Maternal Separation in the Rat

Long-term Effects of Early Life Events on Emotionality, Drug Response and Neurobiology

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Abstract

Exposure to early stress and emotional trauma in humans are associated with an increased risk to develop psychiatric disorders, for example, anxiety, depression and drug abuse. Furthermore, disruptions in stress hormone and neurotransmitter levels as well as structural changes in the brain have been connected to early adversities. Animal models have been developed to experimentally investigate early experiences. In the rat, maternal separation of pups in early ages have been linked to anxiety-related behavioral, endocrinological and neurochemical disruptions in the rat. The aim of the thesis was to investigate these proposed disruptions in pups exposed to repeated maternal separation (MS; 3-4 h/day) during the first two weeks in life relative to controls exposed to brief (3-5 min) daily handling procedure. Behaviorally, anxiety-related behaviors, voluntary alcohol intake and sensitivity to amphetamine were investigated in adult Wistar rat offspring. Furthermore, brain opioid peptides, monoamines, corticosterone levels and weight of adrenal and thymus glands were measured. When separating pups as intact litters kept in incubators, there were mainly no alterations in emotionality, amphetamine-induced locomotor activity, opioid peptide levels or in plasma corticosterone levels in MS offspring, either in males or females. Alcohol intake was, however, intitially decreased in MS females, although total alcohol intake for one week was not affected. When separating pups in intact litters, MS males showed increased weight of adrenal glands, which may reflect a disruption of the HPA axis. When changing the experimental protocol, and separating pups in isolation, the manipulation caused decreased anxiety-related behavior in the offspring. Animals that experienced a temperature challenge while separated (i.e. isolated in room temperature instead of isolated in incubators) showed even more signs of decreased emotionality. There were no significant changes in alcohol intake or in brain monoamine and plasma corticosterone levels compared to controls with this protocol. Maternal care behavior has been reported to be disrupted by prolonged separation episodes. However, when studying the dams' retrieval behavior of the pups in the present thesis, no negative effects were observed. With respect to the MS protocols used in the present thesis, the results do not provide support for the suggestion that MS manipulations causes enhanced anxiety or disruptions in endocrinology and neurochemistry in the adult rat. These findings could reflect a parallel to human conditions as relatively good psychosocial functioning is sometimes seen despite serious adverse experiences in childhood.

Key words: Alcohol intake, Anxiety, Corticosterone, Early deprivation, Emotionality, HPA axis, Maternal care, Maternal separation, Monoamines, Opioid peptides

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3. Marmendal, M., Eriksson, C.J.P., & Fahlke, C. Early deprivation in male Wistar offspring. Part 1: Long-term increased locomotion and exploratory behavior in novel settings. *Manuscript submitted for publication.*

4. Marmendal, M., Eriksson, C.J.P., & Fahlke, C. Early deprivation in male Wistar offspring. Part 2: Further evidence for long-term reduced emotionality in novel settings. *Manuscript submitted for publication.*

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INTRODUCTION

In recent years there has been an increased awareness of the fact that children are exposed to violence, and it has been pronounced as a public health problem (reviewed in Margolin & Gordis, 2000). A Swedish study by Sundell (1997) reported that 3 % of the children (1-6 years of age) in public nursery schools were at risk for maltreatment. The earlier children in this situation are recognized the better are the possibilities of getting help, but unfortunately, most of these cases of possible maltreatment were not reported by the nursery schools (which they are legally obliged to do) to the authorities (Sundell, 1997). The forms of child maltreatment in that study were neglect due to parental psychiatric illness or drug abuse, suspicions of sexual or physical abuse and marital discord. When studying police reports of physical child abuse (in Sweden between the years 1986 and 1996), Lindell and Svedin (2001) discovered a considerable increase in the number of cases. The authors, however, interpret this finding cautiously as the increased reports of child abuse could reflect both an actual rise in violence or a greater tendency to report abuse (or a combination of both). It has, indeed, been reported that physical child abuse has decreased over time, however, 4 % of children (10-12 years of age) and 7 % of young adults (20 years of age) still report that they have been severely physically abused earlier in life (Janson, 2001).

Stressful life events are suggested to play a role in the development and maintenance of psychiatric disorders, for example, depression and anxiety (reviewed in Kessler, 1997; and Margolin & Gordis, 2000). Stress-related disorders are common, affecting approximately 5-20 % of patients in Western industrialized countries (Hamet & Tremblay, 2005; Mayer & Fanselow, 2003). In Sweden it has been claimed that psychiatric illness is one of the largest health problems among youngsters, 10-25 % of the Swedish children have been reported to suffer from some kind of psychiatric problem (reviewed in Olsson, Hagekull, & Bremberg, 2003). Psychiatric illness among adolescents is thought to have increased during the past years, which is reflected by the two-fold increase in sales of antidepressants (Barnombudsmannen, 2005). Estimates by the World Health Organization predict that by 2020, depression will be the second leading global burden of illness (Mayer & Fanselow, 2003).

Clinical studies of stress-related disorders

In humans, evidence for the psychopathological effects of stress derives from non-experimental research. Retrospective studies have shown that early stress and emotional trauma are associated with an increased risk to develop psychiatric disorders (reviewed in Heim & Nemeroff, 2001; Heim & Nemeroff, 2002; and Nemeroff, 2004). For example, prolonged separation from parents early in life or emotional abuse has been shown to be associated with major depression later in life (Chapman et al., 2004; Faravelli et al., 1986; Hällström, 1987; Kendler, Neale, Kessler, Heath, & Eaves, 1992; Oakley Brown, Joyce, Wells, Bushnell, & Hornblow, 1995; Roy, 1985; Young, Abelson, Curtis, & Nesse, 1997). Adverse experiences (e.g. psychological, physical or sexual abuse, neglect and parental separation) in childhood are also proposed to be risk factors for increased mortality and morbidity from a variety of other disorders during adult life, including for example suicide attempt, substance abuse, cardiovascular disease and obesity (Felitti et al., 1998; Hope, Power, & Rodgers, 1998; Kendler et al., 1996). Institutional experience in orphanages have been shown to exert harmful and long-term effects on social, behavioral and emotional development, for example, not to have a normal interest in attachments or exaggerated attention seeking behavior, anxiety, fearfulness and aggression (Frank, Klass, Earls, & Eisenberg, 1996; O'Connor et al., 2003). School-aged maltreated children have been found to be more aggressive, more withdrawn and less cooperative than non-maltreated children (Manly, Kim, Rogosch, & Cicchetti, 2001). Women who report childhood physical or sexual abuse are at increased risk for developing psychiatric disorders in adulthood, and these forms of abuse are also hypothesized to be causally related to increased risk for psychiatric and substance abuse disorders (Kendler et al., 2000; McCauley et al., 1997; Moncrieff, Drummond, Candy, Checinski, & Farmer, 1996; Young et al., 1997).

Emerging evidence from longitudinal studies suggest that exposure to early life stress may be associated with risk for psycho- and physiopathology (reviewed in Maughan & McCarthy, 1997; and Rutter, 1991). For example, a study by Russek and Schwartz (1997) showed that perception of parents as emotionally cold and distant increased the risk of chronic illness (e.g. hypertension and drug abuse) in adulthood compared to children with normal parental relationships. There is evidence of impairment of the hypothalamic-pituitary-adrenal (HPA) axis

following various forms of child abuse and neglect, which may have long-lasting consequences (reviewed in Glaser, 2000). Children exposed to high levels of stress (e.g. maternal depression, maternal parenting and financial stress), and with a history of these stressors in infancy, have shown elevated stress hormone levels (cortisol) relative to children in only one of these conditions (Essex, Klein, Cho, & Kalin, 2002). Furthermore, high levels of cortisol in children have been suggested to predict greater emotional and behavioral difficulties throughout the school transition period (Essex et al., 2002). When exploring early adversity and its relation to psychiatric illness, Phillips and colleagues (2005) found a strong connection between adversities and anxiety disorders in adolescents. Child maltreatment has been reported to have effects on structural, functional and chemical changes in the brain, for example, decreased volume of various brain areas, limbic system dysfunction and higher catecholamine activity (reviewed in Glaser, 2000). Examples of other forms of early-life stress are accidents, surgeries and chronic illness, natural disasters and war. Any such situation occurring during the developmental period may be classified as early-life stress in humans. These stressors typically occurs as chronic adversity and various forms of stressors often coexist (Gilmer & McKinney, 2003; Heim, Plotsky, & Nemeroff, 2004).

The findings in non-experimental research suggest that early stressful life events may lead to psychopathology later in life. However, the ability to make causal inferences about the effects of early stress on psychopathology from these studies is difficult due to methodological problems (Kendler et al., 2000). Besides the difficulty to control for all possible influential factors for psychopathology in retrospective studies, it may be difficult for the subjects to recall life events accurately and to confirm whether stressful events occurred before the onset of, for example, depression. In other words, it is sometimes hard to decide whether the stressor is cause or consequence of the illness (Hardt & Rutter, 2004; Kessler, 1997; Rutter, 2002; van Praag, 2004).

Manifestation of psychiatric illness

Several factors may contribute to the manifestation of psychiatric illness in relation to early-life stress (Figure 1). Both genetic predisposition and environmental factors are hypothesized to be related to the cause of psychiatric illness (reviewed

in Agid, Kohn, & Lerer, 2000; Heim & Nemeroff, 2001; Nemeroff, 2004). The genetic component in psychiatric illness has been reported to be 20-70 %, and the wide range is suggested to depend on, among other things, type of illness (e.g. subtype of depression, schizophrenia and bipolar disorder) and different methodologies used to investigate the heritability (McGue & Christensen, 2003; Nestler et al., 2002; and Sullivan, Neale, & Kendler, 2000). Gender seem to affect the clinical characteristics of psychiatric disorders, as women are more likely to develop anxiety disorders and depression (Gater et al., 1998; Hankin & Abramson, 1999; Pigott, 1999; Savoie, Morettin, Green, & Kazanjian, 2004; Wittchen & Hoyer, 2001). However, there has been increased awareness in the idea that the gender difference may be partly attributable to women's greater exposure to stressors, for example, that females are more likely to be victims of sexual abuse and assault than males (reviewed in Hammen, 2005). Ongoing stress may determine individual stress responsiveness and the manifestation of psychiatric disorders and, for example, therapy or coping styles may buffer the effects of early life stress (Heim & Nemeroff, 2001; Nemeroff, 2004, Figure 1).

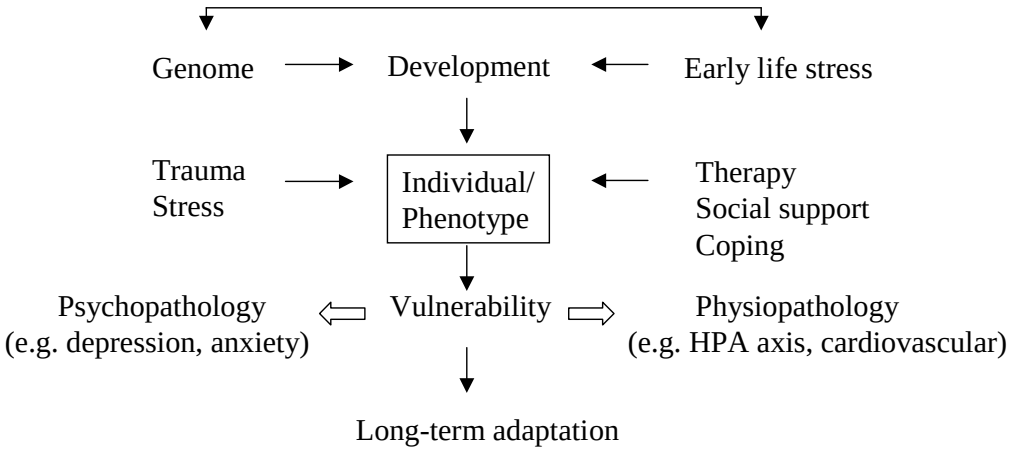

Figure 1. Model of the interaction between genetic disposition and early environment leading to a vulnerable individual phenotype. Stress exposure or trauma may induce pathology based on the underlying vulnerability. Therapy or coping styles may buffer the effects of early life stress on vulnerability (figure modified from Heim and Nemeroff, 2001; Nemeroff, 2004).

The neurobiology of stress

The effects of early-life stress are believed to be mediated by the plasticity of the developing central nervous system (CNS). During critical periods, certain brain regions are particularly sensitive to adverse experiences, which may lead to abnormalities (reviewed in Andersen, 2003; Heim & Nemeroff, 2001, 2002; and Weiss & Wagner, 1998). For example, adults that had experienced childhood abuse with the diagnosis of post-traumatic stress disorder (PTSD), had a smaller hippocampal volume in relation to matched controls (Bremner et al., 1997). Stress or emotional trauma during early development may thus permanently shape brain regions that mediate stress and emotion, leading to an altered emotional processing and a higher sensitivity to stress. In genetically vulnerable individuals, this may then evolve into psychiatric disorders, such as depression and anxiety.

Stress response systems

One major stress response system in the body is the HPA-axis, which consists of the brain, the pituitary gland and the adrenal gland (Figure 2). This endocrine system regulates the release of the steroid hormone cortisol (corticosterone in rats) from the adrenal glands into the bloodstream in response to stress. The HPA-axis activates and coordinates the stress response by receiving and interpreting information from the amygdala and the hippocampus. The hypothalamus secretes corticotropin-releasing factor (CRF) into the bloodstream. CRF stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH in turn enters circulation and travels to the adrenal glands where it subsequently stimulates cortisol release into the blood circulation. The rate of secretion of cortisol is regulated by negative feedback at several levels (hippocampus, hypothalamus and pituitary), and thereby shuts off the system, maintaining it at an optimal level (Bear, Connors, & Paradiso, 2001; Shea, Walsh, Macmillan, & Steiner, 2004).

Aside from the role as a hormone in the HPA-axis, CRF mediates the autonomic and behavioral stress response in its role as a CNS neurotransmitter (Francis, Caldji, Champagne, Plotsky, & Meaney, 1999; Nemeroff, 2004). CRF neurons are distributed in several brain areas (e.g. hypothalamus, amygdala and cortex) and project to locus coeruleus within the brain stem and increase the firing rate of locus coeruleus neurons. This results in activation of the autonomic nervous system

(ANS) involved in the stress system and is seen in the release of catecholamines (primarily adrenaline and noradrenaline) from the adrenal glands (Bear et al., 2001; Meaney, 2001).

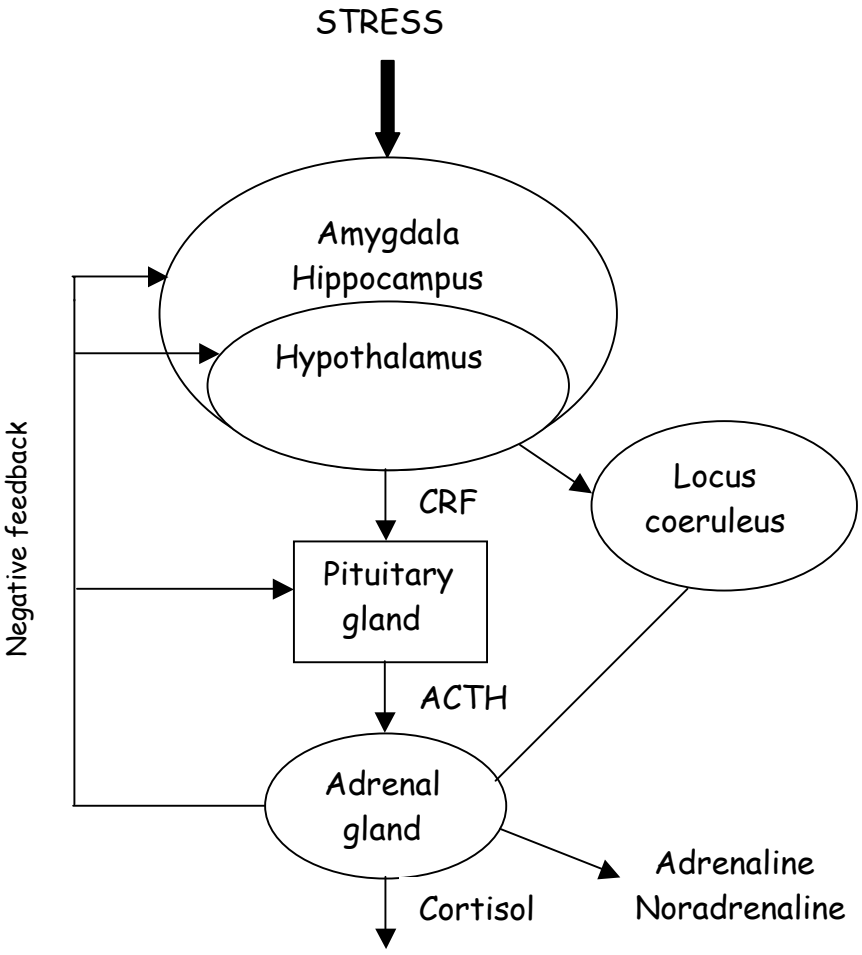

Figure 2. In response to stress, corticosterone (CRF) is released in the hypothalamus and stimulates adrenocorticotropic hormone (ACTH) release in the pituitary gland. ACTH in turn affects adrenal glands to release cortisol. Inhibition of the activated system occurs via negative feedback at several levels. Adrenaline and noradrenaline is released from the adrenals in response to stress (figure modified from Shea et al., 2004).

CRF in several brain structures is also responsible for the behavioral components of the stress response (Meaney, 2001; van Praag, 2004). For example, administration of CRF in the animal brain increases behavioral activation (decreases fear-related behaviors), but overproduction or administration of higher doses of CRF is

associated with increased fearfulness (Eaves, Thatcher-Britton, Rivier, Vale, & Koob, 1985; Heinrichs, Menzaghi, Merlo Pich, Britton, & Koob, 1995; Meaney, 2001). Other neurotransmitters involved in the stress response include serotonin, dopamine and opioid peptides. These neurotransmitters have a variety of effects, for example, opioid peptides lead to pain relief and dopamine release results in increased blood pressure and heart rate. Furthermore, various neurotransmitters can affect the body more indirectly by inhibiting or enhancing the activity of the HPA-axis (Brady & Sonne, 1999). The neurochemical and hormonal responses to stress do not act independently of each other, instead they are tightly interconnected. Thus, CRF release in the hypothalamus is regulated by neurons releasing serotonin or endogenous opioids. Furthermore, CRF release results in ACTH release within the HPA-axis, but also in the release of endogenous opioids, which may contribute to the behavioral and emotional consequences of stress (Brady & Sonne, 1999).

The biological mediators in the stress response are associated with both adaptation and pathology. Both hormones and neurotransmitters are important protectors of the body in the short run, in response to acute challenges. As seen in figure 3, an adaptive stress response increases hormone levels in response to acute challenges, and then returns to basal levels. The stress response focuses our attention, facilitates the mobilization of substrates for energy use, increases cardiovascular tone and suppresses nonessential systems for immediate survival (e.g. immunity, growth, digestive and sleep functions and reproduction).

However, the mediators in the HPA-axis also seem to participate in physio- and psychopathology when we are chronically challenged over long periods of time. Research in both humans and animals have shown negative effects of stress in, for example, hippocampal damage, immunosuppression, obesity, hypertension and atherosclerosis (de Kloet, Rosenfeld, Van Eekelen, Sutanto, & Levine, 1988; McEwen, 2000a; Vythilingam et al., 2002). Chronic activation of the stress system has also been associated with depression and anxiety, cognitive impairments and sleep disorders (Essex et al., 2002; Heinrichs et al., 1995; Nemeroff, 1998; Rosen & Schulkin, 1998). Thus, for an adaptive stress response, both rapid activation and rapid inhibition of the stress system are necessary. In the literature on stress, this process is called allostasis and the term allostatic load refers to the process of chronic activation of the stress system (McEwen, 1998, 2000b).

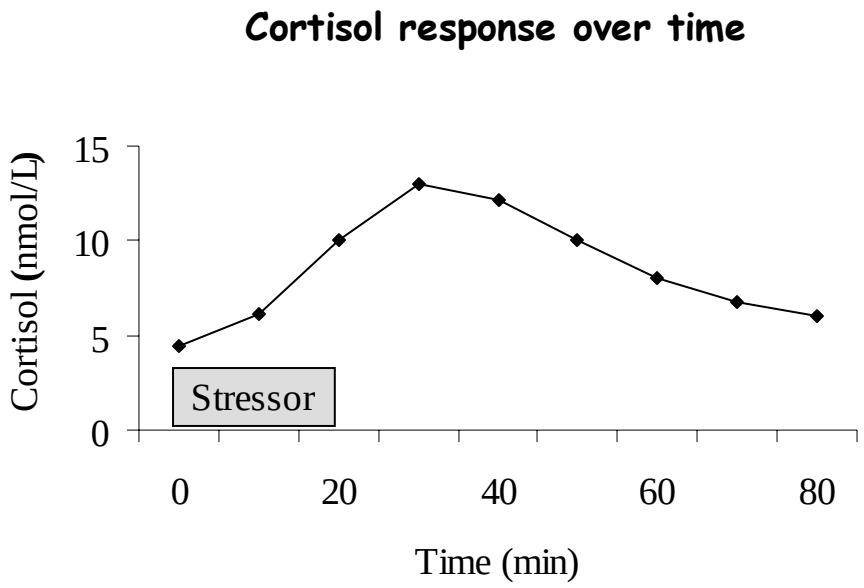

Figure 3. Cortisol response to acute stressors over timein humans. Negative feedback regulation of the HPA-axis shuts the stress response off, maintaining the system at an optimal level (figure modified from Shea et al., 2004).

Serotonin and the HPA-axis

Psychiatric disorders, for example, depression and anxiety are related to chemical imbalances in the CNS that alter interpretations of stimuli and influence behavioral responses to potentially stressful situations. There are multiple transmitters that may be subjected to such imbalances, but cortisol, CRF and serotonin (5-hydroxytryptamine; 5-HT) are particularly relevant for discussions of stress, anxiety and depression (reviewed in McEwen, 2000a; Nemeroff, 1998; and Nestler et al., 2002). The neurotransmitter serotonin has been a major focus in depression research. Serotonin-containing neurons are mostly clustered within the raphe nuclei in the brain stem. These neurons project extensively to all levels of the CNS (Bear et al., 2001). Serotonergic neurons appear to play an important role in the brain systems that regulate mood, emotional behavior and sleep (Bear et al., 2001). Research has shown that depression is connected to decreased serotonin metabolism and down-regulation of serotonin receptors in the brain (van Praag, 2004). Lowered serotonin neurotransmitter function is also suggested to be associated with excessive alcohol consumption (LeMarquand, Pihl, & Benkelfat, 1994a).

Research also focuses on investigations of the HPA-axis in relation to psychiatric disorders. It has, for example, been shown that patients with depression or anxiety

have an excessive activation of the HPA-axis (increased levels of cortisol, CRF and ACTH, reviewed in Arborelius, Owens, Plotsky, & Nemeroff, 1999; Holsboer, 2001; Nemeroff, 1996). The relation between increased CRF release and depression is furthermore strengthened by the fact that exogenously administered CRF in animals causes behavioral effects also seen in symptoms of depression, for example, decreased appetite, disrupted sleep, decreased sexual behavior and altered locomotor activity (Nemeroff, 1996). It has been hypothesized that in genetically vulnerable individuals, adverse childhood experiences sensitize the HPA-axis. Subsequent stressors could then evoke a pathologically hyperactive response in this axis (Nemeroff, 1996).

Increased stress hormone levels are not, however, present in all depressed patients, as subjects with depression have been reported not to differ from healthy controls in this respect (Bao et al., 2004; Peeters, Nicholson, & Berkhof, 2003; Peeters, Nicolson, & Berkhof, 2004), although secretion patterns of cortisol were erratic in patients with more severe symptoms of depression (Peeters et al., 2004). In addition to elevated or no changes in levels of cortisol in relation to depression, prolonged stress reactions have been reported in depressed patients (Burke, Davis, Otte, & Mohr, 2005). Furthermore, decreased levels of corticosterone have been found to be related to depression (Burke et al., 2005; Gur, Cevik, Sarac, Colpan, & Em, 2005; Levitan, Vaccarino, Brown, & Kennedy, 2002; Zarkovic et al., 2003). The HPA-axis reactivity in relation to depression may resemble the schematic patterns outlined in figure 4. The various HPA-axis responses in relation to depression have been suggested to be explained by, for example, the severity and subtype of depression, the age and number of subjects included in the study, possible stress experienced in subjects coming to the laboratory for testing and at what time in the circadian rhythm measures were taken (Burke et al., 2005; Levitan et al., 2002; Stewart, Quitkin, McGrath, & Klein, 2005). Burke et al. (2005) furthermore acknowledge the difficulties in their meta-analysis to determine whether the prolonged stress reaction precedes, accompanies or follows a depressive episode.

The normal physiological reaction to stressors is seen in an elevation of hormones from baseline levels and a decline over time to adaptive levels (Figure 4). In some individuals, the HPA-axis has been shown to have difficulties in normalizing cortisol levels in response to stress exposure and a prolonged physiological response is seen. The prolonged activity of the HPA-axis may subsequently result

in a further impaired axis, with decreased cortisol production and no normal circadian variations of the hormone (McEwen, 1998).

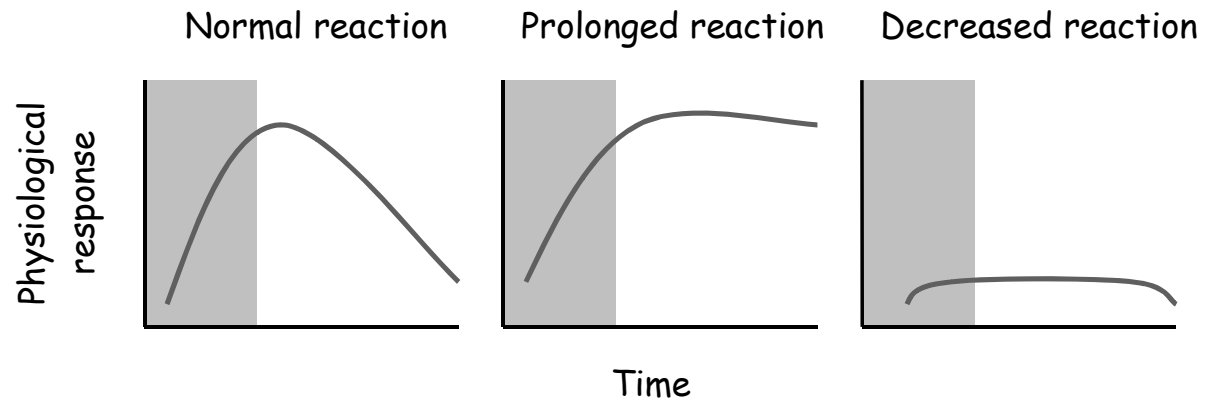

Figure 4. Schematic views of HPA-axis activation to stressors. Normal physiological responses to challenges is shown in an increase in hormone levels and a decline over time, prolonged reaction does not show a decline in response to stressors and this condition may subsequently result in an inadequate reaction where hormone levels are decreased over time. Period of stress activation is shown in the shaded area (figure modified from McEwen, 1998).

Catecholamines

Catecholaminergic neurons (dopamine, noradrenaline and adrenaline) are found in regions of the CNS involved in the regulation of movement, mood, attention and visceral function (Bear et al., 2001). There are dopamine-containing neurons throughout the CNS, but two of the systems involved in movement and emotions are the nigrostriatal pathway and the mesocorticolimbic pathway. These pathways arise from substantia nigra and the ventral tegmental area (VTA) within the brain stem, respectively. The substantia nigra projects to the striatum (caudate nucleus and putamen) within the basal ganglia. This pathway is involved in locomotor activity and degeneration of these neurons is associated with Parkinson's disease. The mesocorticolimbic pathway projects to limbic and cortical regions and the nucleus accumbens within the basal ganglia. This pathway is involved in the brain reward system and it is associated with drug abuse and psychiatric disorders (e.g. schizophrenia). Experiments in laboratory animals have shown that the mesocorticolimbic system is activated in response to even mild stressors (e.g. novelty exposure) and the increased dopamine activity has been connected to

sensitivity to drugs of abuse (Koob, 1992; van der Elst et al., 2005; van der Kam, Coolen, Ellenbroek, & Cools, 2005).

Noradrenergic neurons are located in locus coeruleus within the brain stem and innervate every main region in the brain (i.e. cortex, thalamus, hypothalamus, hippocampus, amygdala, olfactory bulb and cerebellum) and the spinal cord (Bear et al., 2001; Heimer, 1995). The locus coeruleus seems to be involved in the regulation of attention, arousal, sleep-awake cycles as well as learning and memory, anxiety, pain, mood and brain metabolism. Clinical findings suggest that noradrenaline, as well as serotonin, might be involved in depression, and several antidepressant drugs act on both of these neurotransmitters (Kolb & Whishaw, 2001).

Opioid peptides

Neuroactive endogenous opioid peptides (e.g. enkephalines and dynorphines) have widespread projections throughout the brain (e.g. amygdala, striatum, hypothalamus, periaqueductal gray, raphe nucleus and substantia nigra). They are involved in several functions, for example, the regulation of responses to pain and stressors, learning and memory, psychiatric disorders, immune function and endocrine functions (Akil et al., 1984; Koob, 1992; Massotte & Kieffer, 1998). In addition, the brain opioid systems are involved in drug dependence (Gerrits, Lesscher, & van Ree, 2003; Koob, 1992). Endogenous opiates seem to be a reward compound in the brain, and their actions appear to be mediated through the dopaminergic neurons of the mesolimbic system, which increases dopamine release. The reinforcing actions of opiates also seem to occur in the absence of dopamine, suggesting the existence of additional reward systems in the brain (Kolb & Whishaw, 2001).

Alcohol and other drugs

Addiction to alcohol or other drugs is a complex problem determined by several factors, including psychological and physiological components. Stress is considered to be a major contributor to the initiation and maintenance of alcohol (or other drug) abuse and dependence (Brady & Sonne, 1999; Pohorecky, 1981; Sinha, 2001). The relationship between stress and drug abuse is proposed to partially be mediated by diverse neurochemical systems (e.g. serotonin, dopamine and opiate

peptide systems) and the HPA-axis (Brady & Sonne, 1999). For example, one likely explanation for the connection between stress and drug abuse is that stress increases the activity of the dopaminergic brain systems that are involved in motivation and reward, and which also mediate drug-induced rewarding effects. Accordingly, stress-induced changes in those systems may enhance the responsiveness to the effects of drugs (Brady & Sonne, 1999). In addition, anxiety and depression are frequently comorbid with alcohol abuse in humans (Hodgins, el-Guebaly, Armstrong, & Dufour, 1999; Kushner, Thuras, Abrams, Brekke, & Stritar, 2001; Regier et al., 1990).

Preclinical studies of early stress

Longitudinal studies of the impact of early stressful experiences in humans are time consuming and environmental factors are difficult to control. As there are ethical limitations associated with conducting experiments in humans, laboratory animal models have been developed to experimentally investigate early environmental influence on adult behavior and neurobiology. Animal studies provide a valuable model for investigating possible mechanisms underlying the human costs of early childhood adversities on behavioral disorders such as, for example, depression and drug abuse.

A number of studies have evaluated the effects of repeated separations of infant non-human primates from their mothers or peers. Infants exposed to maternal separation, like human infants, demonstrate acute behavioral and physiological reactions to separations (reviewed in Sanchez, Ladd, & Plotsky, 2001). When tested as adults, non-human primates exposed to prolonged periods of maternal or social deprivation exhibit marked behavioral changes, for example increased fearfulness and anxiety, social dysfunction, aggression, altered ingestion and anhedonia. Also neurochemical, endocrine and immune function are changed in non-human primates as an effect of maternal separation (see Gilmer & McKinney, 2003; Pryce, Rüedi-Bettschen, Dettling, & Feldon, 2002; and Sanchez et al., 2001 for a review of the literature). For example, basal and stress-induced HPA-axis activation has been reported to increase after maternal separation in rhesus monkeys, and in these cases alcohol preference was positively correlated with the increased cortisol concentrations (Fahlke et al., 2000; Higley, Hasert, Suomi, &

Linnoila, 1991). As in humans, research on animals strongly indicates that the serotonergic system may mediate alcohol intake, since decreased serotonergic functioning increases alcohol intake and *vice versa* (Higley & Bennett, 1999; LeMarquand, Pihl, & Benkelfat, 1994b). Moreover, it has been shown that maternally separated monkeys' excessive alcohol intake, aggression and anxiety can be reversed by administration of a serotonin reuptake inhibitor, which increases the effect of serotonin in the brain (Higley, Hasert, Suomi, & Linnoila, 1998). It is possible that early environment may influence genes for serotonergic functioning (and thereby decreased serotonin levels in the CNS), as maternal separation in monkeys has found to cause changes in a gene regulating this neurotransmitter (Bennett et al., 2002).

The laboratory rat has proved to be a useful experimental model of early experiences as many aspects of neuronal and physiological development and measures of emotional reactivity in rats are predictive of events in humans (Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001; Kuhn & Schanberg, 1998). In order to investigate effects of early-life stress in the rat, the maternal separation (MS) paradigm has been developed, which is proposed to be a model for child abuse or neglect (de Kloet, Sibug, Helmerhorst, & Schmidt, 2005; Shea et al., 2004). Early adverse life events during neuronal maturation have been shown to affect brain development and to produce persistent changes in brain function and to increase the vulnerability to psychiatric disorders (Ellenbroek & Riva, 2003). The MS paradigm covers an area of diverse methods used in postnatal separations of the pup from their dam, ranging from a single 24-h period to repeated 1-12 h periods of separation after birth until weaning. Furthermore, the separated pups are either kept in litters or separated from one another during the separation period (reviewed in Gutman & Nemeroff, 2002; Lehmann & Feldon, 2000; and Pryce et al., 2002).

MS-induced alterations in the laboratory rat

The newborn rat pup is dependent on care from the mother to survive. Normally pups spend their first week in the nest with littermates and the mother suckles them almost continuously for the first two days, and then she gradually takes longer and longer absences (Ader & Grota, 1970; Calhoun, 1962). During the first week, pups are unable to regulate their body temperature, and cannot hear or see. In the second week of life, pups' eyes and ear canals have opened and they are able to thermoregulate and they show more ambulation. At weaning age (postnatal days

20-25) the pups are fully able to live on their own (reviewed in Kuhn & Schanberg, 1998). When conducting early separations of pups from the dam, several factors are involved in the process. Usually pups are removed from the mother and kept in another room during the separation period. During this period pups are deprived of several types of stimuli, for example, tactile and olfactory (from the mother and in some cases also from the littermates). Also thermal, nutritional and auditory stimuli are changed during the separation period, all of which seem to play a role in regulating the pups' physiology and behavior (Hofer, 1994; Kuhn & Schanberg, 1998). The lack of important stimuli in early ages is proposed to have long-term effects on neurobehavioral outcome in adult offspring. Although results are not consistent within the MS paradigm, it has been shown that the MS manipulation affects a large number of behavioral and neurobiological variables (see Anand & Scalzo, 2000; Gutman & Nemeroff, 2002; Hall, 1998; Ladd et al., 2000; Lehmann & Feldon, 2000; Pryce & Feldon, 2003; and Sanchez et al., 2001 for a review of the literature).

Behavior. Behavioral measurements in different test situations have frequently been used to explore rats' emotional behavior. Emotionality is thought to be multi-dimensional, represented by, for example, locomotor activity, anxiety, exploration, risk assessment and arousal (Archer, 1973; Ohl, Toschi, Wigger, Henniger, & Landgraf, 2001). Beside these terms, emotional reactivity, anxiety-related behavior and fearfulness are also used in the literature (and in the present thesis) to describe dimensions of emotionality. As adults, maternally separated pups have shown increased emotionality/anxiety in several different test situations (reviewed in Ladd et al., 2000). Common models for measuring emotionality in rodents are based on the conflict existing between the natural tendency to explore a new environment and the potential risk for unpredictable occurrences (reviewed in File, 1992). Studies reporting increased emotionality in MS animals have used, for example, the elevated plus maze (Daniels, Pietersen, Carstens, & Stein, 2004; Huot, Thrivikraman, Meaney, & Plotsky, 2001; Kalinichev, Easterling, Plotsky, & Holtzman, 2002; Madruga, Xavier, Achaval, Sanvitto, & Lucion, 2005; Wigger & Neumann, 1999), two compartment exploratory test (Biagini, Pich, Carani, Marrama, & Agnati, 1998) and the open field test (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000). The plus maze apparatus is elevated and in the shape of a plus sign with two open and two enclosed arms. The rat has free access to all arms and the amount of time spent and entries into open and closed arms are

measured and comprise an index of emotionality (the more time spent and entries into closed arms, the more emotionally reactive the animal and *vice versa*). The two compartment exploratory test has a small darkened place set in a brightly lit open field and locomotion and transitions between the compartments are measured. The critical measure for emotionality in the open field test is the time the animal spends exploring the inner area of the novel arena (decreased time indicates more anxiety). In addition to these behavioral tests, acoustic startle response (a protection reflex; increased startle response reflects a more fearful animal), sucrose preference (decreased motivation to obtain sucrose reward reflects anhedonia) and defecation (increased defecation reflects fearfulness) have also been measured and indicated increased emotional reactivity in MS animals (Caldji, Francis et al., 2000; Daniels et al., 2004; Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002). Furthermore, as reported in non-human primates (Higley et al., 1998), treatment with a serotonin reuptake inhibitor eliminated the enhanced emotionality found in MS rats (Huot et al., 2001).

Endocrinology. A functional HPA-axis is essential for survival in adulthood, and fluctuations in the activity of the developing axis during infancy may be maladaptive to the development of the immature brain (Gutman & Nemeroff, 2002). In rats, it is known that pups are protected from external stressors during early development (i.e. the stress hyporesponsive period; SHRP). During this period (postnatal days ~ 2-12) endocrine responses to a variety of stressors (e.g. surgery, handling, ether and thermal challenges), which normally elicit corticosterone elevations in adults, are attenuated in the young offspring (de Kloet et al., 1988; Rosenfeld, Suchecki, & Levine, 1992; Sapolsky & Meaney, 1986; Vazquez, 1998). The protective role of SHRP is thought to act on several levels, both on the adrenal and at brain level (Rosenfeld et al., 1992; Sapolsky & Meaney, 1986). However, the reduced responsiveness of the HPA-axis during SHRP appears not to be absolute, as sufficiently potent stressors (including maternal separation of pups) have been shown to overcome this barrier (reviewed in Anisman, Zaharia, Meaney, & Merali, 1998; Francis et al., 1999; Gutman & Nemeroff, 2002; and Ladd et al., 2000).

It is hypothesized that both extremely high and low levels of hormones during SHRP are associated with abnormal neural development in the CNS (e.g. reduced brain weight, suppression of cell division and myelination of neurons) that subsequently may affect behavioral development, for example, altered social behavior and impaired learning (Sapolsky & Meaney, 1986). In MS studies, disruptions of the HPA-axis have been seen in enhanced secretion of CRF, ACTH and corticosterone in both basal and stressed conditions (Biagini et al., 1998; Daniels et al., 2004; Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002; Nemeroff, 1996; Plotsky et al., 2005) and an impaired inhibition of the HPA-axis (Ladd, Huot, Thrivikraman, Nemeroff, & Plotsky, 2004). These disruptions of the HPA-axis in MS animals indicate that they have an increased endocrine stress response. In order to measure HPA-axis responses to stressors, several different stimuli have been used. In the studies above, the acute air-puff startle stress, restraint stress (placing the animal in a confined holder for a few minutes) and brief human handling exposure were used. As with enhanced anxiety and increased alcohol intake in MS animals, administration of a serotonin reuptake inhibitor eliminated the increased HPA-axis response to stressors in adult MS offspring (Huot et al., 2001).

Neurochemistry. During the early postnatal period, the rat brain is undergoing neural development, for example, neurogenesis, synaptogenesis, dendritic development and apoptosis (programmed cell death, reviewed in Gutman & Nemeroff, 2002). Repeated MS has been reported to produce structural disruptions in the brain, for example, delays in the synaptic development in the hippocampus and increased cell death of neurons and glia (Andersen & Teicher, 2004; Kuma et al., 2004; Mirescu, Peters, & Gould, 2004; Zhang et al., 2002).

Maternal separation affects transmitter systems in the rat brain (reviewed in Ladd et al., 2000; Meaney, Brake, & Gratton, 2002). For example, it has been found that early separations change the sensitivity of serotonin receptors and/or serotonin transporters, and similar effects in humans are suggested to contribute to psychiatric illnesses (Arborelius, Hawks, Owens, Plotsky, & Nemeroff, 2004; Gartside, Johnson, Leitch, Troakes, & Ingram, 2003). Other changes in the serotonin system in the brain have also been reported in MS animals, for example, increased tissue levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), decreased tissue levels of serotonin and both increased and decreased

turnover ratios of serotonin (Daniels et al., 2004; Matthews, Dalley, Matthews, Tsai, & Robbins, 2001).

The levels of the neurotransmitter noradrenaline in hypothalamus and hippocampus have been found to be markedly decreased in MS animals in response to restraint stress, indicating a suboptimal noradrenergic system, which also has been proposed to mediate for example depression in humans (Daniels et al., 2004). Liu, Caldji, Sharma, Plotsky and Meaney (2000) have shown that MS increased levels of noradrenaline in the hypothalamic paraventricular nucleus, indicating that early life events serve to influence the differentiation of noradrenergic neurons, and thus alter HPA stress responses in adulthood.

The MS manipulation has also been suggested to alter the mesolimbic dopamine system (Meaney et al., 2002). Dopamine transmission is partly regulated by the reuptake of dopamine from the synapse, and reuptake occurs via the dopamine transporter system. MS has been found to decrease dopamine transporter levels in some brain areas and thereby increase dopamine responses to stress and sensitivity to cocaine and amphetamine (Meaney et al., 2002). A decreased number of different dopamine receptors in the brain has also been reported in MS animals (Ploj, Roman, & Nylander, 2003a).

Moreover, the endogenous opioid system is activated by different kinds of stressors and is suggested to play an important role in brain reward pathways implicated in drug abuse (reviewed in Koob, 1992; Lu, Shepard, Scott Hall, & Shaham, 2003). Studies indicate that early separation manipulation affects the endogenous opioid system (Ploj, Roman, & Nylander, 2003b). In that study, MS caused long-term alterations of dynorphin and enkephalin in several different brain areas (hypothalamus, amygdala, substantia nigra, neurointermediate pituitary lobe and the periaqueductal gray).

Sensitivity to drugs of abuse. It has been proposed that alcohol consumption is strongly related to the HPA-axis in the rat, as decreased levels of corticosterone are associated with decreased alcohol intake and *vice versa* (reviewed in Hansen, Fahlke, Hård, & Engel, 1994; and Pohorecky, 1990). Early adverse experience, in the form of MS, has been associated with increased voluntary alcohol consumption (Huot et al., 2001; Jaworski, Francis, Brommer, Morgan, & Kuhar, 2005; Ploj et

al., 2003a; Roman, Gustafsson, Hyytiä, & Nylander, 2005). As reported in monkeys, treatment with a serotonin reuptake inhibitor eliminated the increased alcohol preference in MS rats (Huot et al., 2001). Altered sensitivity to other drugs of abuse, for example, cocaine, morphine and amphetamine has also been reported in MS studies (Chretien & Gratton, 2002; Kalinichev, Easterling, & Holtzman, 2002; Matthews, Hall, Wilkinson, & Robbins, 1996; Matthews, Robbins, Everitt, & Caine, 1999; Meaney et al., 2002; Vazquez et al., 2005; Zhang, Sanchez, Kehoe, & Kosten, 2005; Zimmerberg & Shartrand, 1992). For example, MS animals have shown both increased and decreased locomotor activity after administration of amphetamine, suggesting that MS affects the animals' sensitivity to the drug (Zimmerberg & Shartrand, 1992).

Some of these changes seen in animals resemble the disturbances that are characteristic of mood disorders in humans, for example, alterations of the HPA-axis and neurochemistry and structural changes in the brain (reviewed in Kaufman, Plotsky, Nemeroff, & Charney, 2000). This has led to the suggestion that the MS-model might be a suitable environmental animal model for studying the mechanisms contributing to psychiatric disorders, such as for example anxiety, depression and drug abuse (reviewed in Anand & Scalzo, 2000; Huot, Ladd, & Plotsky, 2000; Huot et al., 2001; Meaney et al., 2002; and Sanchez et al., 2001).

Handling of pups

Pups experiencing a handling procedure are commonly added in MS studies, along with other comparison groups. In contrast to the proposed negative effects of prolonged MS, rat pups provided with early stimulation, in the form of handling and a short separation period (~20 min/day) during the postnatal period, have been found to reduce stress reactivity in adulthood compared to non- handled offspring. For example, early handled animals show decreased fearfulness in novel environments and lower HPA responses to stress (Levine, Haltmeyer, Karas, & Denenberg, 1967; Meaney, 2001; Meaney et al., 1996; Ploj et al., 1999; Ploj, Roman, Bergström, & Nylander, 2001; Plotsky & Meaney, 1993). The behavioral and neurobiological alterations associated with early handling have proposed to be essentially the opposite of those reported in maternally separated rats (reviewed in Francis & Meaney, 1999; Gutman & Nemeroff, 2002; Huot et al., 2000; Kaufman & Charney, 2001; and Roman & Nylander, 2005), although this view has been argued to be an oversimplification (Lehmann & Feldon, 2000). In addition, as in

the present thesis, some have used a briefly (3-5 min) handled group to control for the effect of the human-pup contact in the separation procedure in MS studies (Biagini et al., 1998; Kaneko, Riley, & Ehlers, 1994; Madruga et al., 2005; Matthews, Hall et al., 1996; Matthews et al., 1999; Matthews, Wilkinson, & Robbins, 1996; von Hoersten, Dimitrijevic, Markovic, & Jankovic, 1993; Zimmerberg & Shartrand, 1992). Whether this short "control" handling lead to handling-like effects in the offspring has not yet been fully investigated. However, early studies by Levine and colleagues (1971) revealed that daily 3 min handling of rat pups in infancy induced less anxiety (more exploration and less defecation) compared to non-handled animals.

Maternal behavior

Maternal separation and handling of pups appear to mediate behavioral and neurobiological changes in the developing pup. Other early life experiences have also been found to elicit profound effects on behavior and brain function. Studies of the quality of maternal care reveal that dams exhibiting high levels of licking/grooming and arched-back nursing during infancy reduces emotionality and HPA-axis responses in the adult offspring (Caldji, Diorio, & Meaney, 2000; Caldji et al., 1998; Francis & Meaney, 1999; Liu et al., 1997). Behaviorally, the reduced emotional reactivity has been noted in, for example, decreased startle response, increased open field exploration and shorter latencies to eat food in novel environments. Reduced endocrine responses in offspring to dams exhibiting high levels of these care behaviors have been found in, for example, decreased ACTH and corticosterone levels. Exactly how the dams regulate the pups' HPA-axis is not completely understood, but is proposed to occur at several levels. For example, feeding appears to regulate adrenal sensitivity to ACTH, whereas tactile stimulation inhibits the activation of centrally controlled components of the axis (Rosenfeld et al., 1992; Suchecki, Rosenfeld, & Levine, 1993). The quality of maternal care behavior differs naturally among rats (Caldji, Diorio et al., 2000; Champagne, Francis, Mar, & Meaney, 2003), but MS and handling manipulations have been reported to alter maternal care behaviors. Early prolonged separations of pups have led to longer retrieval latencies in MS dams upon reunion, longer time to begin to feed, lick and groom the pups. Dams exposed to the handling manipulation, on the other hand, exhibited increased maternal behaviors, such as arched-back nursing and licking/grooming the pups (Huot et al., 2000; Liu et al., 1997; Pryce, Bettschen, & Feldon, 2001). Furthermore, emotional alterations reported in

maternally separated rats were reversed when MS pups were raised by high-licking and -grooming adult females (reviewed in Kaufman et al., 2000). Thus, it is possible that the quality of maternal care is a critical factor within the MS-paradigm, and in fact may mediate (at least in part) the effects of maternal separation and handling manipulations.

In summary, several studies suggest that adverse early life experiences are risk factors for psychiatric illness in humans, for example, anxiety, depression and substance abuse. Experimental animal models have been developed to further investigate the underlying mechanisms of adverse early experiences. The MS model in rats has revealed profound effects of early separations on adult behavioral, neurochemical and endocrine responses, and thereby is proposed to offer a model for psycho- and physiopathology in humans. In addition, the effects of early adversities in the MS model is induced without drugs which makes it possible to study subsequent pharmacological treatments for these conditions without possible cross reactivity between drugs.

AIM OF THE THESIS

The general aim of the thesis was to investigate long-term behavioral, neurochemical and endocrine effects of maternal separation in adult rat offspring.

It has been shown that early maternally separated rat pups show increased emotionality in adulthood, which is proposed to model human psychiatric disorders (reviewed in Anand & Scalzo, 2000; Hall, 1998; Huot et al., 2000; Ladd et al., 2000; Meaney et al., 2002; Pryce & Feldon, 2003; and Sanchez et al., 2001). The present investigations within the MS paradigm further investigated the environmentally induced emotionality in the rat. Adult animals' behavior was studied in several different test situations, which are assumed to represent dimensions of emotionality: risk assessment (Study 1, 3 and 4), exploration (Study 1-4), fleeing and freezing (Study 2-4) and locomotor activity (Study 2-4). High ambulatory and exploratory scores, more time spent in exposed zones in different testing apparatuses (i.e. activity boxes, hole-board and canopy test) and a higher number of stretched attend postures were assumed to reflect less anxiety-related behaviors (reviewed in Boissy, 1995; Grewal, Shepherd, Bill, Fletcher, & Dourish, 1997). In the fleeing and freezing test (responses to a sudden auditory signal), more ambulatory behavior (fleeing) and longer duration of freezing reflected a more fearful animal (Boissy, 1995). It was furthermore hypothesized that MS animals would show less competitive behavior due to MS-induced anxiety or fear and was tested in a situation where motivated animals had to compete for the same goal (Study 2). Since anxiety and corticosterone levels are suggested to play an important role in reactivity to drugs and drug intake, animals were also tested for amphetamine-induced locomotor activity (Study 2) and preference for alcohol in a two-bottle free-choice test (Study 1-4, Cador, Dulluc, & Mormede, 1993; Hansen et al., 1994; Paré, Paré, & Kluczynski, 1999; Piazza et al., 1991; Pohorecky, 1990).

The MS manipulation is further proposed to cause disruptions in central neurotransmitter systems (e.g. Daniels et al., 2004; Matthews et al., 2001; Ploj et al., 2003b). The consequences of MS on neurotransmitter systems were examined by measures of endogenous opioid peptides (dynorphin B and Met-enkephalin-$Arg^{6}Phe^{7}$; Study 2) and monoamines (noradrenaline, dopamine, serotonin and their metabolites; Study 3 and 4) in different brain areas. Furthermore, possible

disruptions of the HPA-axis were studied by analysis of plasma corticosterone levels at the end of the experiment (Study 1-4; also in young animals in Study 2), as a large body of evidence exists for MS-induced disruptions of the HPA-axis in the offspring, for example, increased corticosterone levels (Biagini et al., 1998; Daniels et al., 2004; Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002; Ladd et al., 2004; Meaney et al., 1994; Plotsky et al., 2005). At the time for decapitation, the weight of thymus and adrenal glands were also measured (Study 1-4), as long-term increased stress hormone secretion has been found to result in thymus atrophy and adrenal enlargement (Akana, Cascio, Shinsako, & Dallman, 1985; Engler & Stefanski, 2003; Selye, 1936).

Most MS experiments are conducted in male rat offspring. However, there are indications that MS may affect emotionality and stress hormone secretion in a sex-dependent manner (de Jongh, Geyer, Olivier, & Groenink, 2005; Genest, Gulemetova, Laforest, Drolet, & Kinkead, 2003; Kalinichev, Easterling, Plotsky et al., 2002; Matthews et al., 1999; McIntosh, Anisman, & Merali, 1999; Wigger & Neumann, 1999). To further investigate possible sex differences, both male and female animals were included in Study 1 and 2 in the present thesis.

As manipulations within the MS paradigm have been reported to impair the quality of maternal care (Huot et al., 2000; Liu et al., 1997; Pryce, Bettschen, & Feldon, 2001), the dams were assessed for retrieval behavior of pups (Study 2 and 3). Pups were furthermore examined for developmental progress in early ages (i.e. body weight [Study 2-4], air righting reflex and fleeing and freezing responses [Study 2]).

Finally, methodological differences in the separation procedure are proposed to have an impact on the results in MS studies (Lehmann & Feldon, 2000; Mintz, Rüedi-Bettschen, Feldon, & Pryce, 2005; Zimmerberg & Shartrand, 1992). In the present thesis animals were either separated as litters in an incubator (Study 1 and 2), housed in isolation in an incubator (Study 3) or housed isolated in room temperature (Study 4), and thereby giving us the opportunity to further explore the effects of different housing conditions of pups during the separation period.

MATERIALS AND METHODS

The experimental designs of studies included in the thesis are presented in time lines in Figure 5. For a more detailed description of the methods carried out, see text and appendix.

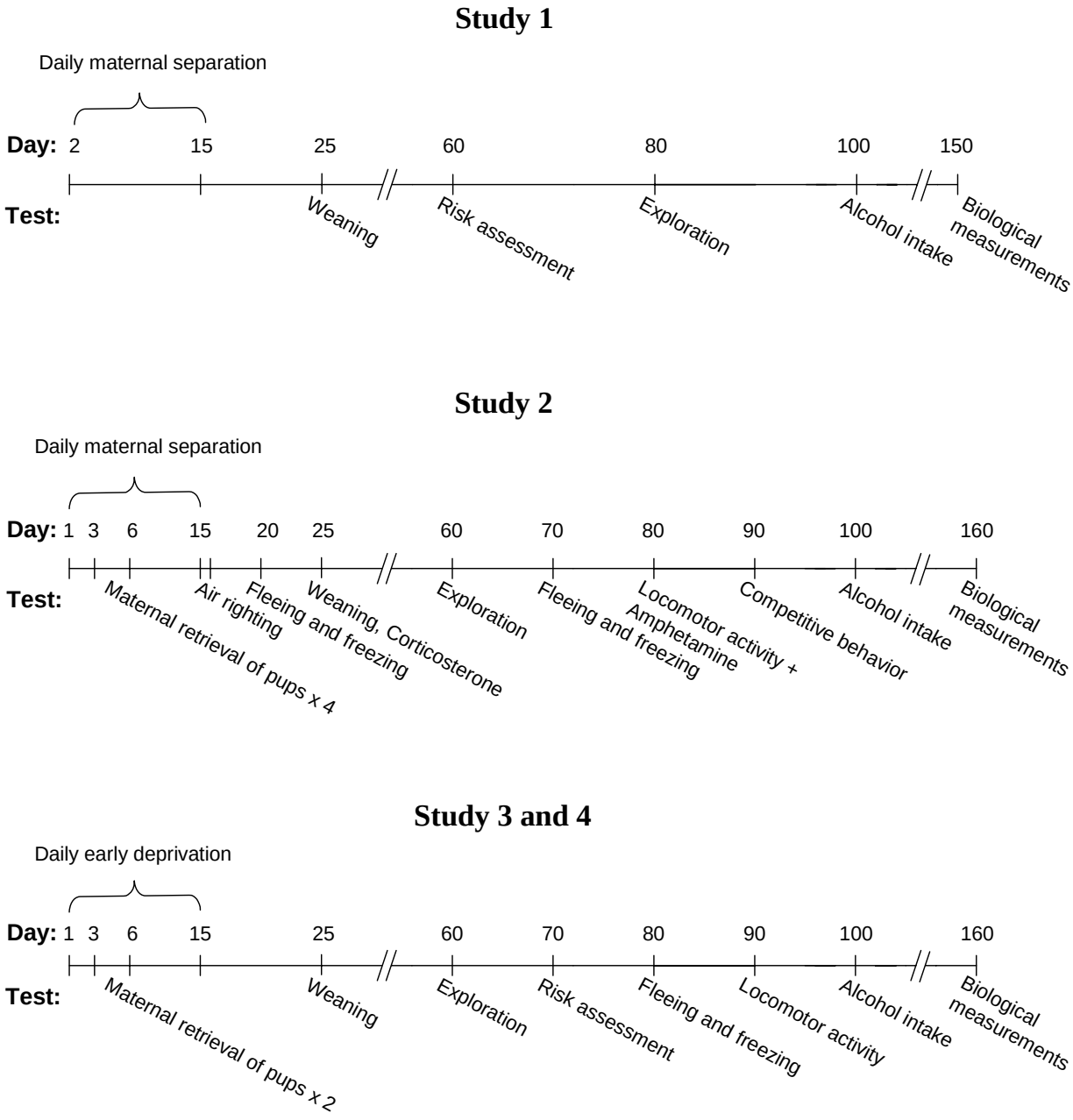

Figure 5. Time lines for the experimental designs of the studies included in the thesis (Study 1-4) indicating the tests and test days used in the experiments.

Animals and experimental manipulations

Animals

Adult male and virgin female Wistar rats from Möllegaard Breeding Center (Denmark; Study 1 and 2) and from Scanbur BK AB (Sollentuna, Sweden; Study 3 and 4) were allowed to adapt to laboratory conditions before the experiments started. A female in estrous was placed overnight with a male in a breeding cage, and thereafter housed in her original group. At the end of pregnancy, females were housed singly and provided with nesting material. In Study 1 and 2, 40 and 20 females respectively, and their offspring, were randomly divided into maternal separation treatment (MS; 20/10 litters) or a brief daily handling procedure (controls; 20/10 litters). In Study 3 and 4, 40 females and their offspring were randomly divided into separation treatment (20 litters) or a brief handling procedure (controls: 20 litters). The litters were culled (when possible) to 8 pups per litter (4 males and 4 females) on postnatal day 1 (day of birth = day 0).

All animals lived in air-conditioned colony rooms (lights off during day time) at a temperature of 23° C and a humidity of 50-60 %. Animals had free access to water and food, except for a period of water restriction in Study 2. The experiments were approved by the local ethical committee of the Swedish National Board for Laboratory Animals.

Maternal separation/Early deprivation and weaning

In all studies, pups were separated daily from their dams for 3 or 4 h during the first period of life (postnatal days 2-15 or 1-15). The dam was first removed from the homecage into a separate cage. Pups were thereafter removed and placed in a plastic box, and transferred to a room adjacent to the colony room. After this separation procedure, the dam was placed back into the maternity cage. At the end of the separation period, the dam was once again removed from the home cage, and the pups were returned to this cage followed by the dam. A main difference between Study 1-2 and Study 3-4 was that in Study 1 and 2, all the pups were removed as *a litter* and placed in an incubator set to maintain an ambient temperature at 30° C (maternal separation; MS).

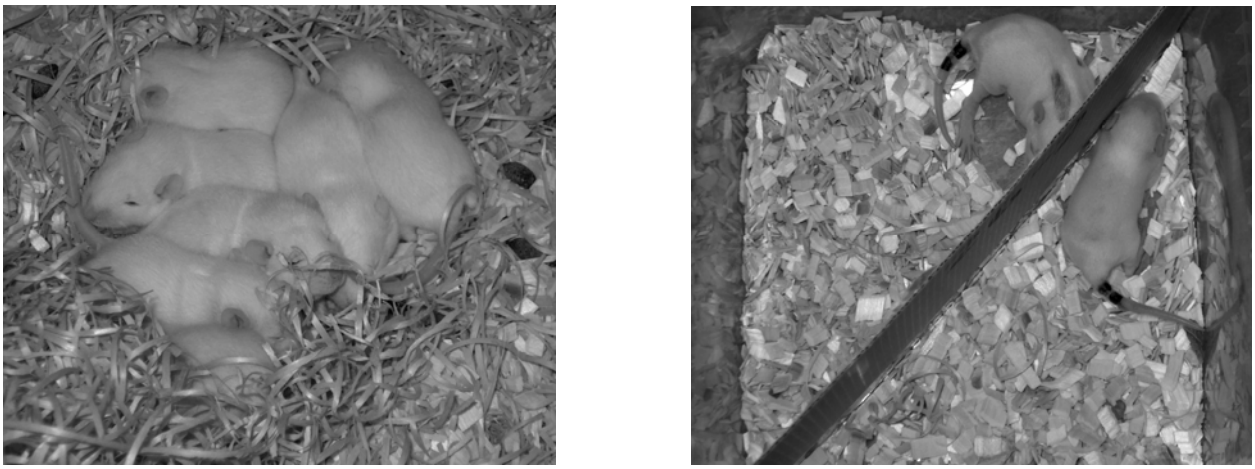

Figure 6. The different separation manipulatons used. On the left: pups kept in litters in incubators (Study 1 and 2). On the right: pups kept in isolation, either in incubators (Study 3) or in room temperature (Study 4).

In Study 3 and 4, two male pups/litter were placed *individually* in plastic boxes, either in an incubator set to maintain an ambient temperature at 30° C (early deprivation; ED_{Inc}), or placed at room temperature (22° C; $ED_{Room;}$ Figure 6; see table 1 for an overview of the methods used). The remaining littermates in Study 3 and 4 (not included in the experiments) were placed as a litter separately from the dam in room temperature during the separation manipulation.

Table 1. *Overview of number of litters and separation treatment in maternally separated (MS), early deprived (ED) and control animals in the four studies included in the thesis.*

	Study 1	Study 2	Study 3	Study 4
MS litters	*n* = 20	*n* = 10	*n* = 20	*n* = 20
Control litters	*n* = 20	*n* = 10	*n* = 20	*n* = 20
MS/ED treatment	3 h days 2-15 Litters in incubator	4 h days 1-15 Litters in incubator	4 h days 1-15 Pups isolated in incubator	4 h days 1-15 Pups isolated in room temperature
Included offspring	One male and one female pup/litter	4 male and 4 female pups/litter	One male pup/litter	One male pup/litter

As proposed by Pryce and Feldon (2003), the terms MS and ED are used to distinguish the different methodologies used in the separation procedure, either separation of pups as a litter from the dam (i.e. MS; Study 1 and 2), or separation of pups in isolation (i.e. early deprivation: ED; Study 3 and 4). The MS term is, however, also used in the literature as well as in the present thesis when generally referring to the paradigm of prolonged separations of pups from their dams, irrespective of the exact separation protocol used.

Pups that served as controls in the studies (1-4) were reared identically to the MS/ED offspring but experienced a brief daily handling procedure during the separation period. The dam was first removed into a separate cage, and the litters were thereafter removed and placed in a plastic box in room temperature. Within 3-5 min, the pups returned to the maternity cage, followed by the dam. During early development all pups underwent weighing and daily marking. Normal cage maintenance began on day 7.

On day 25, offspring were weaned and housed in same treatment groups (MS/ED or controls). On the same day in Study 2, one randomly selected pup from each gender, litter and treatment group was decapitated and blood samples were collected for analyses of plasma corticosterone levels.

Behavioral tests

All behavioral tests were conducted sequentially with the same animals and were always performed during the dark phase of the light/dark cycle. In order to control for possible altered behavior due to estrus (Scimonelli, Marucco, & Celis, 1999), female offspring were examined for occurrence of behavioral estrus at the time for each test (Study 1 and 2). This was done by testing the female's reaction (i.e. lordosis, ear wiggling and crouching) to manual palpation of her flanks and lower back.

Maternal retrieval of pups

To examine dams' maternal behavior towards pups, all dams were assessed for retrieval behavior of the pups (modified from Hård et al., 1985) when offspring were 3 and 6 days of age. In Study 2 the testing was conducted for 15 min immediately before and after the maternal separation procedure (MS group), and twice a day (approximately at the corresponding times) for the control group. In Study 3 the testing was conducted immediately after the separation period, and approximately at the corresponding time of the day for the control group. The dam and her pups were removed from the home cage into separate cages for 5 min. Thereafter the pups returned to the home cage, on the opposite side of the nest, followed by the dam. The time for retrieval of the first pup to the nest, retrieval of the whole litter and the time the dam spent in the nest with pups were measured. If the dam started to build a new nest in the home cage upon reunion, no measures were recorded.

Air righting

In Study 2, pups were examined for the performance of the air righting response, according to a method by Hård and Larsson (1975). The pups were dropped from a height of 30 cm, landing upon a platform of cotton covered by plastic. A positive response was recorded if the pup landed on the platform with all feet simultaneously making contact with the floor.

Fleeing and freezing

Testing for fleeing and freezing (Figure 7), in response to a sudden auditory signal, was performed on postnatal day 20 in Study 2, and at adult age in Study 2-4. The test occurred in a circular Plexiglas cage (Hård, Ahlenius, & Engel, 1983). During a 5-min adaptation period, the following open field behaviors were observed: first crossing (latency to leave the sector were the animal was first placed), locomotor activity (number of lines crossed), number of rearings, cumulative time grooming and defecation (number of fecal boli deposited). A doorbell was then turned on for 6 sec. During the signal the rat would first attempt to flee and thereafter, typically at the end of the signal, freeze. The ambulatory behavior (number of lines crossed) during the signal and the duration of the freezing response were used as measures of flight distance and freezing, respectively.

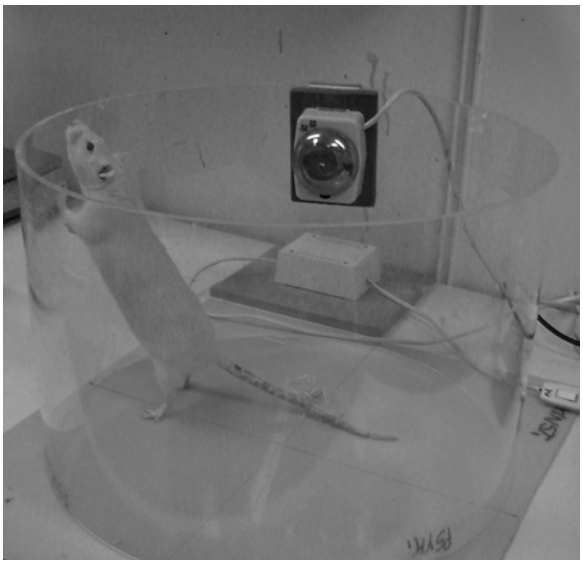

Figure 7. The fleeing and freezing test. During testing the apparatus was equipped with a roof.

Exploration

The test apparatus for exploratory behavior (modified from File & Wardill, 1975) consisted of a wooden, brown-painted hole-board (Figure 8). The floor was divided into 16 squares by white lines and each square contained a hole. The rat was placed in the middle of the arena, and during 5 min (Study 1 and 2), or 2 x 5 min (Study 3 and 4), the observer registered the number and cumulative duration of nose pokes into the holes and locomotor activity (number of lines crossed). In Study 1, the animals were habituated to the testing arena for 5 min before testing. The test was conducted in an illuminated test room.

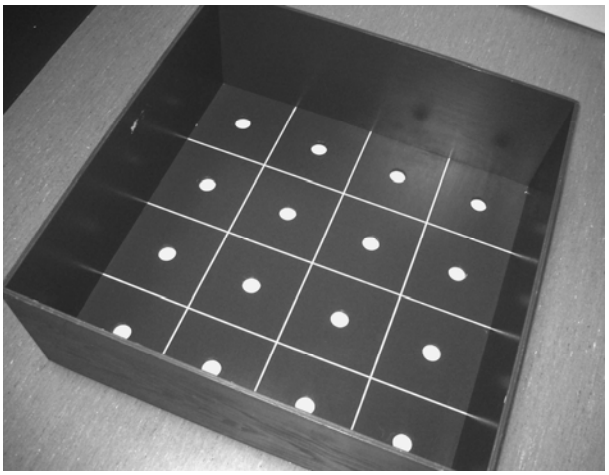

Figure 8. The hole-board for measures of exploratory behavior.

Risk assessment

The test apparatus for risk assessment (Study 1, 3 and 4, modified from Grewal et al., 1997) comprised an elevated deep green circular platform with a smaller red Perspex circular canopy supported 10 cm above the platform by a central pillar (Figure 9). The test apparatus was thus divided into an inner, dimly lit covered zone, and an outer, brightly lit exposed zone. During the 10-min testing period the following behaviors were recorded by two observers: the number of stretched attend postures, ambulatory behavior (number of lines crossed) and the time spent in the outer exposed zone (defecation was also noted in Study 1). A stretched attend posture (SAP) was defined as flexed hind limbs and a flattened lower back position with extended forelimbs when the animal is either standing still or moving slowly.

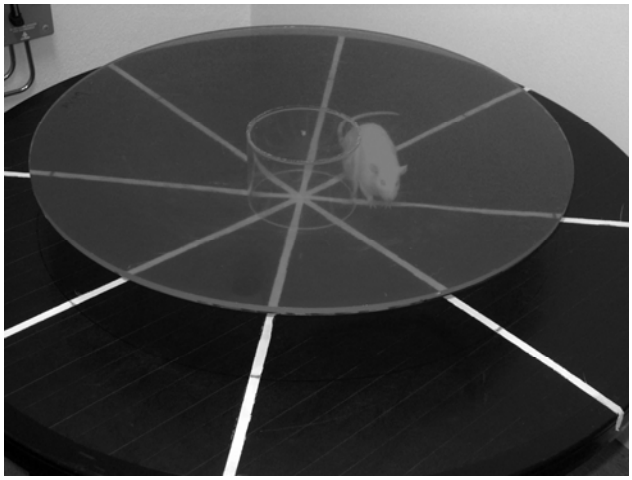

Figure 9. The risk assessment test.

Spontaneous and amphetamine-induced locomotor activity

Locomotor activity was investigated in test chambers made of Plexiglas boxes (Study 2, 3 and 4; Kungsbacka Mät och Reglerteknik AB; Figure 10). The test box contained two series of invisible infrared photocell beams to measure the following variables: the lower grid of infrared beams registered forward locomotion (consecutive interruptions of two beams). Rearing was registered by the high-level infrared beams, which registered every interruption of the beams as a rearing count when the rat raised itself onto its haunches. Total peripheral locomotion was recorded by measuring the animal in the periphery of the box (counts registered for every beam break). Spontaneous locomotor activity was measured for 1 h. In

addition, immediately after the first testing hour in Study 2, animals were given 1.0 mg/kg *d*-amphetamine sulphate in order to measure amphetamine-induced locomotor activity during a second testing hour.

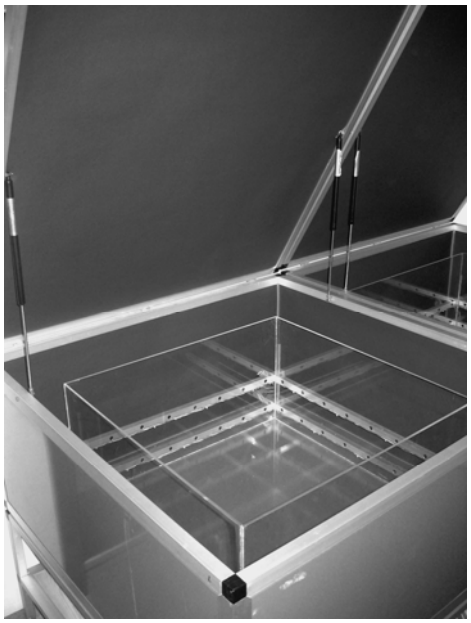

Figure 10. The test chamber for measuring locomotor and amphetamine-induced activity.

Competitive behavior

Animals were studied for displayed behaviors in a situation were they had to compete for the same goal (Study 2, modified from Albert, Dyson, & Walsh, 1987). After 23 h water restriction, animals were moved to individual cages with free access to water and their drinking time during 4 min was recorded (i.e. recording baseline). After a new period of 23 h water restriction, a randomly selected pair of rats (one male MS and control, and one female MS and control) had to compete for waterspout access. The spout had a suspended cone at the point where the waterspout entered the cage, which gave only one rat at a time the opportunity to drink (Figure 11). During 4 min the following measures for each competing pair were recorded by two observers: which animal that initially started to drink, the time each rat spent drinking and number of pushes towards the other animal.

Figure 11. The competition test occurred in a cage were the waterspout had a suspended cone which gave only one rat at a time the opportunity to drink.

Voluntary alcohol intake

Animals' voluntary ethanol intake was tested by giving them continuous access to a bottle containing an ethanol solution in addition to the water bottle (Figure 12). The ethanol concentration (vol/vol) was gradually increased (2-4-6 %) over a 3-week period in Study 1 and a 9-day period in Study 2-4. Thereafter, the animals had continuous access to two bottles one containing 6 % ethanol solution and the other tap water for a one-week period in Study 1 and a 3-week period in Study 2-4.

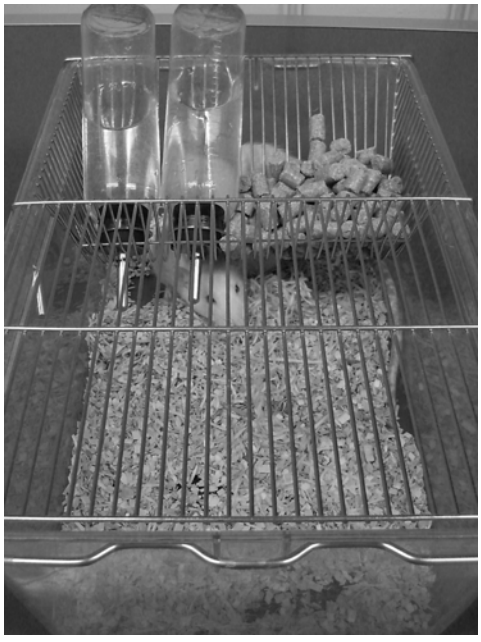

Figure 12. Voluntary ethanol intake in the two-bottle free choice paradigm.

Biological measurements

Tissue dissection and blood samples

After an alcohol washout period animals were decapitated. The thymus and adrenal glands were dissected out and blood samples were collected for radioimmunoassay analyses of plasma corticosterone levels.

Brain dissection and opioid peptide extraction

In Study 2, brain regions (anterior pituitary lobe, neurointermediate pituitary lobe, hypothalamus, frontal cortex, medial prefrontal cortex, nucleus accumbens, caudate putamen, hippocampus, amygdala, substantia nigra, ventral tegmental area, periaqueductal gray) were dissected and analyzed for opioid peptide concentrations of dynorphin B (DYNB) and Met-enkephalin-Arg^{6}Phe^{7} (MEAP) using radioimmunoassay.

Brain dissection and monoamine extraction

In Study 3 and 4, brain areas were taken out for analysis of monoamine content: basal forebrain (medial frontal cortex, nucleus accumbens, olfactory tubercle, septum), dorsal striatum (caudate putamen), hippocampus, amygdala and remaining cortical tissue. The tissues were analyzed for noradrenaline (NA), dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) with high-pressure liquid chromatography with electrochemical detection (HPLC-ED).

Statistics

Due to skewed behavioral data in combination with few animals, non parametric statistcal analyses were used in Study 2, that is, Mann-Whitney *U*-test and Wilcoxon matched-pairs signed-ranks test. In Study 1, 3 and 4 the number of animals was enough to justify parametric statistical analyses, and Student's unpaired *t*-test and ANOVA were used. In Study 3 and 4, the level of significance was adjusted due to multiple comparisons (Hochberg, 1988). Data analyzed with non parametric tests are presented as median ± median absolute deviation (MAD; i.e. the median of the set of differences between each data point and the median of the data), and data analyzed with parametric tests are presented as mean ± SD.

RESULTS

Maternal retrieval of pups

Results of the dams' retrieval behavior of the pups (Study 2 and 3) revealed that on day 6, after the separation procedure, the MS/ED dams spent significantly more time in the nest with pups, compared to control dams ($p < 0.02$ and $p < 0.05$, respectively).

Postnatal development (days 1-25)

For the statistical analysis of the body weights, only litters containing 8 pups were included. In Study 2, group x age interactions were found: MS male offspring (at trend level) and MS females significantly ($p < 0.02$) increased their body weight more over time than their respective controls. In Study 4, ED_{Room} pups had a significantly lower body weight ($p < 0.01$), and the rate of weight gain was slower compared to controls ($p < 0.01$). No significant differences were seen in ED_{Inc} animals compared to controls in Study 3. In adult animals, there were no significant differences in body weight between the experimental and control groups (Study 2-4).

In young pups (Study 2), air righting and fleeing and freezing responses did not reveal any significant differences between MS offspring and controls (either in males or females). Nor were there any significant differences in corticosterone levels on day 25 among these groups.

Adult behavior, endocrinology and neurochemistry

All MS and control females were included in the statistical analyses, since there was no effect of behavioral estrous on any of the tested variables (Study 1 and 2).

Maternal separation (Study 1 and 2)

Maternally separated adult males and females (separated days 2-15, 3h/day as litters in incubator) did not show any significant differences relative to their respective controls in the risk assessment test or in the exploration test (Study 1). As seen in Figure 13, MS females had a lower intake of ethanol relative to control females on day 1 and 2, although total intake for one week was not significantly affected. The other variables (i.e. ethanol preference, water intake and total fluid intake) between the female control and MS animals were not significantly affected. In males, none of the measurements in voluntary ethanol intake were significantly different between control and MS animals during the one-week consumption period.

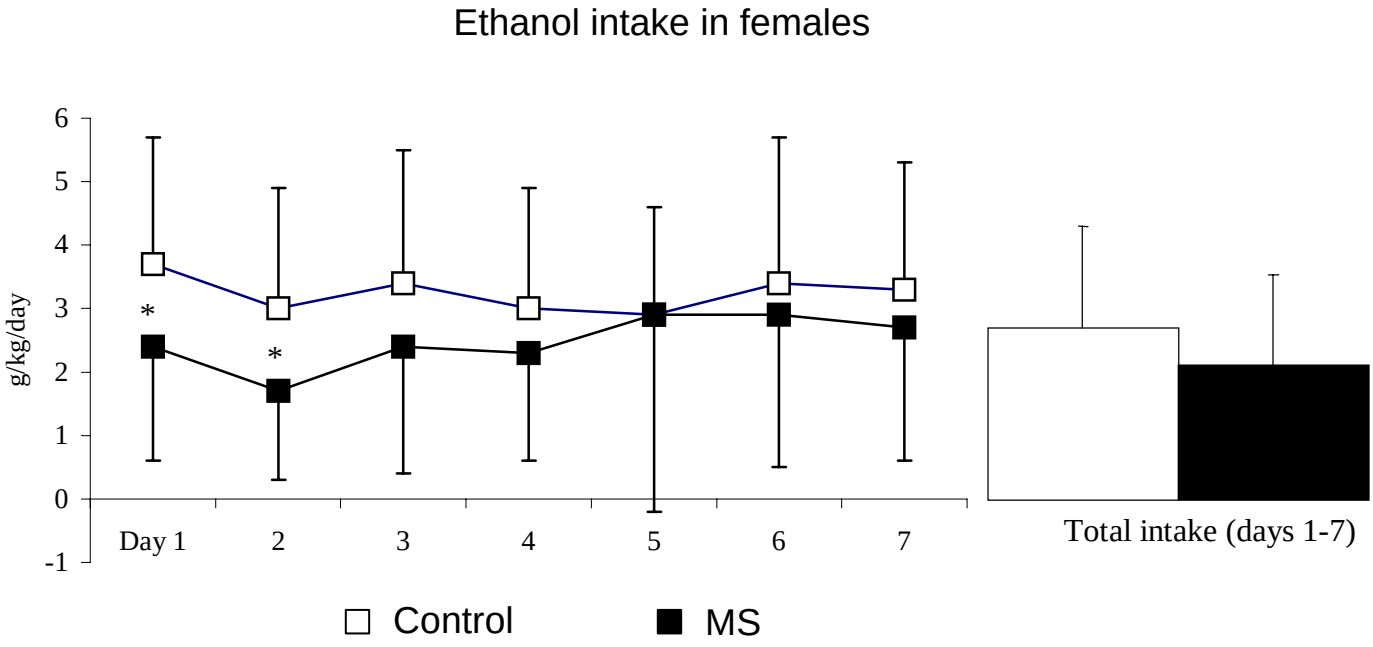

Figure 13. Mean ± SD values for ethanol intake (g/kg/day of absolute ethanol) on days 1-7 and total mean for one week in female control and maternally separated (MS) animals (n = 19-20/group). *$p < 0.05$ vs. Controls (Student's unpaired t-test).

At the time for decapitation, the weight of adrenal glands (mg/g body weight) was increased in male MS animals compared to male controls ($p < 0.01$). No differences were found in the female groups in corticosterone levels, weight of thymus and adrenal glands.

Overall, separation of pups as a litter in incubators during the separation procedure for 4 h did not produce any significant alterations in female and male emotional behavior in the adult offspring (Study 2). Biological measures showed no disruptions of the HPA-axis or the immune system and there were no alterations in the opioid peptide content in the brain in adult male and female offspring relative to their respective controls.

Early deprivation (Study 3 and 4)

When changing the housing conditions, and separating pups individually in incubators or in room temperature (i.e. ED_{Inc} and ED_{Room} animals), behavioral alterations emerged. Early deprivation in both temperature conditions caused behavioral changes in the test for exploratory behavior (Figure 14). In the second 5-min testing period, ED_{Inc} and ED_{Room} animals showed significantly more nose pokes, longer cumulative duration of nose pokes and more crossings compared to controls.

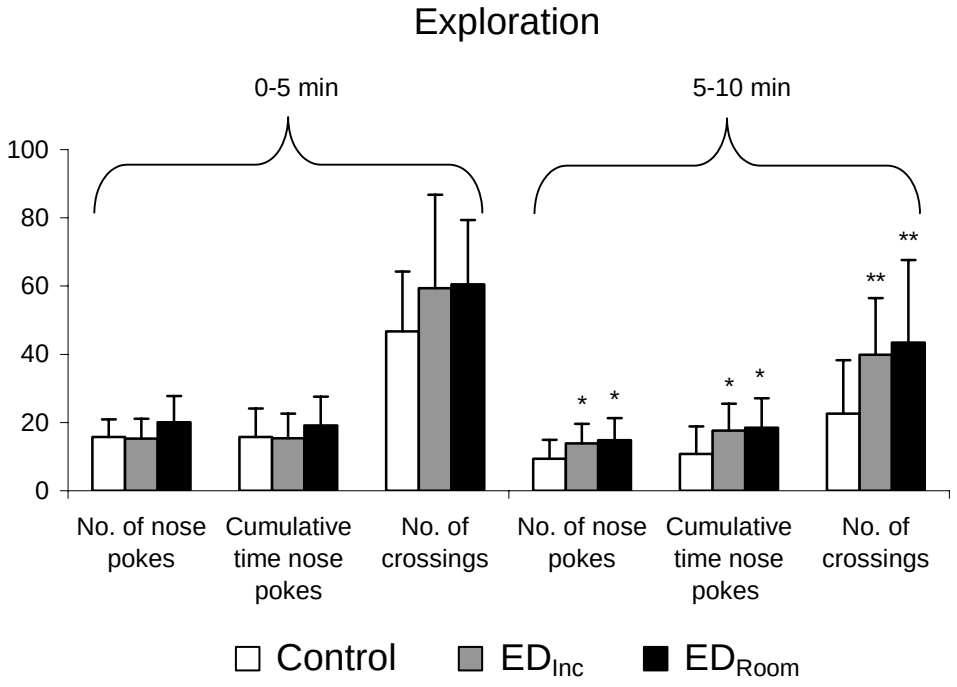

Figure 14. Mean ± SD values for exploration (the number of nose pokes and squares crossed and cumulative duration [sec] of nose pokes) in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; n = 16-20/group). The test was performed for 2 x 5 min. * $p < 0.05$ and ** $p < 0.01$ vs. Control (Student's unpaired t-test).

When early deprivation occurred in room temperature, even more behavioral alterations were observed (Study 4). ED_{Room} animals performed significantly more stretched attend postures (SAPs) in the risk assessment test ($p < 0.01$). Furthermore, when measuring ambulation for a longer period of time (i.e. 60 min), significantly increased forward locomotion was seen in ED_{Room} animals compared to controls (Figure 15). The significantly increased locomotion in ED_{Room} animals was already present for the first 15 (data not shown) and 30 min of the test.

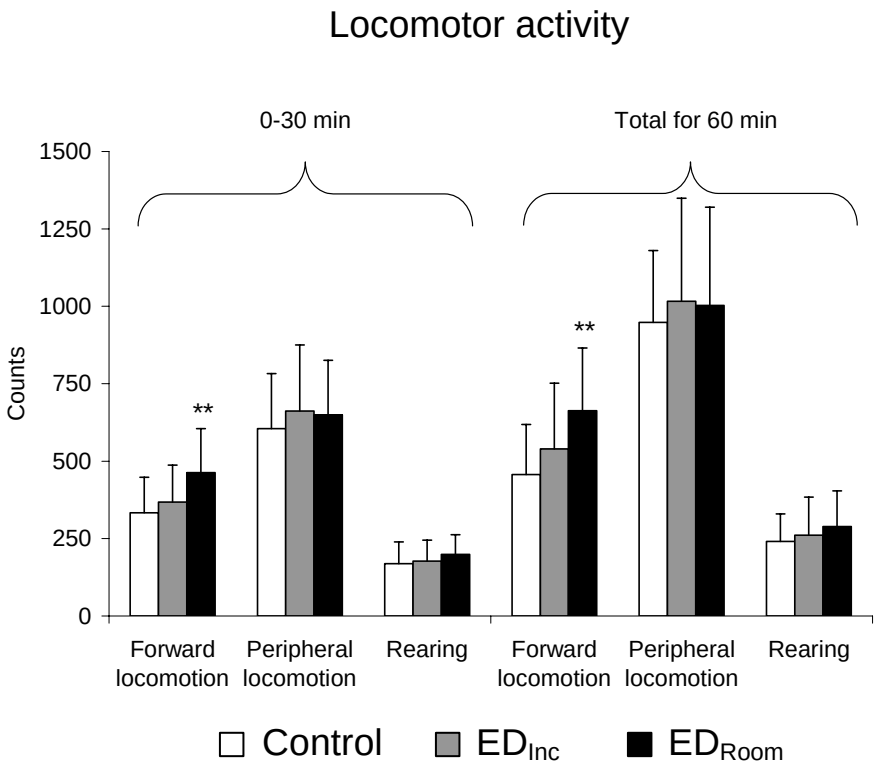

Figure 15. Mean ± SD values for locomotor activity counts (1 h) in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; n = 18-20/group). ** $p < 0.01$ vs. Control (Student's unpaired t-test).

Behaviors in response to a sudden auditory signal (i.e. the fleeing and freezing test) revealed no significant differences between the groups. The animals' voluntary alcohol intake did not significantly differ between ED_{Inc} /ED_{Room} and control animals.

Analyses of the biological variables did not differ among the groups in Study 3 and 4. Thus, the measures of possible HPA-axis and immune system disruptions (corticosterone levels, weight of thymus and adrenal glands) or changes in monoamines (DA, NA, 5-HT) or their metabolites did not reveal any significant differences between ED_{Inc} /ED_{Room} and control animals.

DISCUSSION

When repeatedly separating rat pups from the dam as intact litters in incubators (Study 1 and 2), few alterations in behavior or biology were detected in adult male and female Wistar rat offspring, although adrenal glands were enlarged in MS male offspring (Study 1; this issue is further discussed in Light/dark cycle-section). However, when separations occurred in isolation from littermates (i.e. ED; Study 3 and 4), the manipulation produced long-lasting behavioral effects in the offspring. The ED pups showed decreased emotionality, reflected in enhanced locomotion and exploratory behavior in several behavioral tests. Furthermore, this characteristic was even more pronounced when a temperature challenge during the individual separation was assessed (i.e. isolation in room temperature).

The results of our different separation techniques on postnatal and adult body weight are mainly in line with the literature (reviewed in Lehmann & Feldon, 2000). Pups that have been separated in isolation or separated in intact litters in room temperature have been shown to reduce body weight at an early age. These animals usually manage to catch up with control animals as adults (Lehmann & Feldon, 2000; Zimmerberg & Shartrand, 1992).

Anxiety, hyperactivity and novelty seeking behaviors are suggested to play an important role in the development of vulnerability to drugs of abuse in animals (Bardo, Donohew, & Harrington, 1996; Hoshaw & Lewis, 2001; Paré et al., 1999; Piazza, Deminière, Le Moal, & Simon, 1989; Piazza & Le Moal, 1998; Pohorecky, 1990; Poulos, Le, & Parker, 1995). Within the MS paradigm, Huot et al. (2001) have reported an increased alcohol preference along with enhanced anxiety in MS animals. Increased alcohol intake in MS animals has also been reported by other researchers (Jaworski et al., 2005; Ploj et al., 2003a; Roman et al., 2005). Alterations in amphetamine-induced activity have been reported, suggesting a changed sensitivity to the drug in MS animals (Zimmerberg & Shartrand, 1992). None of our separation protocols elicited any changes in alcohol intake or behavioral changes in relation to amphetamine in these directions, rather a lower ethanol intake was initially observed in female MS animals in Study 1. However, methodological factors differ between the studies and may have contributed to the results. For example, sweetened alcohol fluid (Huot et al., 2001; Jaworski et al.,

2005) and restraint stress (Ploj et al., 2003a) during the alcohol testing period have been used, as well as a strain of alcohol-preferring rats (Roman et al., 2005). Thus, it is possible that the results in the present thesis could have differed if animals had been exposed to stress during the alcohol consumption period.

Measurements of brain neurotransmitters were also conducted in non stressed conditions in the present thesis, and this may have affected the lack of significant results. For example, Liu et al. (2000) have shown that basal levels of noradrenaline were not affected in MS animals, but restraint stress increased noradrenaline responses in these animals. Furthermore, results of brain tissue levels of monoamines in the thesis may have revealed differentially if more specific subregions were dissected and analyzed. For example, Matthews et al. (2001) have reported changed basal levels of dopamine, noradrenaline and serotonin in MS animals when more specific brain areas were dissected than inte the present thesis (Study 3 and 4).

The MS model has been proposed to model human psychiatric disorders and to produce long-lasting behavioral and neurochemical disruptions in the rat (e.g. Biagini et al., 1998; Caldji, Francis et al., 2000; Daniels et al., 2004; Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002; Wigger & Neumann, 1999). However, these findings are not conclusive as no effect of MS, or even reduced emotionality, have also been reported (Crnic et al., 1981; Estanislau & Morato, 2005; Kaneko et al., 1994; Lehmann, Stöhr, & Feldon, 2000; Lehmann et al., 1998; Marin & Planeta, 2004; Matthews, Hall et al., 1996; McIntosh et al., 1999; Ploj, Roman, & Nylander, 2002; Pryce, Bettschen, Bahr, & Feldon, 2001; Renard, Suarez, Levin, & Rivarola, 2005; Shalev & Kafkafi, 2002; Stanton, Crofton, & Lau, 1992; Suárez et al., 2004). The rat pups are protected from stressors during the first two weeks of life (SHRP, de Kloet et al., 1988; Rosenfeld et al., 1992; Sapolsky & Meaney, 1986; Vazquez, 1998). It may well be that the MS protocols used in Study 1 and 2 did not overcome this protective barrier and thereby did not cause any disruptions in stress hormones, neurotransmitters or behavior in the offspring. The quality of maternal care also seems to be an important protective factor in the developing pup (Caldji, Diorio et al., 2000; Caldji et al., 1998; Francis & Meaney, 1999; Liu et al., 1997). The study of maternal retrieval behavior in the present studies was, however, not sufficient to conclude if any permanent alterations were induced by MS (see Maternal behavior-section for further

discussion). In addition, another possible protective factor has been proposed by Greisen and colleagues (2005). The protein brain-derived neurotrophic factor (BDNF) is widely distrubuted in the CSN and plays a critical role during brain development and is required for the survival of neurons (Cirulli, Berry, & Alleva, 2003; Rang, Dale, & Ritter, 1999). While reporting no effect of MS on anxiety, Greisen and colleagues noted increased levels of BDNF in the hippocampus in MS animals compared with a handled control group. They propose this to be a mechanism in MS animals to compensate for stress-induced decreases in neurogenesis.

The major finding in Study 3 and 4 was that maternally separated pups showed decreased anxiety in adulthood compared to controls, which also has been found by other researchers (Kaneko et al., 1994; McIntosh et al., 1999; Ploj et al., 2002; Suárez et al., 2004). Thus, under certain conditions the postnatal MS experience does not seem to be followed by negative consequences or it may even lead to behaviors that could reflect enhanced ability to cope with novelty and reduced vulnerability to stressor-induced behavioral impairments. This could reflect a parallel to humans as research on humans has revealed individual differences in response to psychosocial risk factors. Relatively good psychological functioning is seen in a considerably large number of cases despite serious adverse experiences (reviewed in Rutter, 2002). Rutter proposes that research in both humans and animals should focus on why stress experiences make individuals either more resistant or more vulnerable to later psychosocial stress (steeling and sensitization phenomena). There is some evidence for the fact that milder stressors or stressors that are accompanied by successful coping tend to foster steeling, and overwhelming stress and unsuccessful coping lead to sensitization (reviewed in Rutter, 2002; Southwick, Vythilingam, & Charney, 2005). It has been proposed that enhancing children's self-esteem and social competence may be important for prevention and treatment of emotional and behavioral problems in children from high-risk groups (Kim & Cicchetti, 2004). Furthermore, a related phenomenon is resilience, referring to the achievement of positive adaptation despite early adverse experiences (Luthar, Cicchetti, & Becker, 2000). Some personality characteristics have been identified as protective factors in maltreated children and thereby generate positive adaptation, for example, internal locus of control and high self-esteem (Cicchetti & Rogosch, 1997; Moran & Eckenrode, 1992). Cicchetti (2002)

proposes the possibility that effects of child adversities on the brain may be either pathological or adaptive, depending on if the child develops in a resilient fashion.
In relation to the results in the present thesis, the separation manipulation could be considered as not being overwhelmingly stressful for the animals. It has indeed been proposed that separations must exceed 6 h to constitute a severe experience in the rat pup (Kuhn & Schanberg, 1998). However, repeated 1 h separations have also been shown to induce alterations in serotonin and dopamine systems in juvenile rats (Kehoe, Mallinson, Bronzino, & McCormick, 2001; Kosten, Zhang, & Kehoe, 2004; McCormick, Kehoe, Mallinson, Cecchi, & Frye, 2002), besides studies already mentioned (usually with 3 h-separations). In addition, irregularly repeated separation periods (which may constitute unpredictable stress) may be a more severe experience in the rat. It is also evident that although exposure to child maltreatment has been found to have negative effects on the HPA-axis, all individuals do not develop psychiatric disorders, suggesting that vulnerable individuals (e.g. genetically and/or exposed to additional trauma in adulthood) may have an increased risk for psychopathology (Nemeroff, 1996; Shea et al., 2004). When considering this model for psychopathology in humans, the genetic origin of the rat and the use of additional stressors in adulthood may be of importance when conducting MS studies (these issues are further discussed later in the thesis).

Maternal behavior

The quality of maternal care seems to be important for neurobehavioral development in rat pups (Caldji, Diorio et al., 2000; Caldji et al., 1998; Francis & Meaney, 1999; Liu et al., 1997). Maternal care is proposed to be negatively disrupted in dams of separated animals and therefore may, at least partly, mediate the neurobehavioral changes in MS offspring (Huot, Gonzalez, Ladd, Thrivikraman, & Plotsky, 2004; Huot et al., 2000; cited in Ladd et al., 2000). However, no changes or even increased maternal care in MS dams have also been reported (Macrí, Mason, & Wurbel, 2004; Pryce, Bettschen, & Feldon, 2001; Rüedi-Bettschen, Feldon, & Pryce, 2004a; Zimmerberg, Rosenthal, & Stark, 2003). Unfortunately, reports of maternal care behavior in MS experiments are still very few and further research on this topic is needed to understand the possible maternal effects on the offspring.

Although we only observed the dams' behavior toward pups for a relatively short period of time (15 min) at each test occasion in Study 2 and 3, and no measures of specific maternal behaviors were conducted (such as licking/grooming and arched-back nursing), no negative disruptions in retrieval behavior and time spent in the nest were observed in the experimental dams. However, it has to be noted that during the separation period MS dams did not have any opportunity to feed any pups, and that the ensuing engorgement of the mammary glands may have affected her behavior towards pups upon reunion. Hypothetically, there is a possibility that dams of separated pups in the present thesis performed maternal care that counteracted the effect of the separation episode, which in turn may have reduced behavioral fearfulness in separated animals in adulthood. This may have occurred by increased maternal care in MS dams, for example, due to receiving relatively cold pups to the home cage after the separation procedure (especially in Study 4). Prolonged maternal attention towards chilled pups has been reported in other studies (Leon, Croskerry, & Smith, 1978; Rüedi-Bettschen et al., 2004a; Stern & Johnson, 1990). On the other hand, it has also been reported that MS pups, who received more maternal care relative to control pups, still showed deficits in their adult behavior, as seen in a reduced motivation to obtain sucrose reinforcement (Rüedi-Bettschen et al., 2004a; Rüedi-Bettschen, Pedersen, Feldon, & Pryce, 2005). Macrí et al. (2004), have also studied the impact of maternal care as a mediating factor in MS studies. In that study, both MS and handling manipulation induced elevated levels of maternal care compared to non-handled animals, but handling resulted in reduced HPA-axis and fear responses in adult offspring compared to non-handling, while MS and non-handling did not differ. The authors conclude that this indicates that maternal care cannot be the only mediator of these effects.

Different methods within the MS paradigm

The variety of results in the MS field has led attention to the methods used in MS experiments. When using repeated periods of separations (as in the present thesis), several different separation techniques within the paradigm have been used and may account for some of the variation in results. For example, duration, timing and number of separations during early development differ among studies, and the effects of MS seem to be dependent on those factors (reviewed in Gutman &

Nemeroff, 2002; Lehmann & Feldon, 2000; Pryce & Feldon, 2003; and Pryce et al., 2002). Other methodological factors varying in MS studies, supposedly affecting the results are, for example, the ambient temperature during the separation procedure (i.e. separation in a "nest-like" warm environment or separation in room temperature), access to tactile and olfactory stimulation during the separation procedure (i.e. separation of intact litters or separation in isolation), the strain of rat used and the choice of control group (Avishai-Ephner, Yi, Newth, & Baram, 1995; Ellenbroek & Cools, 2000; Lehmann & Feldon, 2000; Zimmerberg & Shartrand, 1992). Since the effects of these factors have not thoroughly been investigated within the MS paradigm, all the various methodologies used make it harder to survey and compare results across studies. These proposed influential factors within the paradigm are discussed below.

Duration, timing and number of separations

The MS animals experience different procedures in duration, timing and number of separations, which may influence the consequences of MS (reviewed in Gutman & Nemeroff, 2002; Lehmann & Feldon, 2000; Pryce & Feldon, 2003; and Pryce et al., 2002). For example, in repeated separations, the number of separations ranges from 5 to 28 and the duration of separation ranges from 1 to 12 h and the separation period are executed at varying postnatal days. Although the separation protocol used in Study 4 (i.e. isolation for 4 h/day in room temperature for 15 days) could be considered as a severe experience for the pups relative to the MS protocols used in the field, it is possible that longer duration of separation or unpredictable separation days may cause more severe consequences in the offspring. It is known that the rat dam normally leaves the nest several times a day for a period of time, and it is proposed that separations must exceed 6 h to constitute a severe experience in the pups, which they normally do not experience in nature (Kuhn & Schanberg, 1998). It has also been shown that continuous separation from both dam and littermates during postnatal days 4-12 (i.e. pups experiencing total absence from dam and litter contact and artificially reared during this period) did not produce any severe alterations in anxiety in adult rats (Kaneko, Riley, & Ehlers, 1996). However, it must be noted that this result may have been affected by the fact that these animals underwent surgery, as the artificial feeding of pups required a feeding tube and electrophysiological recordings in adulthood required electrodes.

The separation manipulation within the MS-paradigm occurs before weaning (before day 28), during a period when rat pups are protected from stressors (SHRP). However, results from isolating offspring after weaning-age (called isolation rearing) indicate changes in, for example, anxiety, learning, alcohol intake, HPA-axis and central neurotransmitter levels in adult animals (Hall, 1998). Isolation rearing is proposed to produce animals which could be characterized as pathological and may model aspects of human psychopathologies (Hall, 1998). Thus, it is possible that the age for separation manipulation could be a critical factor for developmental impairments. This could reflect human conditions, as it has been shown that the earlier the age at which severely deprived children were adopted from institutional care, the better their physical growth and cognitive function were found to be at a later age (O'Connor et al., 2000; Rutter & the English and Romanian Adoptees (ERA) Study Team, 1998).

Housing and ambient temperature during separation

The present thesis used four different protocols when assessing the separation manipulation in pups. In Study 1 and 2, separated animals were housed as litters and kept for either 3 or 4 h in incubators. In Study 3 and 4 separated animals were housed isolated from littermates and either kept in incubators or in room temperature for 4 h. Other factors were (more or less) held constant across these experiments, for example duration, timing and number of separations, reversed light/dark cycle, briefly handled controls, rat strain, animal husbandry procedures and some of the behavioral tests. It seems as if housing condition (as litters or in isolation) and ambient temperature during the separation manipulation may have affected the outcome of our results. Pups isolated (e.g. without tactile stimulation) in room temperature during the separations were the most behaviorally affected (ED_{Room} animals; Study 4). A study by Mintz, Rüedi-Bettschen, Feldon and Pryce (2005), also revealed that ambient temperature during separation might be of importance, since reduced social motivation was found in MS animals kept in room temperature, but not in MS animals kept in warmth. The importance of tactile stimulation at early ages to recovery from hippocampal damage has been reported by Rodrigues et al. (2004). Interestingly, that study reveals that MS (1 h during days 8-21) also managed to recover from the brain damage when measured at 3 months of age.

Light/dark cycle

The majority of MS studies are conducted with a normal light/dark cycle (i.e. lights on during day time). In our laboratory, a reversed cycle was used, and the separation manipulation and behavioral testing were conducted during the dark phase of the light-dark cycle (i.e. the normal period of wakefulness in the rat). Besides the differences in rats' activity level during the day and night, it has been shown that lactating females spend the greatest amount of time with the litters in the light phase, and least amount of time during the dark phase (Ader & Grota, 1970). Thus, it is possible that separations during the light period may constitute a more severe experience in the developing pup. Investigations conducted during the dark period by Pryce and colleagues revealed that both early separation and handling manipulation led to decreased emotional reactivity relative to non-handled animals (Pryce, Bettschen, Bahr et al., 2001; reviewed in Pryce & Feldon, 2003). However, the same research group has recently examined separated pups' adult behavior in both light and dark conditions (Rüedi-Bettschen et al., 2005). Separations performed during the light phase did not cause any behavioral or endocrine effects relative to non-handled animals in adulthood when assessing behavioral tests reflecting helplessness, fear/anxiety and anhedonia and measurements of basal and stress-induced ACTH and corticosterone levels. Separations performed during the dark phase, on the other hand, led to decreased motivation to obtain sucrose reward (reflecting anhedonia) but no effects on fear/anxiety and no learning deficit in the test for helplessness-like behavior or stress hormone levels. The authors conclude that separations performed during the dark phase and at room temperature are the most relevant postnatal treatments when developing a model for investigating impaired affective systems (Rüedi-Bettschen et al., 2005).

The light/dark phases may also be considered when comparing results of measurements of the HPA-axis mediators in the rat since it has been shown that a higher increase of corticosterone in response to stress occurs when the hormone is at its lower circadian level, and the minimum responses occurs at the peak level (Retana-Marquez et al., 2003). In that study, stressors induced different effects on plasma corticosterone levels depending on the stressor and the time of the day at which the stressor was applied, suggesting that the HPA-axis is more sensitive to stressors in the light phase than in the dark phase in nocturnal animals. It has also been shown that measurements of basal hormone levels may not differentiate

groups in MS studies, but when a stressor (e.g. restraint stress or air-puff startle stress) was added before measurements, hormone levels were higher in MS animals (Ladd et al., 2004; Ladd, Thrivikraman, Huot, & Plotsky, 2005; Plotsky et al., 2005). These facts may have had an impact on the present results (no differences in corticosterone levels among the groups), as basal levels of corticosterone were measured in the dark phase and in non stressful conditions. However, the MS manipulation did affect the weight of adrenal glands in male offspring in Study 1, implying that the HPA-axis in MS animals might have been affected, although this was not reflected in basal corticosterone levels. Furthermore, conducting repeated measures of corticosterone might be advantageous for determining the circadian rhythm of the hormone. If using stressors before measurements, the difference between stress-induced and baseline levels of hormones could also be valuable to calculate.

Testing apparatus

When measuring emotionality in rats, several different models have been used in MS investigations. However, the components of anxiety-related behavior assessed by these models have been described as beeing poorly defined and different models may cover different aspects of anxiety (Archer, 1973; File, 1992; Ramos, Berton, Mormede, & Chaouloff, 1997; Walsh & Cummins, 1976; Yilmazer-Hanke, Wigger, Linke, Landgraf, & Schwegler, 2004). It has for example been proposed that two distinct forms of fear seems to be generated in the elevated T-maze (Graeff, Guimaraes, De Andrade, & Deakin, 1996). The T-maze apparatus is derived from the widely used elevated plus-maze, and is suggested to generate both conditioned and unconditioned fear in the same rat (i.e. both learned and innate fear). The often used open field test is thought to induce moderate anxiety by confronting rodents with a novel environment with no possibility to escape. By contrast, anxiety is reduced if the open field apparatus provides a safe enclosure for the rat (e.g. the two-compartment exploratory test), and therefore is proposed to predominantly reflect exploratory behavior (Dulawa, Grandy, Low, Paulus, & Geyer, 1999).

Results of several studies have clearly shown that animals' behavior is affected by minor changes in the testing apparatus (e.g. circular or square testing arena and illumination), as well as by species, strain and sex (reviewed in Archer, 1973; Boissy, 1995; Lister, 1991; Shekhar et al., 2001; and Walsh & Cummins, 1976).

For example, high levels of illumination are associated with diminished locomotor activity (Walsh & Cummins, 1976). Research indicates that no single measure or model is sufficient to identify emotionality or anxiety in animals, and it is suggested that several behavioral measures from each test are observed, as well as conducting more than one behavioral test (File, 1992; Walsh & Cummins, 1976). Although animals in the present thesis were tested in several behavioral tests for emotionality, they were repeatedly used. This may have influenced their behavioral outcome as behavioral reactions decrease with repeated exposure to a previously novel environment (Boissy, 1995; Lister, 1991; Walsh & Cummins, 1976). Trying to overcome this possible influential factor in the present investigations, behavioral tests were conducted with ~10 days between each test and the animals were naive for each test situation (except for the fleeing and freezing responses in Study 2). Other improvements when recording data in animal studies could be by assessing measurements with observers who are not aware of the animals' history of treatment, by using video recording and the use of soundproof testing chambers to avoid possible human interference.

Strain of rat

It has been proposed that certain rat strains (i.e. Lewis and Spontaneous Hypertensive Rats) would be suitable for studying neurobiological mechanisms underlying differences in anxiety (Ramos et al., 1997). Furthermore, it has been suggested that the Wistar Kyoto rat strain represents a useful animal model for depressive behaviors (Paré, 2000). In MS experiments, the choice of rat strain has also proven to be an influential factor and contradictory behavioral results have been reported depending on the rat strain used. When using the Fischer strain of rats, early separations led to a predisposition for high levels of helplessness-like behavior (expressed as escape/avoidance deficit), which is proposed to model a depression-like state (Rüedi-Bettschen, Feldon & Pryce, 2004b). This finding was, however, not present when using the Wistar strain of rats (Rüedi-Bettschen et al., 2005), the strain used in the present thesis. Furthermore, an anhedonia-like trait was found in early separated animals of the Wistar strain (Rüedi-Bettschen et al., 2005), but this was not replicated when using Fischer rats (reference to submitted manuscript in Rüedi-Bettschen et al., 2004b). The importance of strain has also been pointed out by other authors (Ellenbroek & Cools, 2000; Neumann et al., 2005), who have reported different long-term effects of MS depending on the rat strain (Wistar, Fischer and Lewis respective hyper- or hypo-anxiety Wistar were

examined). Research has shown that even sublines of the same rat strain (i.e. the use of different vendors) may represent an important source of variability in stress reactivity (Paré & Kluczynski, 1997).

Choice of control group

In addition to different manipulations of the experimental group, several different control groups (or comparison groups) have been used in the MS literature. The most widely used controls are animals that are left completely undisturbed with their dams until the MS period is executed (i.e. a non-handled group; NH). A second type of control group comprises animals that are left undisturbed in the maternal cage, except for normal cage changing and refilling of food and water (animal facility rearing; AFR). Usually one of these two groups is selected along with an additional comparison group, an early handled group (H), which consist of ~ 20 min daily maternal separations during the MS period. There is a discussion in the literature about choosing the most appropriate control group when investigating the effects of MS. Since both NH and H groups could be considered to constitute experimental manipulations, it is suggested that the AFR group could be an appropriate control (reviewed in Lehmann & Feldon, 2000; and Pryce & Feldon, 2003). Choosing the AFR group is, however, not without shortcomings, as husbandry procedures vary among laboratories (Pryce & Feldon, 2003). The NH group has also been proposed to be an ultimate reference group in experiments of early experience (Denenberg, 1977). In the case of MS experiments, addition of this group to MS and H groups could reveal possible effects of the human-pup contact in MS studies. In addition, it has been argued that manipulations of the rat (other than normal care) yields rats that are more similar to those in nature, indicating that *"…normal laboratory animals do not receive sufficiently varied stimulation during infancy to allow them to respond to adult stress situations in the most adaptive manner"* (Levine & Mullins, 1971, p. 173). This must be considered when comparing results across MS studies, since both AFR and/or NH groups are usually used.

Since the MS manipulation includes human-pup contact, we have chosen a briefly handled group as a comparison group in the present investigations, which would control for the effects of unavoidable handling of MS animals occurring during the course of the experiments. The possible effect of daily human-pup contact is not taken into account when using NH or AFR groups as controls in MS studies,

although the AFR group experience human contact during cage changing (usually two times/week). The briefly handled control group is, however, not commonly used in the MS field, and proper comparisons of results become more difficult for several reasons. Firstly, when finding a similar control group in the literature, other factors still differ between studies (e.g. rat strain, light/dark cycle and access to tactile and olfactory stimulation during separation). The ability to compare MS results is, however, a general difficulty in the MS field since many diverse protocols are used. Secondly, if trying to compare results across studies when using the briefly handled group, it is hard to determine which group it resembles the most, the H, AFR or NH group. Considering the briefly handled control group as corresponding to the H group in other laboratories or, alternatively, an AFR group, one would expect it to yield neurobehavioral differences relative to MS groups, although results are not conclusive (Caldji, Francis et al., 2000; Daniels et al., 2004; Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002; Ladd et al., 2000; Wigger & Neumann, 1999). The corresponding group to briefly handled animals has, however, not yet been sufficiently investigated (Lehmann & Feldon, 2000).

Comparison of MS studies using the briefly handled control group

For reasons mentioned above it is difficult to compare results within the MS field. One factor that limits our comparison of results with other MS studies is the choice of control group. As mentioned earlier we have used a briefly handled (i.e. 3-5 min separation/handling during the MS period) control group, whereas most other studies have used NH or AFR groups as controls. There are, however, studies that have used a similar control group to ours which make comparison of results easier in this respect (Table 2). Studies included in the table are those conducting behavioral tests of emotionality and sensitivity to drugs in MS relative to briefly handled male rats.

Various results are presented in the MS field, and even when strictly comparing our results only with studies using a similar control group as ours, the results are still far from conclusive. MS animals have exihibited both significantly enhanced

anxiety (Madruga et al., 2005; Matthews, Wilkinson et al., 1996; von Hoersten et al., 1993), less anxiety (Study 3 and 4), or no effect of MS on anxiety (Kaneko et al., 1994; Matthews, Hall et al., 1996; Matthews, Wilkinson et al., 1996; von Hoersten et al., 1993; Zimmerberg & Shartrand, 1992 and Study 1 and 2, and partly in Study 3 and 4). Animals' sensitivity to amphetamine have also differed among these studies, both significant differences and no differences among the groups have been reported (Matthews, Hall et al., 1996; Zimmerberg & Shartrand, 1992 and Study 2). When significant differences between MS and briefly handled controls were found in amphetamine responses, the effect of the drug seemed to be dependent on pups' ambient temperature during separation, as MS pups kept in incubators were less active, and pups kept in room temperature were more active than controls (Zimmerberg & Shartrand, 1992).

As seen in table 2, few studies have found significantly enhanced anxiety-related behaviors in MS animals in relation to briefly handled controls. Six out of ~50 measured variables were significantly negatively affected by MS, and about the same number of results have been found in the opposite direction. In most of the variables there were no significant effects of MS, although effect sizes were relatively high in some cases. Possible explanations for the absence of significant results in the presence of high effect sizes could be that the number of animals was low and/or high variance. Furthermore, methodological factors that may be of relevance for the outcome of MS investigations have differed among these studies. For example, the duration of separation ranges from 2-6 h and number of separations ranges from 10-28. Also the choice of rat strain, conducting experiments during the light or dark phase and housing condition during separation (in litters or in isolation) has differed among these studies. In addition, although keeping pups in intact litters, von Hoersten et al. (1993) differed in that they removed the dam from the home cage during the separation period, instead of removing the pups, as usually are done. Moreover, although normally conducting tests in the light period, some of the tests were performed during the rats' dark phase in Kaneko et al. (1994) and von Hoersten et al. (1993). Besides different testing equipment used in the studies, the animals' habituation to the test situation and prior experience have differed (e.g. surgery and possible prenatal stress due to transportation of pregnant dams).

Table 2. *Summary of studies using briefly handled animals (i.e. daily 3-5 min separation/handling during the separation period) as controls in MS studies. Results of behavioral tests of emotionality and drug response in male animals are included in the table. The "MS manipulation" column consists of information on the duration, timing and number of separations, housing conditions during the separation and if experiments were conducted during the animals' light or dark phase. The effect sizes are based on mean differences between MS and control animals and the SD for controls*[1].

Study	MS manipulation	Rat strain Animals/ group	Test for emotionality/ drug response	Signi-ficant result	Direction for emotionality/ drug response	Effect size
Kaneko et al., 1994	6 h days 2-6 and 9-13 Litters in incubator Light phase	Sprague-Dawley *n* = 10	• Open field (5 min) • Activity boxes *crossings (12 h)* • Elevated plus maze (5 min)	No No No		
Madruga et al. 2005	3 h days 1-10 Litters in incubator Dark phase	Wistar *n* = 14-16	• Open field (5 min) *crossings* *entries to center arena* *time exploring center arena*	 Yes Yes Yes	 More in MS More in MS More in MS	 1.1 0.9 0.9
Matthews, Wilkinson et al., 1996	6 h, 10 occasions during days 5-20 Litters in incubator Light phase	Lister-Hooded *n* = 8	• Food reward • Activity boxes *crossings (10 min)* *crossings (30 min)* • Sucrose preference	No Yes No No	 More in MS More in MS Less in MS	 1.4 0.1 1.8
Matthews, Hall et al., 1996	6 h, 10 occasions during days 5-20 Litters in incubator Light phase	Lister-Hooded *n* = 7 - 8	• Activity boxes (2 x 120 min) *crossings (basal)* *with amphetamine* • Activity boxes (4 x 90 min) *basal with reduced food intake*	 No No No		
Matthews et al., 1999	6 h, 10 occasions during days 5-20 Litters in incubator Light phase	Lister-Hooded *n* = 15	• Cocaine self-administration *several doses and test sessions*	 No		
von Hoersten et al., 1993	2 h days 1-28 Litters in incubator Light phase	Wistar *n* = 11	• Open field (3 x 3 min) *crossings* *exploring center arena* *rearing* • Two compartment exploratory test (3 min) • Freezing	 No Yes No Yes No	 More in MS More in MS More in MS More in MS More in MS	 0.2 - 0.5 0.5 - 0.7 0.8 - 1.0 1.5
Zimmerberg and Shartrand, 1992	6 h days 2-15 a) Isolated in incubator Light phase	Long-Evans *n* = 14	• Activity boxes *crossings (basal; 15 min)* *with amphetamine (45 min)*	 No Yes	 MS less active	
	b) Isolated in room temperature Light phase		• Activity boxes *crossings (basal; 15 min)* *with amphetamine (45 min)*	 No Yes	 MS more active	

Table 2. Continued.

Study	MS manipulation	Rat strain Animals/ group	Test for emotionality/ drug response	Signi-ficant result	Direction for emotionality/ drug response	Effect size
Study 1	3 h days 2-15 Litters in incubator Dark phase	Wistar ***n*** **=** 10-20	• Risk assessment (10 min)			
			SAP	No	More in MS	0.2
			Crossings	No	More in MS	0.4
			Exposed zone	No	More in MS	0.2
			Defecation	No	Less in MS	0.3
			• Hole-board exploration (5 min)			
			nose pokes	No	More in MS	0.1
			duration nose pokes	No	Less in MS	0.1
			crossings	No	More in MS	0.6
			• Alcohol preference (1 week)	No	Less in MS	0.1
Study 2	4 h days 1-15 Litters in incubator Dark phase	Wistar *n* = 9 - 10	• Hole-board exploration (5 min)			
			nose pokes	No	More in MS	0.3
			duration nose pokes	No	More in MS	0.3
			crossings	No	Less in MS	0.5
			• Freezing	No	Less in MS	0.0
			• Activity boxes (60 min)			
			basal activity	No	More in MS	0.1 - 0.4
			with amphetamine	No	MS more active	0.1 - 0.4
			• Competition test (4 min)	No	More in MS	1.1
			• Alcohol preference			
			week 1	No	More in MS	1.2
			week 2	No	More in MS	0.1
			week 3	No	Less in MS	0.2
Study 3	4 h days 1-15 Isolated in incubator Dark phase	Wistar *n* = 16-20	• Hole-board exploration			
			first 5 min	No	Less in MS	0.1 - 0.7
			second 5 min	Yes	Less in MS	0.8 - 1.1
			• Risk assessment (10 min)			
			SAP	No	Less in MS	0.6
			Crossings	No	More in MS	0.1
			Exposed zone	No	Less in MS	0.0
			• Freezing	No	More in MS	0.8
			• Activity boxes (60 min)	No	Less in MS	0.2 - 0.5
			• Alcohol preference	No	Less in MS	0.2 - 0.3
Study 4	4 h days 1-15 Isolated in room temperature Dark phase	Wistar *n* = 16-20	• Hole-board exploration			
			first 5 min	No	Less in MS	0.4 - 0.8
			second 5 min	Yes	Less in MS	1.0 - 1.3
			• Risk assessment (10 min)			
			SAP	Yes	Less in MS	1.4
			Crossings	No	Less in MS	0.7
			Exposed zone	No	Less in MS	1.0
			• Freezing	No	More in MS	1.0
			• Activity boxes (60 min)			
			Forward locomotion	Yes	Less in MS	1.3
			Peripheral locomotion	No	Less in MS	0.2
			Rearing	No	Less in MS	0.5
			• Alcohol preference	No	Less in MS	0.1 - 0.2

[1]Effect size; rules of thumb by Cohen (1988): 0.20 is a small effect, 0.50 a medium effect and 0.80 a large effect.

With no doubt, the MS field has become very complex, and there are few (if any) identical MS techniques used in the laboratories. Additional considerations to take into account when comparing results and designing MS studies are, for example, possible confounding effects of prenatal stress (e.g. transportation of pregnant dams), litter effects (siblings are treated as independent observations), effects of litter size, gender composition of the litter and the pups' age at weaning (Denenberg, 1977; Gray & Lalljee, 1974; Lehmann et al., 2000; Novakova, 1966; Spear & File, 1996). Furthermore, pups are usually not brought up in the same family group (as in the present thesis), instead they are cross-fostered. That is, all pups are delivered on the same day and are randomly assigned to different rearing conditions.

With regard to the various results obtained in the MS field, the effects of environmental manipulation in the form of MS might be sensitive to slight differences in the methods used. As a consequence, results may have low external validity. That is, results in one study are hard to replicate in other MS experimental settings (Mitchell & Jolley, 1996). Although animal models are useful for comparisons with human conditions, a low degree of ability to generalize within the MS paradigm in rats, or within different animal species, does of course raise the question to what degree the results could be generalized to humans (Hall, 1998; Lehmann & Feldon, 2000). Variations in statistical power, that is the ability to find significant differences when differences truly exist (Mitchell & Jolley, 1996), could also be a possible explanation why effects are found in only some MS experiments. A high number and more homogenous rats in combination with more extreme manipulations imply higher power (Mitchell & Jolley, 1996). Lack of analyses of power in MS studies leaves the question of to what degree variations in power can account for lack of significant effects in some MS experiments open. One way to further investigate the results in the MS field could be by the use of meta-analytical techniques. Here several studies are aggregated and the total number of rats will become higher. As a consequence the chance to find a significant effect will increase, if there is an effect to discover (Andersson, 2003). Furthermore, multiple regression analysis could be used to explain the various results in different MS studies, for example to what degree the choice of rat strain and duration of separation are contributing factors to differences in emotional reactivity.

Although preclinical research on animals is valuable for understanding stress mechanisms in humans there are difficulties when inferring from animals to humans. For example, there are differences in the time periods of brain development between rats and humans and different research methods are used when analyzing neurobiological systems, which complicate the direct comparison of results (Heim & Nemeroff, 2001). It is also difficult to model typical stressful experiences of human children in animal models (Heim & Nemeroff, 2001). For example, although MS is proposed to be a model for child abuse or neglect (de Kloet et al., 2005; Shea et al., 2004), research in children has shown that the number and combination of risk factors is of importance for the development of psychiatric problems (Rutter & Quinton, 1984). Furthermore, it has been proposed that non-human primates could be a more suitable animal model for examining early life stress and its effects on development in humans (Heim et al., 2004; Sanchez et al., 2001).

Summary

Taken together the results in the present thesis, a battery of behavioral tests indicated that MS did not induce increased anxiety-related behavior in the adult rat offspring. On the contrary, MS seemed to produce beneficial effects in the offspring, as signs of reduced anxiety-related behaviors were observed when pups were kept in isolation. Thus, under certain conditions the MS manipulation seemed to enhance these animals' ability to cope with the novelty experienced in behavioral tests. It has also been proposed in research in humans that not all kinds of risk and stress exposures should be considered to be negative, challenges we meet may also create a positive development and an appropriate dose of risk is a necessity to develop resilience (Inger & Borge, 2005). Endocrine measurements showed a possible disruption of the HPA-axis in male MS offspring (Study 1), this finding was, however, not reflected in corticosterone levels and was not replicated in further studies.

The model for vulnerability to psychiatric illness in humans (Figure 1 in the Introduction) proposes an interaction between genetic factors and early environment. Subsequent stress exposure in adult ages may induce pathology based

on the underlying vulnerability (Heim & Nemeroff, 2001; Nemeroff, 2004). Implementing this view in MS studies could be a further development of the MS-model. Thus, early separation manipulations could be combined with later stress exposure in the adult animal. It has, for example, been reported that MS seem to induce changes in the hippocampus that remain latent until activated by stressful episodes in adulthood (Stewart, Petrie, Balfour, Matthews, & Reid, 2004). This is in line with the observed activation of the HPA-axis in MS animals, as this often is a result when assessing stressful manipulations in adulthood (Huot et al., 2001; Kalinichev, Easterling, Plotsky et al., 2002; Ladd et al., 2004; Ladd et al., 2005; Plotsky et al., 2005). However, exposure to chronic stress in adulthood has also been shown to reverse some (but not all) neuroendocrine effects of MS (Ladd et al., 2005) and to have no effect on anxiety-related behavior (Renard et al., 2005). Referring to the model for vulnerability in humans, the choice of rat strain in MS experiments may also be considered, as genetic origin is proposed to be an important factor in vulnerability to psychopathology. Furthermore, a possible protective factor in the MS model may be the quality of maternal care behaviors. It is possible that increased maternal care in MS dams may buffer the effects of the separation episodes in the offspring and further research on maternal care is much needed in MS experiments.

With respect to the MS protocols used in the present thesis, the results do not provide support for the suggestion that MS manipulations causes enhanced anxiety or disruptions in endocrinology and neurochemistry in the adult rat. These findings could reflect a parallel to human conditions as relatively good psychosocial functioning is sometimes seen despite serious adverse experiences in childhood.

SAMMANFATTNING PÅ SVENSKA
(Summary in Swedish)

Bakgrund

Den första tiden i livet genomgår hjärnan en antal betydande utvecklingsfaser, vilket innebär att individen under denna period är extra påverkbar. Såväl arv som uppväxtmiljö påverkar hjärnan, och därmed individens fortsatta utveckling. Kunskapen om de tidiga uppväxtvillkorens påverkan på människans fortsatta utveckling kommer av nödvändighet från icke-experimentella studier. Retrospektiva och longitudinella studier har visat att tidiga stressfyllda händelser och emotionella trauman är kopplat till en ökad risk för att utveckla psykisk sjukdom, t ex depression, ångest och missbruk av droger (Heim & Nemeroff, 2001; Maughan & McCarthy, 1997; Nemeroff, 2004; Rutter, 1991). Exempel på tidig stress i det här sammanhanget kan vara psykisk och fysisk försummelse, fysiska och sexuella övergrepp eller drogmissbruk hos föräldrarna. Andra former av tidig stress hos barn kan vara olyckshändelser, kronisk sjukdom, naturkatastrofer och krig. Den genetiska komponentens betydelse för utvecklande av psykisk sjukdom är i dagsläget inte helt klarlagd, men den antas förklara någonstans mellan 20 och 70 % av de psykiska sjukdomstillstånden (McGue & Christensen, 2003; Nestler et al., 2002; Sullivan et al., 2000).

Det faktum att stressrelaterade sjukdomar idag är ett stort hälsoproblem bland unga människor (Barnombudsmannen, 2005; Olsson et al., 2003) motiverar att studera sambanden mellan tidig stress och senare stressrelaterade tillstånd. Existerande studier på människa ger en bild av att det finns en stark koppling mellan tidig stress och senare psykisk sjukdom, även om orsakssambanden inte alltid är möjliga att fastställa. Av etiska skäl är det självfallet inte möjligt att utföra kontrollerade försök för att studera hur stress hos barn påverkar utvecklingen senare i livet, istället har olika prekliniska modeller (dvs. djurmodeller) utvecklats. De prekliniska studierna fyller en viktig funktion i forskningen då man har möjlighet att utföra experimentella studier och därmed kontrollera olika miljöfaktorer samt fastställa vad som orsakar vad.

Laboratorieråttan har visat sig vara en användbar experimentell modell för att undersöka hur tidig stress kan resultera i förändringar i hjärnans funktion samt ökad sårbarheten för psykisk sjukdom (Ellenbroek & Riva, 2003). En experimentell metod som används för att studera uppväxtmiljöns betydelse är maternal separation (MS). Under den första tiden i livet är ungar helt beroende av mammans omvårdnad för att överleva och få en normal utveckling. MS-modellen har sin utgångspunkt i att man separerar ungarna från mamman under den tidiga känsliga perioden, därmed kan man framkalla en otrygg uppväxtmiljö för att sedan undersöka eventuella förändringar i neurobiologi och beteende.

Inom MS-paradigmet utförs separationerna någon gång under perioden direkt efter födelsen och före avvänjningen. Det finns flera olika metoder för separationerna, det kan antingen vara en lång (24 h) eller flera kortare (1-12 h) separationer. Sammanfattningsvis har forskning visat att separationer från mamman i tidig ålder påverkar många beteende- och neurobiologiska variabler hos vuxna MS-djur (Anand & Scalzo, 2000; Daniels et al., 2004; Huot et al., 2000; Ladd et al., 2000; Matthews et al. 2001; Meaney et al. 2002; Ploj et al. , 2003b; Pryce & Feldon, 2003). När det gäller mätning av ångestlikt beteende har MS-djur visat ökad nivå i flera olika testsituationer (Ladd et al., 2000). Vidare har MS-djur visat sig ha förhöjda nivåer av stresshormoner, både basalt och i stressande situationer (Biagini et al., 1998; Daniels et al., 2004; Huot et al., 2001; Plotsky et al., 2005). Alkoholkonsumtion antas vara starkt kopplat till nivåer av stresshormoner, där höjda nivåer ökar alkoholintag och *vice versa* (Hansen et al., 1994; Pohorecky, 1990). Förutom ökade nivåer av stresshormoner hos MS-djur har ökat frivilligt intag av alkohol visats hos dessa djur (Huot et al., 2001; Jaworski et al. 2005; Ploj et al., 2003a; Roman et al., 2005). En förändrad preferens för andra droger hos MS-djur har också rapporterats, t ex kokain, morfin och amfetamin (Chretien & Gratton, 2002; Kalinichev et al., 2002; Matthews et al., 1999; Vazquez et al. 2005; Zhang et al., 2005). MS har också visat sig påverka transmittorsystem i hjärnan, bland annat ge förändringar i nivåer av opioida peptider och monoaminer (Daniels et al. 2004; Koob, 1992; Ladd et al., 2000; Matthews et al., 2001; Meaney et al., 2002).

När man separerar ungar från mamman påverkas flera faktorer. Ungarna flyttas oftast från mamman och djurrummet till ett separat rum och miljön förändras på flera sätt, de mister t ex taktil kontakt med mamman (och ibland med kullsyskon).

Andra förändringar i miljön under MS är omgivande temperatur, näringstillförsel och syn- lukt- och ljudintryck. En faktor som starkt antas bidra till ungars utveckling är vilken kvalitet på omvårdnad de får av sin mamma. Mammor som ger sin avkomma en högre kvalitet (mycket putsande och en bra position vid diande) får ungar med lägre nivå av ångestlikt beteende och stresshormonhalter (Caldji et al., 1998, Francis & Meaney, 1999). Att genomföra MS har visat sig förändra mammors vårdnadsbeteende till det sämre: dessa mammor tar längre tid på sig att samla in sin kull och de tar längre tid på sig att börja dia och putsa sina ungar (Huot et al., 2000; Liu et al., 1997). I MS-studier kan mammans omvårdnad alltså vara en kritisk faktor som helt eller delvis medierar effekterna av separationerna.

Den här avhandlingen omfattar ett antal MS studier där uppväxtmiljöns betydelse har undersöks för att kartlägga om tidig stress, i form av tidiga separationer, kan resultera i ökat ångestlikt beteende, förhöjda nivåer av stresshormoner samt ökat intag av alkohol i vuxen ålder. Även eventuella förändringar av nivåer av transmittorsubstanser (monoaminer och opioida peptider) i hjärnan har studerats.

Sammanfattning av delstudierna i avhandlingen

Metod

Separationerna har utförts under 3-4 timmar varje dag under de första 2 veckorna efter födelsen (tabell 1). I Studie 1 och 2 var ungarna samlade som kull under separationerna från mamman i en inkubator med värme. I Studie 3 var ungarna i kullen separerade från mamman och isolerade från varandra i en inkubator med värme. I Studie 4 var ungarna separerade från mamman och isolerade från varandra i rumstemperatur. För att särskilja dessa olika metoder i avhandlingen benämns separationerna i studie 1 och 2 för MS, och när man separerar ungar isolerat benämns det early deprivation (ED). I vuxen ålder har sedan djuren testats i olika beteendesituationer och funktionen av hjärnans monoaminerga och opioida system har också analyserats.

Mödrabeteende

Resultaten från testet av mödrabeteendet (Studie 2 och 3) visade att MS-mammor, efter att ha samlat in ungarna i boet, stannade signifikant längre tid hos sina ungar i boet (dag 6).

Beteende, endokrinologi och neurokemi i vuxen ålder

Maternal separation (Studie 1 och 2). När ungar separerades som kull i värme visade de inga signifikanta skillnader i ångestlikt beteende som vuxna (Studie 1 och 2). I Studie 1 visade vuxna MS-honor ett lägre intag av alkohol under de första dagarna vid alkoholtestet, även om totalt intag under en vecka inte påverkades signifikant (Figur 13). Det endokrina systemet var inte påverkat, och inte heller nivåer av opioida peptider i olika hjärndelar mellan MS och kontroller.

Early deprivation (Studie 3 och 4). När ungarna separerades isolerat från varandra (antingen i värme eller i rumstemperatur) resulterade detta i beteende-förändringar. ED-behandlingen vid båda temperaturförhållandena visade att dessa djur hade ett minskat ångestlikt beteende jämfört med kontroller i testet för explorativt beteende (Figur 14). När ED-djur isolerades i rumstemperatur uppvisade dessa djur förändringar i samma riktning även i andra test för ångestlikt beteende (Figur 15 och SAP-testet). De biologiska variablerna (stresshormon- och monoaminhalter) visade ingen skillnad mellan ED-djuren och kontroller.

Diskussion

När ungar separerades från mamman som kull i värme (Studie 1 och 2) fanns få förändringar i beteende och biologiska variabler när djuren testades i vuxen ålder. När separationerna däremot utfördes med isolerade ungar (isolerade från mamman och kullsyskon; Studie 3 och 4), uppvisade dessa djur ett minskat ångestlikt beteende i vuxen ålder. De biologiska variablerna visade däremot ingen skillnad mellan ED-djur och kontroller.

MS-modellen har föreslagits vara en modell för psykisk sjukdom hos människa och för att inducera varaktiga beteende- och neurokemiska förändringar hos råtta. Resultaten inom MS-paradigmet är dock inte entydiga, då även minskat ångestlikt

beteende har rapporterats i flera studier (Kaneko et al., 1994; McIntosh et al., 1999; Ploj et al., 2002; Suárez et al., 2004). Detta kan antyda att även mindre gynsamma uppväxtvillkor kan leda till relativt stabil psykisk hälsa. Detta kan vara en parallell till mänskliga förhållanden där man sett att tidiga negativa erfarenheter faktiskt kan leda till psykologisk sett relativt välfungerande individer (Rutter, 2002). Dessa resultat kan möjligen förklaras inom ramen för teorier om att svagare stressorer eller stessorer som hanteras adekvat leder till "härdande" effekter hos individen, medan överväldigande stress eller stress som upplevs inte kunna hanteras leder till en ökad känslighet hos individen (Rutter, 2002; Southwick et al., 2005). Med facit i hand, kan de experimentella manipulationerna i Studie 1-4 kanske anses vara alltför svaga. Det har föreslagits att separationer av ungar från mamman måste överstiga 6 h för att utgöra en allvarlig upplevelse hos ungarna (Kuhn & Shanberg, 1998).

Med utgångspunkt från de MS-manipulationerna som har använts i denna avhandling ger inte resultaten stöd för antagandet att tidiga separationer orsakar ökad ångest eller störningar i endokrinologiska och neurokemiska system hos den vuxna råttan. Resultaten kan ha en motsvarighet hos människan, då relativt god psykosocial funktion kan observeras trots svåra och stressfyllda händelser i barndomen.

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APPENDIX

1. Marmendal, M., Lindqvist A-S., Eriksson, C.J.P., & Fahlke, C. Maternal separation in male and female Wistar offspring: effects on emotionality, ethanol intake and corticosterone levels. *Manuscript.*

2. Marmendal, M., Roman, E., Eriksson, C.J.P., Nylander, I., & Fahlke, C. (2004). Maternal separation alters maternal care, but has minor effects on behavior and brain opioid peptides in adult offspring. *Developmental Psychobiology, 45,* 140-152.

3. Marmendal, M., Eriksson, C.J.P., & Fahlke, C. Early deprivation in male Wistar offspring. Part 1: Long-term increased locomotion and exploratory behavior in novel settings. *Manuscript submitted for publication.*

4. Marmendal, M., Eriksson, C.J.P., & Fahlke, C. Early deprivation in male Wistar offspring. Part 2: Further evidence for long-term reduced emotionality in novel settings. *Manuscript submitted for publication.*