agency for Animal Welfare, general board of Veterinary Medicine, in compliance with the European Community Council Directive of September 22, 2010 (2010/63/UE). All procedures involving animals were conducted by FELASA C graded researchers, and all efforts were made to minimize the number of animals used and their suffering.

2.2 Maternal separation procedure

MS procedure consisted in a physical separation between the dam and the pups for 2h per day, during two different developmental periods, specifically between the 2nd and the 6th PND or between the 10th and the 14th PND. The mothers were removed from the home cage and put into another single cage (the same cage every day for each mother). Then the pups were removed from the home cage and put in another cage (the same cage every day) on heated sawdust at 30°C, in another room, so as to prevent vocalization and auditory communication with the mother.

To further understand the effects of environmental manipulations, we compared the behavior of rats submitted to MS period under enriched or standard conditions. In this context, during MS period rats were housed in different environmental groups: standard or enriched conditions. The EE consisted in nest material such as paper towels, cardboard tubes and cotton.

According to this, the litters were randomly divided in 5 different experimental groups:

- **MS 2-6 SE,** Maternal separation from PND 2 to 6 in standard environment
- **MS 2-6 EE,** Maternal separation from PND 2 to 6 in enriched environment
- **MS 10-14 SE**, Maternal separation from PND 10 to 14 in standard environment
- **MS 10-14 EE,** Maternal separation from PND day 10 to 14 in enriched environment
- **Control** pups from control litters were left undisturbed with their dams until weaning (PND 21)

After the two hours of MS, pups were returned to the home cage. At PND 22 all pups were weighed and housed in groups of two littermates of the same sex per cage until they achieve adolescence and the behavioral tests start.

2.3 Behavioral Tests

All testing procedures were conducted in the dark phase of the light/dark cycle between 13:00 and 20:00h under dim light (15–20 lx). All behavior tests were performed during the rat adolescent period when rats show a particularly strong tendency to be social (around 40 PND) (Terranova, Cirulli, & Laviola, 1999). They were designed to access the social recognition and the social interaction.

2.3.1 Social Recognition Abilities And Social Preference

Sociability is defined as the time that the animal spends with another rat, comparing to time spent alone in an identical but empty chamber (Kaidanovich-Beilin et al., 2011). Social memory is commonly examined in rodents through various social recognition tasks that utilize the innate preference of adult rodents to spend more time with novel over familiar conspecifics (Macbeth, Edds, & Young, 2009). One test that is used is the three-paradigm test or Crawley's sociability and preference for social novelty test. This test can be used to study social affiliation and social memory with novel conspecifics. Rats tend to spend more time interacting with a novel rat versus one they have previously encountered. The basic measure is the amount of time the subject spends with either a completely novel animal or an animal to which they have been introduced to in a previous session, or the animal can also choose to be in the middle section, that is, alone. The apparatus for Crawley's sociability and preference for social novelty test consists in a rectangular,

three-chamber box. Each chamber is 40 x 60 cm and the dividing walls are made from clear Plexiglas, with an open middle section, which allows free access to each chamber (Figure 7). Both tests were conducted as described by Kaidanovich-Beilin (2011).

Social Affiliation test (session I)

At 45 PND, each experimental rat started the test in the central compartment and was left there for 5 min for habituation. After this period the experimental rat was removed and another animal (Stranger I) was placed in one of the containers within the compartment of the apparatus while the other compartment remained empty. Then, the experimental animal was again placed in the center compartment and videotaped for 10 minutes. The widely spaced wire bars of the container permit olfactory, visual, auditory and some tactile contact while preventing aggressive and sexual interactions, thus ensuring a pure measure of simple interest in approaching and remaining in physical proximity to another.

Social Preference test (session II)

After social affiliation test, an additional rat (Stranger II) was placed in the empty container and the behavior of the experimental rat was again videotaped for additional ten minutes to record its behavior.

Videotapes were analyzed using the Noldus Observer® software (Observer 5; Noldus Information Technology, Wageningen, Netherlands). The following parameters were recorded: duration and number of direct (active) contacts between the experimental rat and Stranger I and empty containment cup or Stranger II. The following behaviors were analyzed 1) direct contact is define as stretching of the body of the experimental rat in an area 3-5 cm around the container; 2) number of other behaviors by the experimental rat in each compartment, including walking, self-grooming, lack of any body movements for more than 5 seconds (freezing); 3) duration and number of entries to each compartment (the animal is considered to be in the chamber when its head and four paws have entered into the chamber).

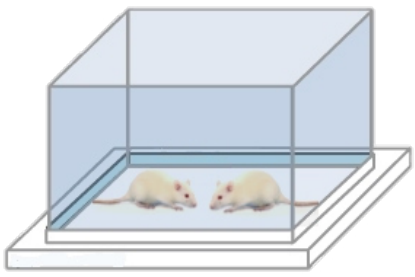

Figure 8 – Illustration of the Social Interaction tests (adapted from http://btc.psych.ucla.edu/openfield.htm).

2.3.2 Social Interaction

Adolescent rats perform two social interaction tests: one with a familiar subject (cage mate), at 40 PND and 48 hours later, another social encounter with an unfamiliar subject, in order evaluate social interaction.

Immediately prior testing, each experimental subject was marked by a vertical line on the back and placed alone in a holding cage for 60 min. This pre-test social deprivation in a novel environment is a standard procedure that has been extensively used to increase baseline levels of social behavior (File, 1993). Each animal was then placed into the testing arena simultaneously with a same age and sex test partner. The arena was constituted by an open arena of (40cm x 40cm x 40cm) that allowed the free interaction between the familiar or unfamiliar subject (Figure 8). During the 30 minutes time session the behavior of animals were recorded by a video camera above of the open arena. The Observer® (Noldus, Netherlands) software system was used for analysis of observational data, scoring time and frequency of each behavior classified in 5 different categories: Investigatory behavior, affilliative behavior, play, non-social activities and maintenance as described in table 4 (based on established behaviors and categories by Terranova, Cirulli, & Laviola, 1999). Only the last 10 min of the 30 min sessions were scored and statistically analyzed, since preliminary observations carried out in three sampled pairs of subjects for each experimental group confirmed that, as found in a previous work (Cirulli, Terranova, & Laviola, 1996), no behavioral differences were detectable during the first portion of the social encounters.

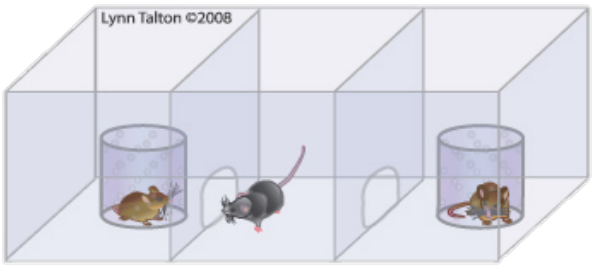

Figure 7 – Illustration of the apparatus for Crawley's sociability and preference for social novelty test (retrieved from http://btc.psych.ucla.edu/socinteract.htm)

Table 4 - Rodent's behavior assessment for social interaction tests

Social Activities	*Investigative*	**Follow** *(Closely following the partner)* **Mutual** Circle *(Partners mutually sniff each other)* **Nose to Nose sniffing** **Anogenital sniffing**
	Affiliative	Social Inactive *(Rat's lying flat or standing still)* Social Rest *(Rat's being groomed)* Allogroom *(One rat grooms the head and flanks of another rat in little nibbles)*
	Play	**Rough and tumble play** *(Both rats exhibit sudden darting movements like pouncing on the back, chasing, wrestling and pinning)* **Play Bout** *(vigorous allogroom)* **Chasing** *(running after avoinding or fleeing the partner)* **Dominant Posture** *(Lock partner in a submissive posture)* **Submissive Posture** *(Lying on back, throat exposed to the partner)* **Avoiding** *(Moving away from the partner, rotating body axe)* **Flee** *(Being chased by ando r running away from the partner)* **Attack** *(Bitting the partner)*
Non Social Activities		**Inactive** **Rearing** *(lifting the forepaws under its hindpaws)* **Digging**
Maintenance Activities		Self Grooming

2.4 Molecular Genetics

Tissue Preparation

At the 49 PND all animals (n=40) were sacrificed by rapid decapitation. The brain was removed and different areas were dissected. Samples were snap frozen by immersion in liquid nitrogen for posterior total RNA extraction.

RNA extraction protocol

Total RNA was isolated from the frozen tissue of the prefrontal cortex, amygdala and hypothalamus using Trizol (Invitrogen, Carlsbad, CA, USA) and the Aurum Total RNA Mini Kit (Bio-Rad, CA, USA) according to manufacturer's instructions. The frozen samples were homogenized with 1000 μl of Trizol using two different gauge needles for a complete homogenization of the tissue. After, a 5 minute incubation at room temperature was performed to facilitate protein dissociation. In order to promote different phases separation, 200 μl of chloroform (Carlo Erba Reagents by Dasit Group, Italy) were added following a vigorously mixed and incubated at room temperature for 3 min followed by a centrifugation at 12 000 rpm, 4°C during 15 minutes. The supernatant in the aqueous phase was collected and dehydrated through the addition of 600 μl of ethanol 60% (Carlo Erba Reagents by Dasit Group) stirring vigorously. The sample was transferred to a RNA binding column adding 700 μl of low stringency wash solution and centrifuged for 30 seconds at 12000 rpm at 4°C. In order to prevent DNA contaminations, each RNA binding column was treated with 80μl of DNase solution (5 μl DNase I dilution + 75 μl dilution solution) during an incubation period of 20 minutes. Posteriorly, 700 μl of high stringency wash solution was added to the RNA binding column, centrifuged at 12000 rpm for 30 seconds discarding the filtrate. For the low stringency wash solution, we repeated the step above, discarding the filtrate as well, centrifuging for additional 2 minutes. RNA binding column was placed in another tube, added 25μl of elution solution waiting 1 minute for the membrane to load, and centrifuge at 12000 rpm for 2 minutes to elute total RNA. Another 25μl of elution solution was added repeating the last step. The quality and quantity of the extracted RNA were evaluated using Nanodrop determining it's concentration and the 260/280 and 260/230 ratios that evaluate nucleic acid contaminations and other contaminations, respectively, as proteins and salts that could result from the extraction protocol.

Quantitative Real Time PCR

To analyze the expression of the OXT and OXTR genes we used the Real – Time Polymerase Chain Reaction (RT-PCR) technique, using the 7500 Fast Real Time PCR System (Applied Biosystems/Life Technologies, USA). First, a reverse transcription into first–strand complementary DNA (cDNA) was performed, using 1μg of the total RNA and a reverse transcriptase - iScript™ cDNA Synthesis Kit (Bio-Rad) -

according to the manufacturer's instructions (cycling profile: 5min at 25°C, 30min at 42°C, and 5min at 85°C).

The EvaGreen supermix (Solis Biodyne, Estonia) was used according to the manufacturer's instructions for a 20 μL total reaction volume (4 μL Master Mix/ EvaGreen, 0.5 μL 10 mM of each forward and reverse primer, 2 μL cDNA as template and 13 μL of water). The cycling parameters were 1 cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, annealing temperature was 60 °C for all genes analyzed for 20 s and 72 °C for 30 s. Primers OXTR *fw* 5' - GTC AAT GCG CCC AAG GAA GCT TC - 3' and *rev* 5' - CTC TGG CTT GAG CTG CGA CGG – 3'; GAPDH *fw* 5' - ATA GAC AAG ATG GTG AAG GT - 3' and *rev* 5' - GGT AGA GTC ATA CTG GAA CA – 3'; OXT *fw* 5' - GCC AGG AGG AGA ACT ACC - 3' and *rev* 5' - CTC GGA GAA GGC AGA CTC - 3'.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes, due to its constitutive expression in the mammalian cells. Data from RT-PCR were expressed according to the cycle threshold (C_t) obtained for each gene. The normalization was performed calculating the ΔC_T values for each sample: ΔCT of the target gene – ΔCT of the housekeeping gene. The housekeeping sample was always used as the baseline for each comparison. To transform these values into absolute values it was calculated the fold change with the formula $2^{-\Delta CT} = 2^{-(\text{Ct target gene - Ct housekeeping gene})}$.

The addition of the reverse transcription step producing the complementary RT-PCR was an outstanding and powerful tool to amplify any type of RNA and quantify gene expression (Heid et al., 1996; Higuchi et al., 1993). To detect RT-PCR products it is included a fluorescent molecule in the reaction that reports the amount of DNA by fluorescent signal. The accumulation of the amplified product is detected and measured as the reaction progresses and we can follow in real time. The main advantages of this technique are the ability to monitor in real time the reaction progress as well to measure the amount of the amplification product at each cycle.

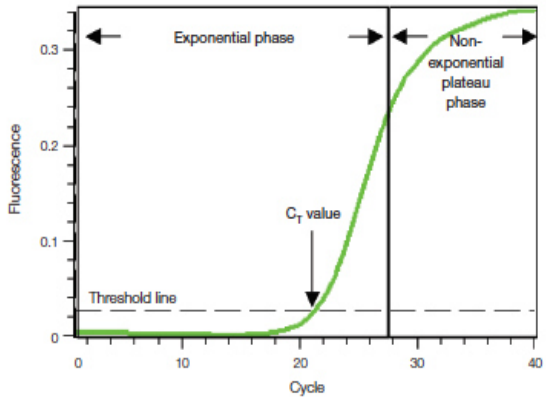

Figure 9 – Amplification plot of a typical real-time PCR output with a baseline of fluorescence for 40 cycles.

The amplification plot has two phases, an exponential phase and a non exponential plateau phase. During the exponential phase, the amount of product almost doubles in each cycle. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs and the curve intersects the CT (is 22.5 in this example). As the reaction proceeds, the reaction components are consumed, and ultimately one or more of the components becomes limiting.

2.5 Statistical analysis

Behavioral and molecular data was analyzed using the IBM SPSS ® software. Statistical tests for normality of the data were applied in order to decide the use of parametric or non-parametric tests. For the social recognition tests, an ANOVA for repeated measures was performed with one within subject factor (familiarity) and group as the between subject effect, followed by Bonferroni's Pairwise comparisons. Regarding social interaction tests and the expression of OXT and OXTR, parametric (ANOVA) or non parametric (Kruskal Wallis) tests were performed and the differences between the groups were assessed using Bonferroni's or Dunn's post hocs tests, respectively. For all tests it was evaluated the EE effect when there was a MS group effect. Correlations between the different behavioral and biological parameters were evaluated by the Pearson coefficient. Statistical significance was accepted for $p < 0.05$ and the results are expressed as mean ± s.e.m.

Results

3.1 Social Recognition

3.1.1 Social Affiliation test (session I)

ANOVA for repeated measures revealed a significant effect of familiarity (F (1, 21) = 18,290, p = 0,000) and no interaction effect between familiarity and group (F (2, 21) = 0,978, p = 0,393). Pairwise comparison analysis revealed that both MS 10-14 SE and Control groups displayed significant higher amount of time spent in the chamber with the stranger I vs an empty chamber (MS 10-14 SE p = 0,045 and Control p = 0,001). In contrast, the MS 2-6 SE group presented similar time spent in both compartments (p = 0,105) (Figure 10A).

Since early MS revealed to have an impact on the time spent between both compartments, we assessed the effect of an enriched environment upon social affiliation. A significant effect of familiarity was observed (F (1, 24) = 27,865, p = 0,000) while the same did not happened for familiarity and group effect (F (2, 24) = 1,051, p = 0,365). The Bonferroni's pairwise comparisons revealed that the MS 2-6 EE group displayed higher amount of time in the chamber that contained Stranger I similarly to the control group (MS 2-6 EE p = 0,002, Control p = 0,001), while the MS 2-6 SE displayed similar time in both compartments, as previously encountered (p = 0,081) (Figure 10B).

3.1.2 Social Preference test (session II)

Considering the social preference test, it was performed also an ANOVA for repeated measures which revealed no familiarity effect (F (1, 19) = 2,471, p = 0,132) and no interaction effect between familiarity and group (F (2, 19) = 2,719, p = 0,092). Despite this, pairwise comparisons demonstrated that the MS 10-14 SE group displayed a significant preference for the compartment that contained the stranger II, which was significantly different from the time spent of the control and MS 2-6 groups in this chamber (MS 10-14 SE p = 0,015; MS 10-14 SE from MS 2-6 SE p = 0,050; MS 10-14 SE from Control p = 0,046) (Figure 11A).

When we assessed the impact of an EE upon social preference, a significant effect of familiarity was observed (F (1, 19) = 10,595, p = 0,004) while it was not demonstrated a familiarity and group effect (F (2, 19) = 3,049, p = 0,071). Pairwise comparisons revealed that MS 10-14 SE

group had a preference to spend more time in the Stranger's II compartment vs. Stranger's I compartment (p = 0,001) while Control and MS 10-14 EE groups did not present any differences (p = 0,535 and p = 0,271, respectively). The higher values of time in the compartment of Stranger II of the MS 10-14 SE were also statistically different from the values of Control group for the same compartment (MS 10-14 SE from Control p = 0,046)(Figure 11B).

The time spent in each compartment does not necessarily indicate a physical contact neither an interaction with the strangers II and I. Concerning this, an ANOVA for repeated measures was performed and revealed a significant effect of familiarity (F (1, 19) = 21,328, p = 0,000) and no interaction effect between familiarity and group (F (2, 19) = 1,161, p = 0,257). Bonferroni's pairwise comparison analysis demonstrated that both Control and MS 10-14 SE groups presented significant higher values of direct contact with the Stranger II (MS 10-14 SE p = 0,001 and Control p = 0,039), while MS 2-6 SE group did not displayed differences (p = 0,090) (Figure 11C).

The effect of an EE upon the direct contact on social preference was assessed also by an ANOVA repeated measures that showed a familiarity effect (F (1, 22) = 18,890, p = 0,000) but no interaction effect between familiarity and group (F (2, 22) = 0,550, p = 0,585). The pairwise comparisons revealed an attenuation from the MS 2-6 EE group (p = 0,002), since it made more direct contact with the stranger II, similarly to the Control group (p = 0,034), while MS 2-6 SE group did not displayed differences as previously encountered (Figure 11D).

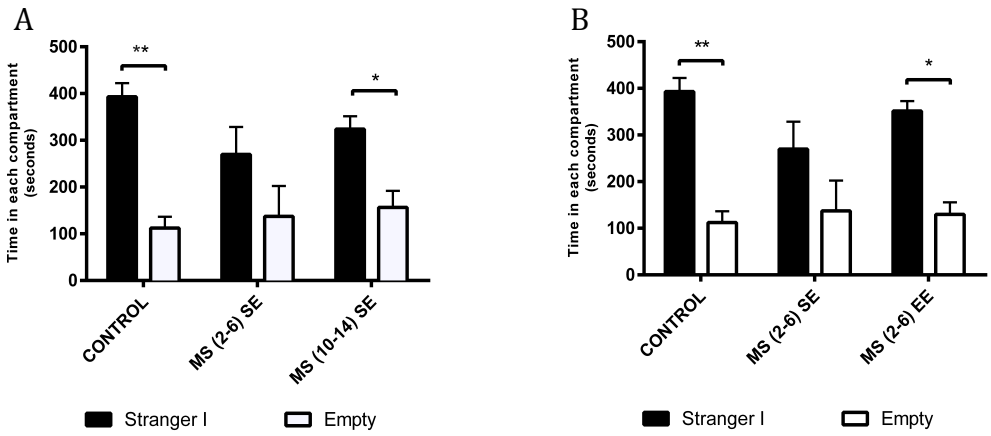

Figure 10 – Effects of MS (A) and different environmental conditions (B) on total time that the experimental rat spent in each compartment during the Social Affiliation test (Mean time ± SEM, n=8). Values with * p<0,05 and ** p<0,01 are significantly different within the Stranger I chamber and the empty chamber.

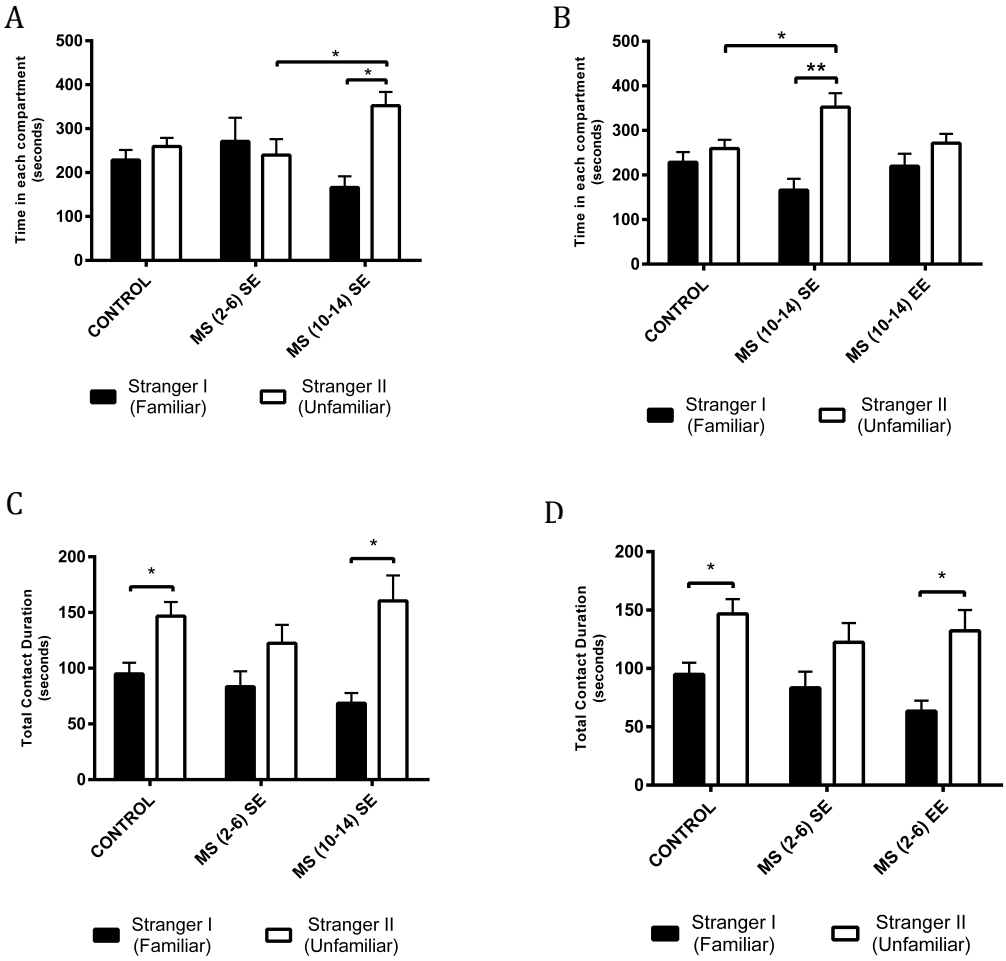

Figure 11 – Effects of MS and different environmental conditions (B, D and E) on total time that the experimental rat spent in each compartment during the Social Preference test (Mean time ± SEM, n=8) (A and B). Total contact duration between experimental rat and the stranger II or stranger I, during the social recognition test (C and D). Significant differences are marked as: * p<0,05 and ** p<0,01 represents significant group or familiarity effects.

3.2 Social Interaction

The Social interaction was assessed with both familiar and unfamiliar individuals. For each, duration and frequency were evaluated for all 5 categories described in table 4. In the investigatory behavior, a one-way ANOVA revealed a significant effect of group towards the familiar individual (F (2, 20) = 3,995, p = 0,034). Post hoc analysis demonstrated that MS 10-14 SE group presented a significant decrease in the total duration of investigatory behavior when compared to the control group, (p = 0,031). No significant effects were found between MS occurring in the early period and Control group (p = 0,165; p = 0,634, respectively). Since there was a MS effect on the duration of the investigatory behavior we assessed the effect of an enriched environment. A one-way ANOVA revealed a significant group effect (F (2, 20) = 4,231 p = 0,029). As previously observed MS 10-14 SE spent significantly less time in investigatory behavior than Control group (p = 0,025), while, MS 10-14 EE did not differ from controls (p = 0,183) nor from MS 10-14 SE (p = 0,532) although displaying intermediating values. No other differences were found for both familiar (H = 4,365, p=0,112 for frequency) and unfamiliar tests (F (2, 20) =2,043, p = 0,155 and F (2, 20) =2,869, p = 0,080, for duration and frequency, respectively (Figure 12 – Investigatory behavior).

Regarding affiliative behavior, a Kruskal Wallis was performed and demonstrated a significant group effect (H = 16,84, p = 0,000). Dunn's post hoc analysis revealed that the MS 10-14 SE displayed a significant increase of time spent in affiliative behavior when compared to Control and MS 2-6 SE groups (MS 10-14 SE vs. Control p = 0,003; MS 10-14 SE vs. MS 2-6 SE, p = 0,000). The same increase occurred relatively to MS 2-6 SE and Control groups, in terms of frequency (H = 15,35, p = 0,00o; MS 10-14 SE vs. Cont. p = 0,004 and MS 10-14 SE vs. MS 2-6 SE p = 0,001). The early MS group did not differ from control group for both total duration and frequency (p > 0,999; p > 0,999, respectively). The impact of an EE on the later MS period was also assessed by a Kruskal Wallis test, which revealed a significant group effect for both duration and frequency (H = 12,79, p = 0,001 and H = 13,94, p = 0,000, respectively). Post hoc analysis demonstrated that both standard and enriched conditions of MS 10-14 displayed significant higher duration and frequency of affiliative behavior when compared to Control group (MS 10-14 SE vs. Control p = 0,001; MS 10-14 EE vs. Control, p = 0,042 for duration, and MS 10-14 SE vs. Control p = 0,010; MS 10-14 EE vs. Control p = 0,001 for frequency), while there were no significant differences between the MS 10-14 groups for duration and frequency (MS 10-14 SE vs. MS 10-14 EE p = 0,818 and MS 10-14 SE vs. MS 10-14 EE p > 0,999,

respectively). No statistical differences were found in the unfamiliar test (H = 2,445, p = 0,294, H = 2,445, p = 0,294 for duration and frequency respectively) (Figure 13 – Affiliative behavior).

Concerning the play behavior in the familiar test, a one-way ANOVA demonstrated a significant group effect for both duration and frequency (F (2, 20) = 4,683, p = 0,021, F (2, 20) = 8,761, p = 0,001, respectively). The Bonferroni's post hoc analysis revealed a significant decrease of this behavior for the MS 10-14 SE group when compared to Control group for duration and frequency, (MS 10-14 SE vs Control p = 0,021; MS 10-14 SE vs. Control p = 0,0063, respectively) and also a significant decrease in terms of frequency for the MS 2-6 SE group (MS 2-6 SE vs. Control p = 0,006). Since MS had a significant effect on both periods we assessed the impact of an enriched environment for each one. A one-way ANOVA for the later MS period revealed a group effect for both duration and frequency (F (2, 20) = 4,484, p = 0,0246, and F (2, 20) = 6,164, p = 0,008, respectively). Post hoc analysis demonstrated that MS 10-14 SE had decreased levels of duration and frequency in comparison to Control group (MS 10-14 SE vs. Control p = 0,035 and MS 10-14 SE vs. Control p = 0,006, respectively), as previously encountered. Furthermore, the MS 10-14 EE group displayed a marginal increase when compared to MS 10-14 SE (p = 0,059) in terms of duration, approaching the Control group. The one-way ANOVA for the early MS period also revealed a group effect in terms of frequency (F (2, 20) = 7,556, p = 0,003). The post hoc analysis demonstrated that both standard and enriched conditions displayed decreased frequency of play behavior when compared to Control group (MS 2-6 SE vs. Control p = 0,006, MS 2-6 EE vs. Control p = 0,009).

The unfamiliar test was tested for duration (H = 5,442, p = 0,129) by a Kruskal Wallis, which revealed no group effect. Concerning frequency it was performed a one-way ANOVA (F (2, 20) = 3,599, p = 0,046) that demonstrated a group effect. Bonferroni's post hoc demonstrated that MS 2-6 SE presented a significant decrease when compared to Control group (MS 2-6 SE vs. Control p = 0,039), while there was no differences for the MS 10-14 SE group when compared to Control or MS 2-6 SE groups (MS 10-14 SE vs. Control p = 0,248; MS 10-14 SE vs. MS 2-6 SE p = 0,641). Because there was a MS effect on the early period we analyzed the effect of the enriched environment. The one-way ANOVA revealed a group effect (F (2, 21) = 5,442, p = 0,012) and post hoc analysis demonstrated that both standard and enriched conditions displayed decreased frequency of play behavior when compared to Control group (MS 2-6 SE vs. Control p = 0,018, MS 2-6 EE vs. Control p = 0,032), while there were no significant differences among the MS 2-6 groups (MS 2-6 SE vs. MS 2-6 EE p = 0,964). No significant differences were displayed for duration in the unfamiliar test (H = 4,948 p = 0,0842) (Figure 14 - Play).

Regarding the non social activities a one-way ANOVA demonstrated significant group effect in terms of frequency for the familiar individual (F (2, 21) = 4,008, p = 0,033). Bonferroni's post hoc analysis revealed a significant decrease for the MS 10-14 SE group (MS 10-14 SE vs. Control p = 0,031). When we analyzed the effect of an enriched environment, a one-way ANOVA demonstrated a group effect (F (2, 21) = 4,158, p = 0,030) and post hoc analysis shown that the MS 10-14 SE presented a significant decrease when compared to Control group (p = 0,048), as previously encountered, while there were no statistical differences for the MS 10-14 EE group (MS 10-14 EE vs. Control p = 0,057 and MS 10-14 EE vs. MS 10-14 SE p = 0,995). No significant differences were observed in duration for the familiar test (H = 2,661 p = 0,264), as well as in duration and frequency for the unfamiliar individual (F (2, 20) = 0,001, p = 0,998, and F (2, 20) = 1,105, p = 3,505, respectively) (Figure 15 – Non Social Activities).

The self grooming behavior was evaluated for duration and frequency towards a familiar individual by one-way ANOVA (F (2, 21) = 1,707, p = 0,205, and F (2, 21) = 2,866, p = 0,079, respectively) (that revealed no group effect. For the unfamiliar one, a one-way ANOVA demonstrated also no group effects in both duration and frequency (F (2, 20) = 0,198, p = 0,821, and F (2, 20) = 0,176, p = 0,839, respectively) (Figure 16 – Self Grooming).

Investigatory behavior

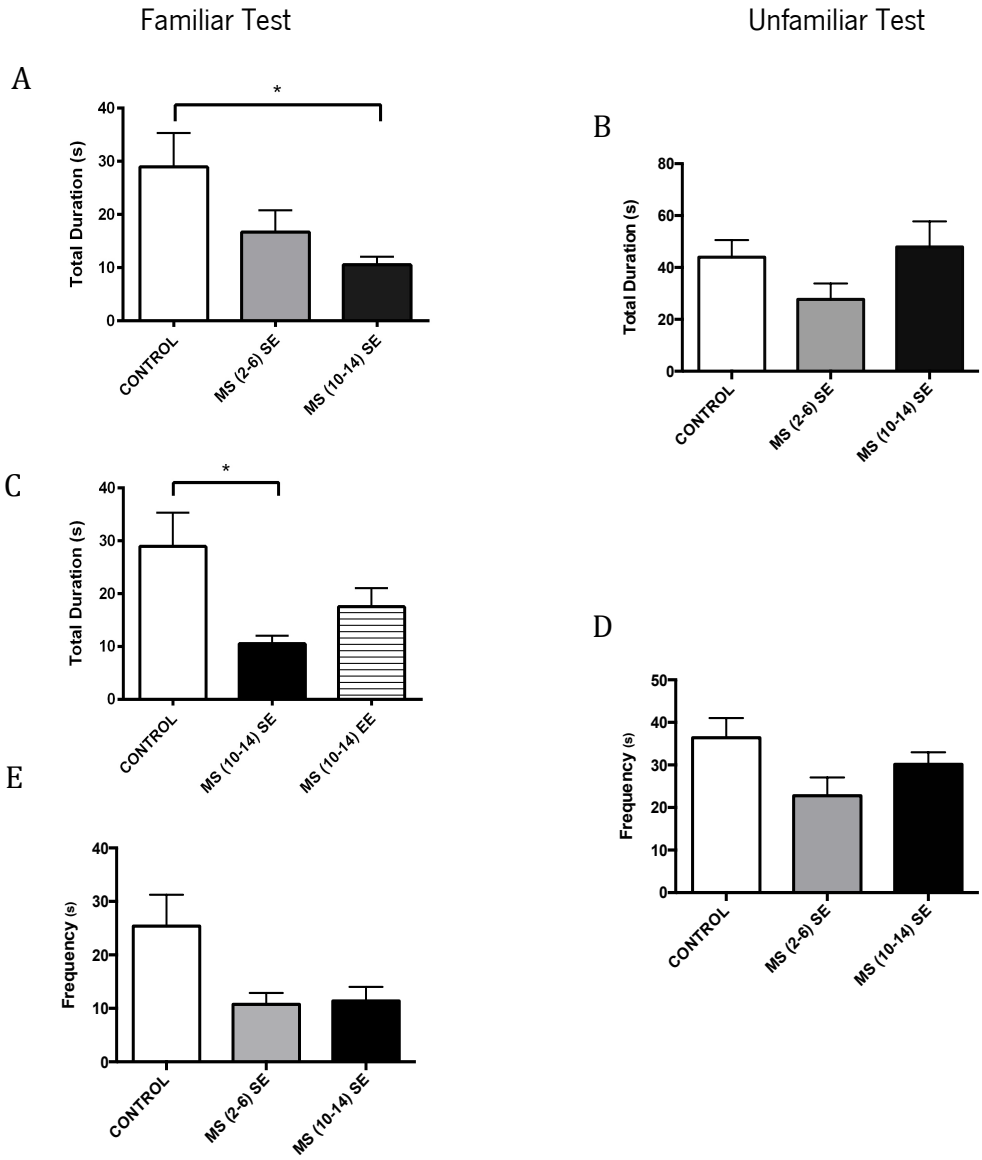

Figure 12 – Effects of MS and different environmental conditions during the social interaction tests: familiar (A, C and E) and unfamiliar (B, D) for the investigatory behavior. A, B and C refers to total duration of investigatory behavior and D and E to frequency of it. Data are means + SEM (n=8 for all groups), * p <0.05 vs control.

Affiliative behavior

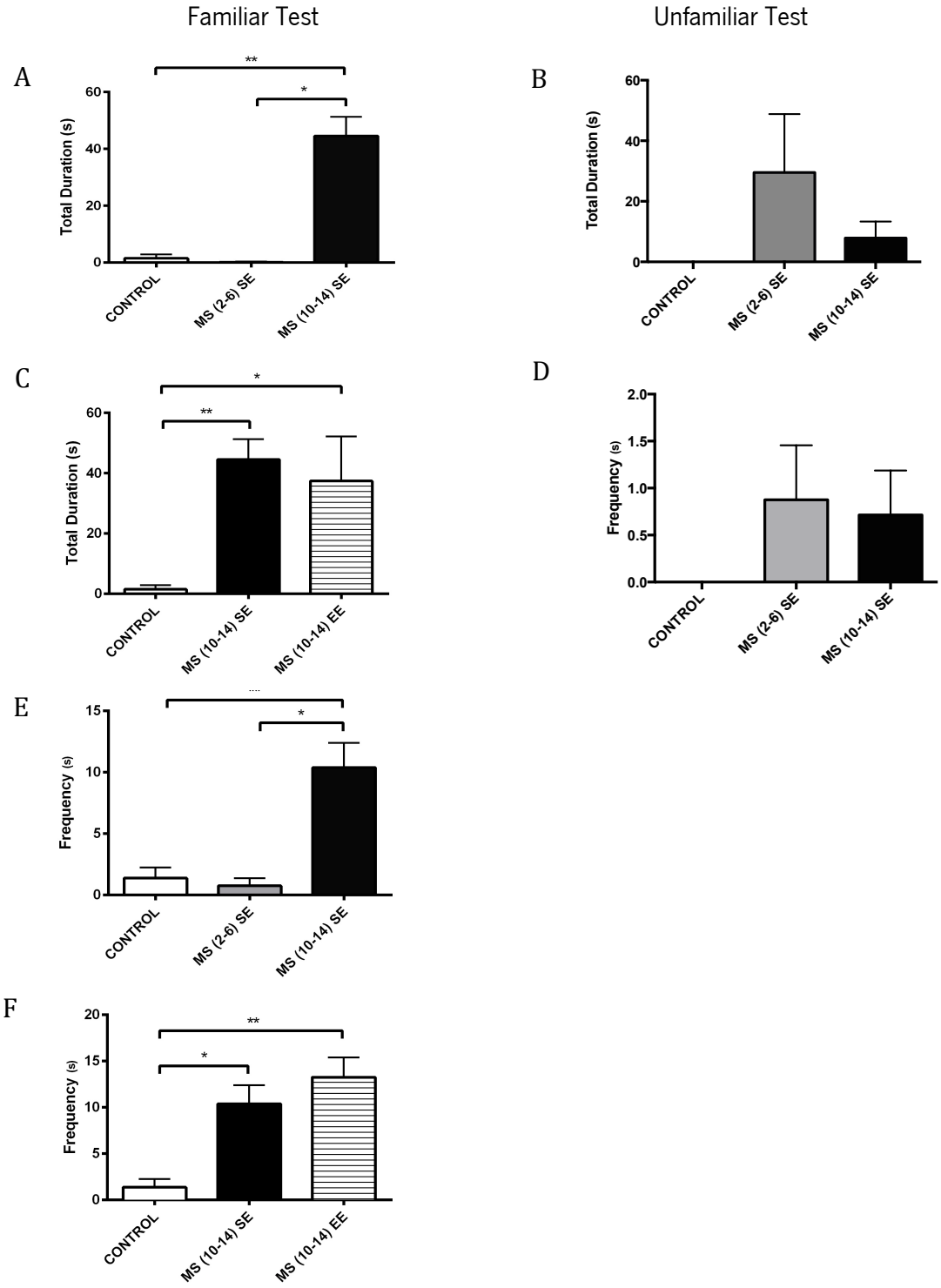

Figure 13 – Effects of MS and different environmental conditions during the social interaction tests: familiar (A, C, E and F) and unfamiliar (B, D) for the affiliative behavior. A, B and C refers to total duration of affiliative behavior and D, E and F to frequency of it. Data are means + SEM (n=8 for all groups), and significant differences are marked as: * p <0,05 and ** p <0,01.

Play behavior

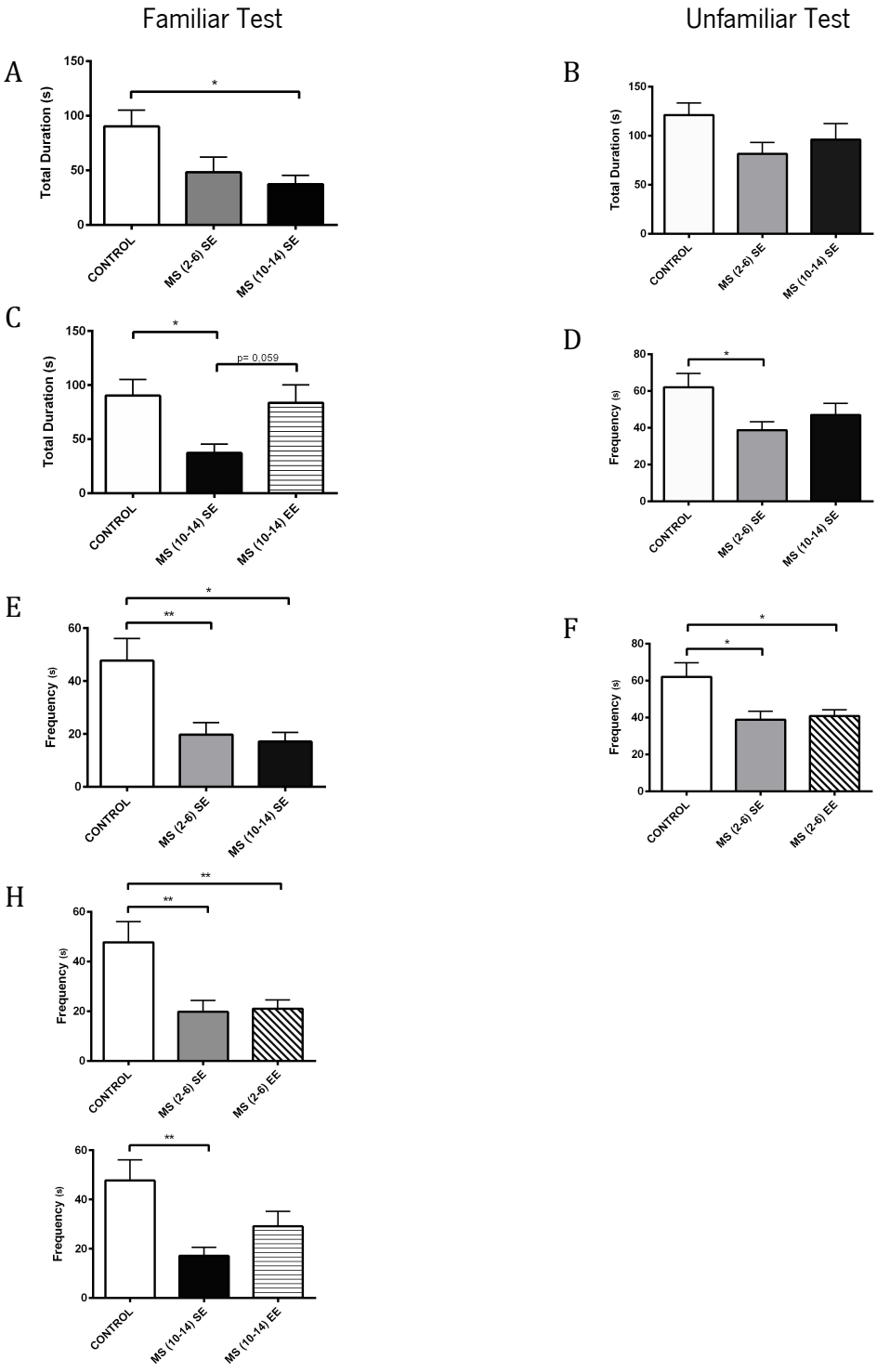

Figure 14 – Effects of MS and different environmental conditions during the social interaction tests: familiar (A, C, E, G and H) and unfamiliar (B, D and F) for the play behavior. A, B and C refers to total duration of play behavior and D, E, F, G and H to frequency of it. Data are means + SEM (n=8 for all groups), * p <0.05 and ** p <0,01.

Non Social Activities

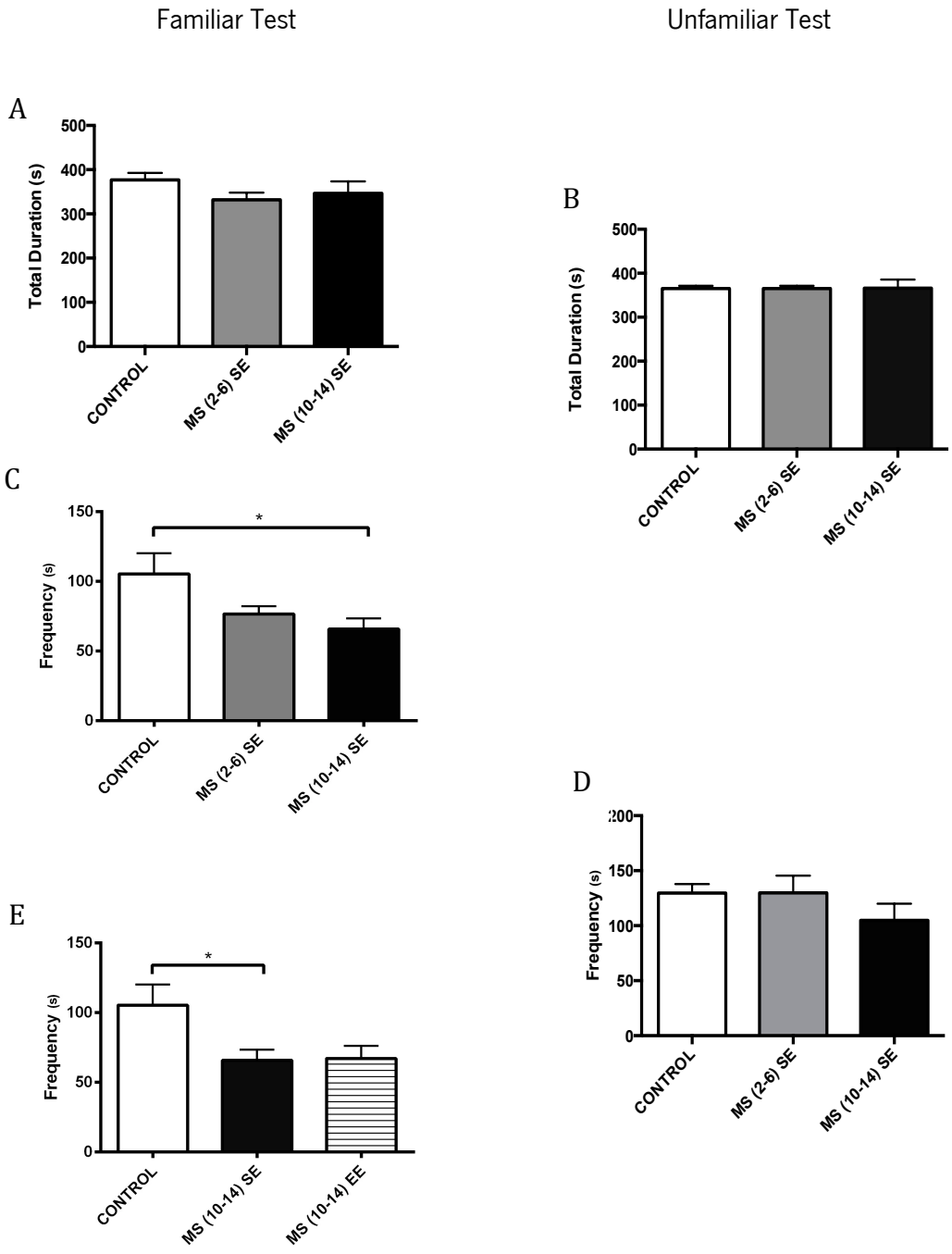

Figure 15 – Effects of MS and different environmental conditions during the social interaction tests: familiar (A, C and E) and unfamiliar (B, D) for the non social activities behavior. A, B and C refers to total duration of non social activities and D and E to frequency of it. Data are means + SEM (n=8 for all groups), * p <0.05.

Self Grooming behavior

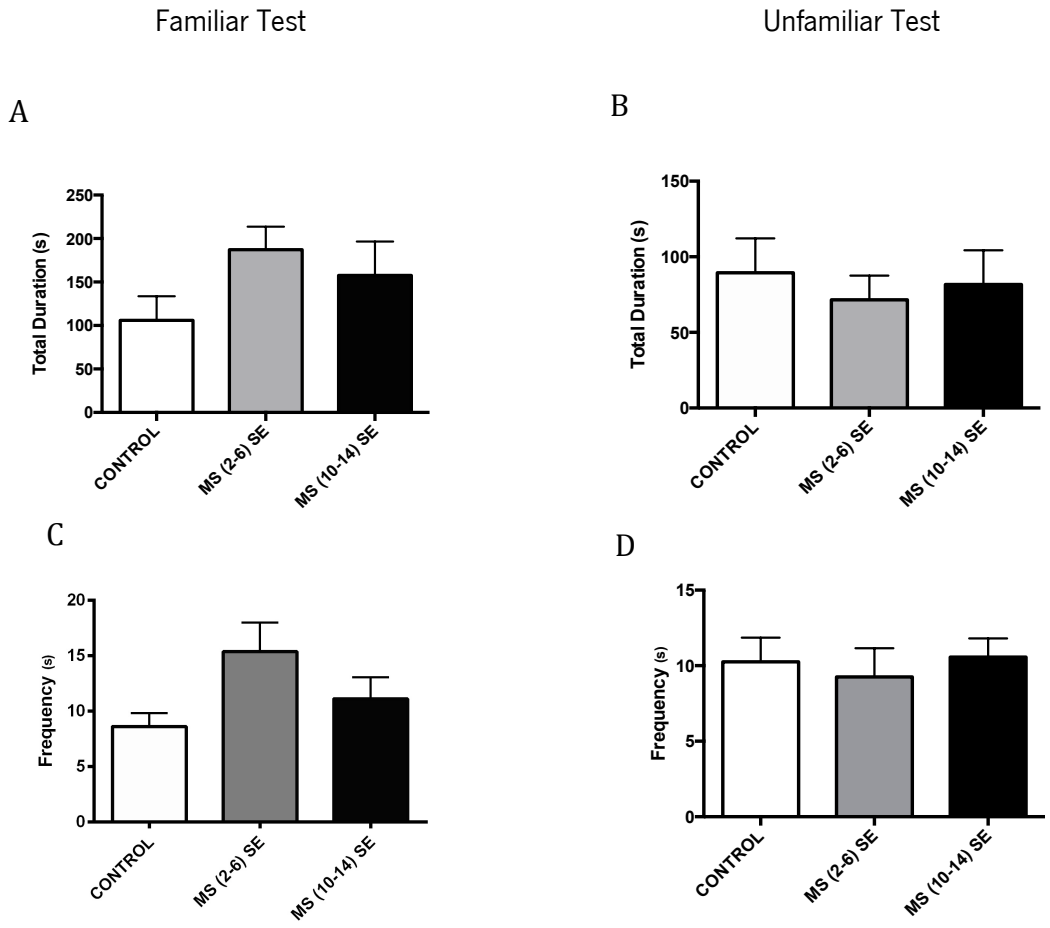

Figure 16 – Effects of MS and different environmental conditions during the social interaction tests: familiar (A and C) and unfamiliar (B and D) for the self grooming behavior. A and B refers to total duration of self grooming behavior and C and D to frequency of it. Data are means + SEM (n=8 for all groups), * p <0.05.

3.3 Molecular Biology

The molecular analysis of the expression levels of the OXTR and OXT genes was evaluated by a one-way ANOVA that demonstrated a group effect (F (2, 21) = 3,589, p = 0,045). Bonferroni's post hoc analysis showed a significant increase of OXTR in the PFC for the MS 10-14 SE group when compared to the Control group (MS 10-14 SE vs. Control p = 0,0362). Since there was a MS effect we evaluated the impact of the enriched environment by a one-way ANOVA, which demonstrated a group effect (F (2, 21) = 4,152, p = 0,030). Post hoc analysis demonstrated a significant increase of MS 10-14 SE (MS 10-14 SE vs. Control p = 0,0362) as previously encountered. There were no other differences between the MS 10-14 EE and Control groups (MS 10-14 EE vs. Control p = 0,023).

Regarding the other brain areas, the amygdala and hypothalamus no significant differences were observed of OXTR and OXT expression levels (H = 1,024, p = 0,6243, and F (2, 20) = 0,07002, p = 0,9326, respectively).

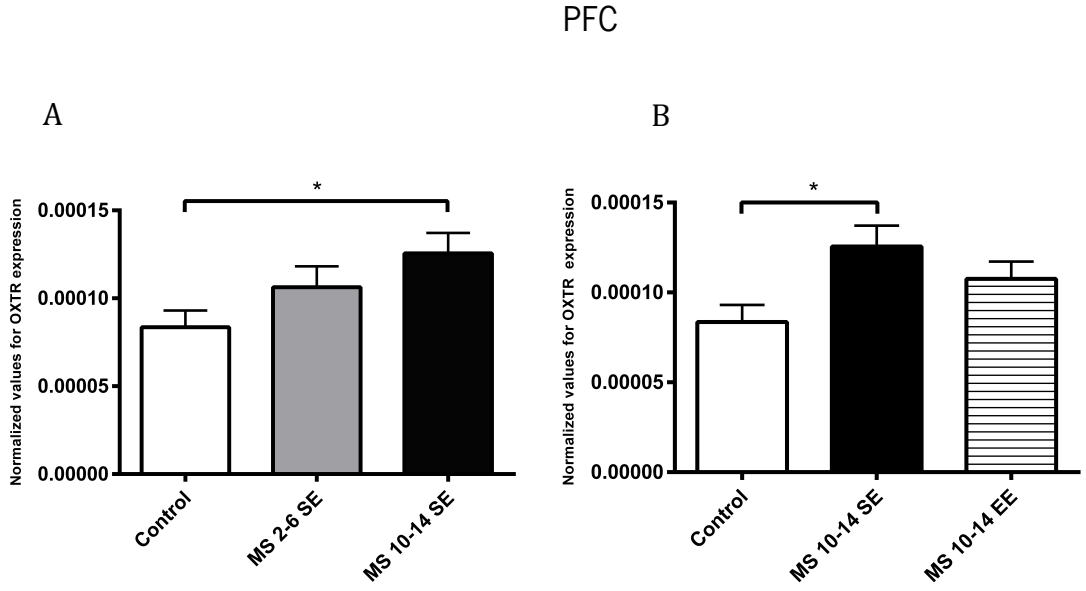

Figure 17 – Effects of MS (A) and different environment conditions (B) in OXTR expression in the PFC (Pre Frontal Cortex). Data are means + SEM (n=8 for all groups, except MS 10-14 SE, n=7), * p <0.05.

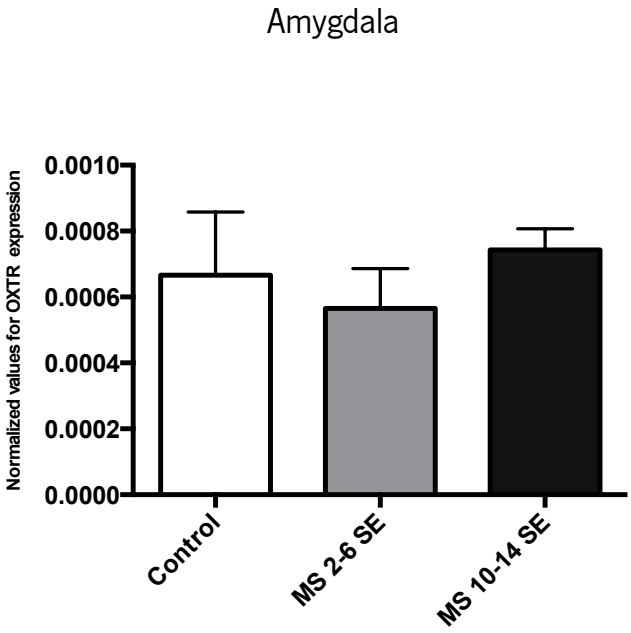

Figure 18 – Effects of MS in OXTR expression in the amygdala. Data are means + SEM (Control n=5; MS 2-6 SE n=4 and MS 10-14 n=8).

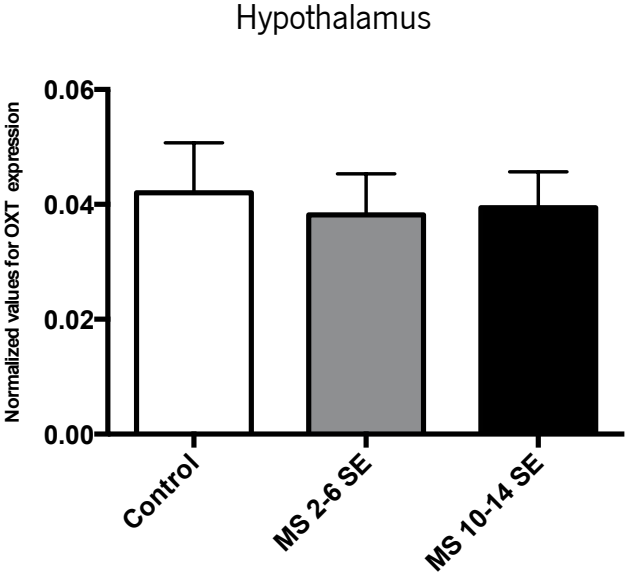

Figure 19 – Effects of MS in OXT expression in the hypothalamus. Data are means + SEM (n=8 for all groups).

In order to assess behavioral and biological measurements relationship, we performed a Pearson correlation. It was revealed a positive interaction, between the affiliative behavior and the OXTR expression in the PFC (r=0,47, n= 24, p=0,01) and a negative one, between the play behavior and the same brain area (r= - 0,47 , n= 24, p= 0,02) for the familiar test.

Table 5 – Correlational results between the familiar test and the molecular biology results from the OXT and OXTR expression.

Familiar correlation results		OXTR		OXT
		PFC	Amygdala	Hypothalamus
Duration	Investigative	-,384	,013	-,005
	Affiliative	**,473***	,166	-,161
	Play	**-,467***	,082	-,249
	Non Social Activities	-,244	,036	-,055
	Self Grooming	,258	-,104	,208
Frequency	Investigative	-,281	,087	-,031
	Affiliative	,374	,232	-,074
	Play	-,392	,104	-,231
	Non Social Activities	-,300	,077	-,156
	Self Grooming	,292	-,263	,077

Table 6 – Correlational results between the unfamiliar test and the molecular biology results from the OXT and OXTR expression.

Unfamiliar correlation results		OXTR		OXT
		PFC	Amygdala	Hypothalamus
Duration	Investigative	,258	,115	-,235
	Affiliative	,093	-,165	-,283
	Play	-,048	-,146	,317
	Non Social Activities	-,049	,029	-,062
	Self Grooming	-,370	,199	,004
Frequency	Investigative	,213	,034	-,107
	Affiliative	,299	-,023	-,259
	Play	-,050	-,147	,318
	Non Social Activities	-,014	-,245	-,001
	Self Grooming	-,043	,157	,102

Discussion

In the present study, we provided new evidence that short periods of maternal absence are able to shape adolescent social behaviors and highlighted that distinct social behaviors are differentially sensitive to MS across ontogeny. Even for subtle MS (2h/day) it was presented impairments in social recognition (MS 2-6 SE animals do not seem to be able to recognize familiar conspecifics) and interaction abilities (MS 10-14 SE displayed augmented affiliative behaviors and decreased play and investigatory behaviors as well as non social activities) besides an increased OXTR expression profile (also for the MS 10-14 SE group). In attempt to correlate both behavioral and neurobiochemical results it was found a positive correlation between affiliative behaviors and the OXTR expression on PFC. Furthermore, we were also able to show that EE during the period of MS acted as a buffer for almost all social behaviors and OXTR expression impairments.

Alterations at Social Recognition

Social recognition test showed that rats exposed to MS 2-6 SE did not expressed social affiliation/motivation neither preference for social novelty (or impairment of social memory). In fact, our data indicate that only rats exposed to the early MS period (MS 2-6 SE) spent less time investigating the social stimuli, which suggests that MS during this early period reduced motivation to social interactions. The stressful experience caused by MS during the neonatal period is known to alter the hypothalamic pituitary adrenal (HPA) axis function, the stress response system of adult rats and also induce hyperresponsiveness to stress in adulthood (Kuhn & Schanberg, 1998; Pihoker, 1993). This hyperresponsiveness to stress situations is more likely to display behaviors related to social avoidance and behavioral inhibition during social encounters (Beery & Kaufer, 2015; Roelofs et al., 2009). The fact that these animals showed a tendency for increased self-centered behaviors (observed by self-grooming behavior) could be a mechanism to deal with particular stressful situations and seem to be in accordance with other studies that demonstrated that induced levels of CRF can increase grooming behaviors in rats and mice (Berridge & Dunn, 1986). However, it is important to be aware that there is growing evidence suggesting that HPA axis response in adolescents may be differentially affected by repeated stressors when compared with that on adults. Although, in

the present study, we didn't assess corticosterone levels, the literature in the field has been showing (Lui and el 2012) that adolescent rats had a higher concentration of corticosterone and greater Fos expression in the paraventricular nucleus of the hypothalamus compared with adult rats in response to repeated stress (Lui et al., 2012).

Regarding social preference MS 2-6 did not show differences in the time spent with the familiar and unfamiliar subject that is in accordance with other studies showing that maternally deprived rats were not able to differentiate between a new and a familiar juvenile conspecific in a social recognition task (Lévy et al., 2003; Lukas et al., 2010). Remarkably, although we performed the MS protocol between the 2nd and 6th post-natal days and only for 2h/day, our results resemble most of the literature in which the MS procedure comprehends almost all days from the designated "stress hypo-responsive" period (PND 1–14) (Lukas et al., 2010; Lukas et al., 2011; Vivinetto et al., 2013).

Importantly, we also found that EE, during MS 2-6 period, completely reverted the effects of MS on both social affiliation and social recognition test. In fact, this is in accordance with some literature showing that EE reverses the effects of early life stress events (Pryce & Feldon, 2003; Francis et al., 2002).

In contrast, the MS 10-14 SE group did not show any impact on social recognition, displaying a clear preference for the novelty, as control animals. Animals at the adolescent period tend to have elevated levels of investigative and play behaviors towards an unfamiliar conspecific, which might be due to a curiosity factor (Francis et al. 2002; Vivinetto et al. 2013; Cirulli et al. 1996).

Alterations in social interaction behavior

The ability to respond to conspecifics is essential to all species, and particularly important for organisms that engage in social behavior and interactions with peers become particularly important during adolescence (reviewed in Spear 2000). However, social behavior often changes in response to stressful life experience such as MS. In the present study, data from the social interaction test – in which the animal interacts with a familiar or an unfamiliar peer in different days – revealed that MS during PND 10-14 modify adolescent social

interaction with familiar peer but not with unfamiliar one, whereas MS during PND 2-6 had more modest effects on social interaction in both cases.

The social interaction test displayed with a familiar conspecific revealed that rats exposed to MS 10-14 reduce play and investigatory behavior as well as non social activities besides an increase in the time spent in affiliative behavior. The increase of affiliative behavior observed, suggest that adolescent rats exposed to MS during PND 10-14 compensate the impacts of MS (stressful experience) by creating a positive, reward experience such as social contact with familiar peers. These results are supported by Panksepp et al (2007) which propose a functional relationship between behavioral approach and reward, based on classical theories of motivation, i.e. affiliative behaviors are driven by a state of reward and can gain value through association with these rewarding experiences (Panksepp et al., 2007). Furthermore, there are studies indicating that effects on affiliative behavior may differ according to partner familiarity, sex, age, species, and affective state (Beery & Kaufer, 2015). Another study highlight the importance of partner identity/familiarity, showing that rats helped trapped strangers by releasing them from a restrainer, but only if it was matched to their own strain, or a strain they had exposure to from birth; they were uninterested in freeing rats of an unfamiliar strain (Bartal, Decety, & Mason, 2011). In rodents, several stressors have been shown to reduce social behaviors (Beery & Kaufer, 2015) and social behaviors can in turn play a role in buffering the effects of that stressor, providing adaptive value of social networks for coping with stress exposure (Beery & Kaufer, 2015). Indeed, studies from Meaney (1979) reported that social isolation induced affiliative behaviors (Meaney & Stewart, 1979)

Since MS 2-6 group did not significantly affect this behavioral category it is suggested that salience of reward value of afilliative behavior may be dependent not only of the partner familiarity but also on the time period during which early stress is experienced. T aken toge ther, these findings suggest that rats experiencing stressful situations (maternally separation) on the second postnatal week exhibit more adaptive response to cope with stress situation in the presence of a familiar peer.

Additionally, the play behavior was decreased for both MS periods, although with differences according to the familiarity of the partner. The MS 2-6 SE had a decrease of play

frequency for both interactions (familiar and an unfamiliar conspecific), while the MS 10-14 had a decrease on play behavior only for the interaction with the familiar peer. The MS impacts on play behavior are not well described and there is not total agreement on this matter. Studies showed that rats experiencing social isolation display less social interest as well as impaired behavioral and endocrine responses in adulthood when confronted with a social stressor. Besides, there are studies describing increased dominant juvenile play-fighting behaviors (Veenema & Neumann, 2009), and reports from Caldji (2010) reveal that long periods of MS increase HPA activity, leading to higher corticosteroids level (Caldji et al., 2010). In line with this, previous studies from Meaney (1982) also reported decreased play behavior due to increased corticosteroids levels (Meaney, Stewart, & Beatty, 1982). Considering this, it is possible that our results observed on play behavior might be due to corticosteroids increase induced by our MS protocol in both groups, something that should be addressed in future studies.

Interestingly, the EE during MS 10-14 had a protective effect on social play behavior, since MS 10-14 EC and control animals showed a similar play time behavior. These results are supported by others reporting that prenatal stress reduced the expression of social play behavior but this effect was markedly reversed following EE (Morley-Fletcher et al., 2003).

Alterations on the oxytocinergic system

OXT system has been implicated in several social behaviors, from affiliative (H. E. Ross et al., 2009; Heather E. Ross & Young, 2009), social recognition (Michael Lukas, Toth, Veenema, & Neumann, 2013). Parental separation has also been linked with profound alterations on the OXT system, namely decreased OXT levels and reduced OXTR binding (Babygirija et al., 2012; Cao et al., 2014; Lehmann & Feldon, 2000; Michael Lukas et al., 2011; Pryce & Feldon, 2003; Veenema, Bredewold, & Neumann, 2007). In our study we found increased levels of OXTR in the PFC of adolescent rats that have been previously separated from their mothers between the 10^{th} to 14^{th} PND. The animals that had EE for the same MS conditions did not differed from the control group, which indicates an attenuation effect by the physical comfort. This indicates that even subtle MS (2 hours/day for only 5 days)

considering that it acts as a non negligence model, induce alterations on OXTR expression levels. The fact that only the later MS group displayed variation can be speculated according to the ontogeny's oxytocinergic system. In fact, the maturation of the OXT receptor differs across the different rat brain areas throughout the developmental period, namely at the 3rd postnatal week and puberty (E. Tribollet et al., 1992). Areas like the *cingulate cortex* (proximal to the PFC)*, retrosplenial cortex*, *caudate, putamen, dorsal subiculum*, *lateral and medial mammillary nucleus,* and *substancia gelatinosa* that present high densities of OXT-binding sites at early age (PND 10) and reducing significantly between postnatal days 10 and 21. On the opposite, some OXT-binding sites only appear at the time of adolescence, between postnatal days 40 and 45, at areas like the *dorsal peduncular cortex, islands of Calleja, ventral pallidum cell groups, bed nucleus of the stria terminalis, ventral subiculum, ventromedial nucleus*, and the *central amygdaloid nucleus* (M. Lukas et al., 2010b; E. Tribollet et al., 1992).

One hypothesis could be that the absence of physical contact with the dam (induced by MS protocol) can lead to reduced circulating OXT levels, which might induce a compensatory effect, leading to the increased expression of the receptor during periods where typically there is a relatively low expression, as demonstrated by Tribollet (1992). Although we didn't address the expression throughout development we speculate that this effect persisted until adolescence, a mechanism that should be further addressed in future studies.

Interestingly, the correlation analysis performed demonstrated that this increased OXTR expression in the PFC is associated with increased affiliative behavior towards the familiar conspecific, highlighting a possible causal relationship between the neurobiochemical alterations and the behavioral phenotype of these animals. Unpublished data demonstrated that the dams of the MS 10-14 SE group displayed increased affiliative behaviors towards pups. This is also the group that presents increased affiliative behavior towards a familiar animal in our social interaction test at adolescent age. Therefore there might be a close relationship between the maternal behavior of the dam and social interaction plus the OXTR expression of pups. This is corroborated by our correlation where an increase of affiliative behaviors is associated with augmented OXTR expression.

In contrast, an increased OXTR expression in the PFC showed to be associated with decreased play behavior towards the familiar conspecific. Taken together these results demonstrated that animals with increased levels of OXTR in the PFC are less engaged in

positive social interactions (such as play behavior) and more in affiliative behaviors.

Amygdala has also been implicated in social behaviors (Felix-Ortiz & Tye, 2014) as well as in the OXT system (J N Ferguson et al., 2001; Gorka et al., 2015). Our results for the amygdala did not presented any alterations. Although there seems to be a tendency for the MS10-14 SE group to have higher OXTR expression levels, our lack of statistically significant results can be due to our small sample size.

In the hypothalamus we did not observed any alteration on OXT expression level contrary to what has been described by other studies (Todeschin et al., 2009; Tsuda & Ogawa, 2012; Veenema et al., 2007). However, most of MS studies used this as a negligence model, which implies more than 3 hours of MS during most of the stress hypo responsive period. Since our protocol consisted in subtle MS (2 hours for 5 days for different weeks of the weaning period) they might not be sufficient to induce long lasting alterations in hypothalamus at OXT level contrary to the increase OXTR expression in PFC, as discussed above. Nevertheless, since this OXT levels have been measure in adolescent rats we cannot exclude the possibility that specific alterations that occurred due to the MS protocol, could be recover by the time of behavior and OXT/OXTR expression assessments.

Conclusion

The present work showed that short MS periods experienced early in life are associated with alterations in social behavior in adolescence that compromise emotional response, and alters important phenotypic profiles with high adaptive significance for this age. This study also demonstrates the importance of EE as a generator of plastic changes in behavioral responses. Both MS groups, MS 2-6 SE and MS 10-14 SE, were differently affected at different levels of social interaction. On one hand, the MS on 2nd to 6th PND lead to impaired social recognition, reduced social motivation and increased tendency for self-centered behaviors, highlighting the lack of reward obtained from social interactions.

In contrast, MS between 10 and 14 PND produces alterations only when the interaction involves a familiar individual, where there is close intimacy and bonding. In stressful context (such as the test arena) MS10-14 animals display increased affiliative behavior towards

the familiar animal, as a coping strategy. This behavioral alteration might be explained in light of the augmented PFC OXTR expression also observed.

Additionally, the EE seem to buffer some the behavior impairments induced by MS, highlighting the relevance of physical comfort as a compensatory mechanism.

With our MS protocol we did not had the purpose of mimicking maternal neglect. Instead, we were interested on mimicking subtle MS that happen for most children when their parents need to leave to work and have to separate for few hours and children go to school for example.

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