Maternal Separation in the Rat

The Short- and Long-term effects of Early-life Experience on Neuropeptides, Monoamines and Voluntary Ethanol Consumption

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Abstract

Early-life experience has profound effects on the individual’s neurobiology and behaviour later in life. The rodent animal experimental model maternal separation (MS) was used to study this more in detail. The MS model involves short and prolonged postnatal separations simulating an emotionally safe and stressful environment, respectively. The aims of the thesis were to examine the impact of individual MS on ethanol consumption and on brain dopamine and serotonin systems in adult male rats. Furthermore, the influence of separation conditions on the short- and long-term consequences of MS on several neurotransmitter systems was examined.

Rat pups were assigned to either litter-wise MS for 15 or 360 minutes (MS15i or MS360i) or individual MS for 15 or 360 minutes (MS15i or MS360i). Control rats were subjected to conventional animal facility rearing (AFR). Ethanol intake was assessed in a two-bottle free-choice paradigm. Neuropeptides were analyzed with radioimmunoassay, monoamines and metabolites with electrochemical detection and gene expression with qPCR.

Using the MSi paradigm, minor effects on voluntary ethanol consumption were observed. However, the monoaminergic responses elicited by ethanol were dependent on the early-life environment.

Furthermore, short- and long-term consequences of MS on serotonin, opioid, oxytocin and vasopressin systems were studied. Multiple neurobiological measurements in one and the same rat offered a unique possibility to examine the effects of duration (MS15 versus MS360) and condition (l versus i) of MS. Time-, region-, sex- and transmitter-specific effects were observed. More pronounced differences were seen in serotonin measures and oxytocin in young rats. In adults these differences in basal levels were normalized. Opioid peptides differed in stress-related brain areas in young rats and in limbic areas in adults. Rats subjected to the MS15i environment that relates to natural conditions generally exhibited a different neurobiological profile than other groups. AFR rats, i.e. conventional control rats, were more similar to the putative most stressful condition MS360. Taken together, the networks examined in the present thesis are important for the establishment of normal social behaviour and derangements in these systems may result in neurobiological changes leading to the susceptibility for psychopathological conditions later in life.

Keywords: Early-life stress, Handling, Serotonin, Dopamine, Oxytocin, Vasopressin, Enkephalin, Dynorphin, Two-bottle free choice, Maternal deprivation

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To

Mama, Papa, Tania, Milia
&
Carl
///...Hold me
Lay your head lowly
Softly then boldly
Carry me there

Hold me
Love me and feed me
Kiss me and free me
I will feel blessed

Carry
Carry me boldly
Lift me up slowly
Carry me there

Save me
Heal me and bathe me
Softly you say to me
I will be there

Lift me
Lift me up slowly
Carry me boldly
Show me you care

Hold me
Lay your head lowly
Softly then boldly
Carry me there

Need me
Love me and feed me
Kiss me and free me
I will feel blessed...///

by Micheal Jackson
(1958-2009)
List of papers

This thesis is based on the following publications and unpublished manuscripts, which will be referred to by their Roman numerals (I-IV) in the text.

I. Oreland S, Raudkivi K, Oreland L, Harro J, Arborelius L and Nylander I. Ethanol-induced effects on central dopamine and serotonin in adult Wistar rats are dependent on early-life experiences. Submitted manuscript.


IV. Oreland S, Gustafsson-Ericson L and Nylander I. Evidence for a strong impact of early rearing environment of immunoreactive oxytocin levels in the brain and pituitary gland of young and adult male rats. Submitted manuscript.

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Abbreviations

5-HIAA 5-hydroxyindoleacetic acid
5-HT 5-hydroxytryptamine; serotonin
5-HTT 5-hydroxytryptamine transporter
AAAD L-amino acid decarboxylase
A.D. Anno domini
AFR Animal facility rearing
AL Anterior lobe of the pituitary
ANOVA Analysis of Variance
A.M. Ante meridiem
AMPA $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
AVP Arginine vasopressin
BLAST Basic local alignment search tool
cDNA Complementary deoxyribonucleic acid
CNS Central nervous system
COMT Catechol-O-methyltransferase
DA Dopamine
DOP Delta opioid peptide
DOPAC 3,4-di-hydroxyphenylacetic acid
DNA Deoxyribonucleic acid
DRN Dorsal raphe nucleus
DYNB Dynorphin B
EDTA Ethylenediaminetetraacetic acid
GABA Gamma-aminobutyric acid
GPCR Guanine nucleotide-binding protein-coupled receptor
HEC High ethanol consuming
HPA Hypothalamic-pituitary-adrenal
HPG Hypothalamic-pituitary-gonadal
HVA Homovanillic acid; 3-methoxy-4-hydroxyphenylacetic acid
i Individual separation
Ir Immunoreactive
KCl Potassium chloride
KOP Kappa opioid peptide
L-DOPA L-dihydroxyphenylalanine
LEC  Low ethanol consuming
MAO  Monoamine oxidase
MAO-A Monoamine oxidase A
MEAP Met-enkephalin-Arg\(^6\)-Phe\(^7\)
MOP  Mu opioid peptide
mRNA Messenger ribonucleic acid
MS Maternal separation
MS15 Maternal separation 15 min
MS360 Maternal separation 360 min
\(n\) Number of rats
NAcc Nucleus accumbens
NH Non-handled
NIL Neurointermediate lobe of the pituitary
OT Oxytocin
PCA Principal component analysis
PLSD Protected least significant differences
P.M. Post meridiem
PND Postnatal day
PVN Paraventricular nucleus
\(q\)PCR Quantitative polymerase chain reaction; real-time reverse-transcriptase PCR
SEM Standard error of the mean
SON Supraoptic nucleus
v/v Volume/volume
VTA Ventral tegmental area
W Water consuming rats
Introduction

In the 1950s, Bowlby (Bowlby, 1954) studied social behaviour and especially social contacts between infant and parent. He postulated the attachment theory stating that a warm and healthy relationship is important for the development of psychologically healthy adults. In addition, he showed that adverse early-life experience, such as parental loss, neglect, physical and/or sexual abuse, may have consequences for brain development (Teicher et al., 2003). Furthermore, such changes may contribute to an increased vulnerability to various mental disorders, such as depression, anxiety and posttraumatic stress disorders (Canetti et al., 1997, Carlson and Earls, 1997, Nemeroff, 1998, Gilmer and McKinney, 2003, Langeland et al., 2004). Later studies have examined the influence of early environment on the genetic make-up and its consequences for mental health later in life. Environmental and genetic factors act in concert as protective or risk factors for the predisposition to psychopathology. Indeed, two studies published in *Science* in the beginning of this century (Caspi et al., 2002, Caspi et al., 2003), suggested that variations in the 5-hydroxytryptamine transporter (5-HTT; serotonin transporter) and monoamine oxidase A (MAO-A) gene in combination with early-life experiences could lead to an increased vulnerability for antisocial behaviour and depression in adult males. Later on, Nilsson and co-workers (Nilsson et al., 2005) reported that polymorphisms in the 5-HTT gene in combination with different early-life environments were related to adolescent alcohol consumption in males. Early environmental factors may induce changes in gene expression by, for instance, activation of transcription factors or by epigenetic processes. The epigenetic modulations may persist for a long time or be permanent and therefore passed on to the next generations (Bronner et al., 2007, Champagne, 2008).

However, the neurobiological consequences of early-life experiences are not understood and more knowledge is required about the effects of early environment on basal neurobiology and its impact on, for instance, behaviour and responsiveness to challenges later in life.
The experimental animal model

Maternal separation

Because of ethical and moral considerations, a manipulation in the early-life environment of human infants and children is not possible. Thus, as early as the 1950s-60s, non-human primates and rodents were used as experimental models to study the impact of early-life experience on physiology and behaviour (Weininger, 1954, Harlow, 1962, Denenberg and Smith, 1963, Lehmann and Feldon, 2000, Levine, 2002). In studies on non-human primates, the consequences of rearing in different environments have been examined. Comparisons between peer-rearing and mother-rearing have for instance revealed neurobiological and behavioural differences in adult monkeys (Higley et al., 1991a, Higley et al., 1991b, Fahlke et al., 2002). In non-human primates with a genetic predisposition for excessive ethanol intake, high ethanol preference was seen only in the peer-reared animals, but not in the mother-reared animals (Barr et al., 2003). Rodents are frequently used to study the effects of early environment on neurobiology and behaviour. The benefits of using rodents are many. For instance, rats and mice are similar to humans in terms of neuronal function. In addition, rodents have a relatively short life cycle as compared to, for example, non-human primates.

The rodent maternal separation (MS) model is a commonly used animal experimental model in studies of the impact of the early environment on physiological and behavioural functions later in life (Ladd et al., 2000, Pryce and Feldon, 2003, de Kloet et al., 2005, Holmes et al., 2005). The model originates from studies by Otto Weininger, who showed that a short handling of the pups caused positive effects in adulthood. He held the newborn pups and stroked their back for ten minutes, which resulted in less fearfulness in the adult rat (Weininger, 1954). Later on, Levine and colleagues reported that three minutes of daily separation of the pups and mother reduced physiological responses to stress (Levine, 1957, Levine and Lewis, 1959). These early reports of altered adult behaviour after early handling procedures were the beginning of the extensive research efforts using rodent experimental models to describe the impact of early-life environment.

Pups are dependent on their mother for nursing and protection. The mother helps her pups to regulate their physiological responses, such as maintaining a normal body temperature, heart rate, sleep/wake cycle, gastrointestinal activity and growth hormone production (Hofer, 1994). After weaning, which occurs at postnatal days (PNDs) 20-25, the pups are able to take care of themselves (Kuhn and Schanberg, 1998). In addition, the early social interactions, including attachment to the mother, are known to be essential, not only for establishment of social behaviour but also for normal physiological development (Hofer, 1994, Krinke, 2000). Manipulations during the three first postnatal weeks such as MS thus alter these developmental
processes. The MS model enables simulation of different environmental settings using controlled experimental conditions and evaluation of short- and long-term consequences. For instance, it is possible to mimic either an emotionally adverse and stressful environment or a safe environment (Ladd et al., 2000, Boccia and Pedersen, 2001, Pryce et al., 2005).

Today, a number of MS protocols are in use. Common MS protocols consist of short MS, where pups are separated 3-15 min, and prolonged MS lasting 180-360 min (Lehmann and Feldon, 2000, Gutman and Nemeroff, 2002, Pryce et al., 2002, Roman and Nylander, 2005, Moffett et al., 2007). Short periods of MS are considered to be more similar to a naturalistic environment where the mother in the wild has to leave her pups to search for food. However, inconsistency between different experimental groups is often seen between studies when comparing the neurobiological, endocrine and behavioural data. One plausible explanation for the large variation in results could be the different protocols used. MS may, for example, be performed during different light cycles and temperatures, repeatedly or at single occasions and can comprise either individual or litter-wise separations with different duration (Lehmann and Feldon, 2000, Pryce and Feldon, 2003, Roman and Nylander, 2005). Of particular importance in MS studies is the choice of control group and the use of different controls may explain the contradictory findings. The most commonly used control groups are non-handled (NH), those handled but not separated (MS0) or those reared as normal animal facility rats (AFR) (Jaworski et al., 2005). Depending on various factors, such as handling of the pups by the experimenter, the neurobiological and behavioural alterations may differ in these rearing groups.

Previously described consequences of MS include effects on stress reactivity. Corticosterone in rodents is released by hypothalamic-pituitary-adrenal (HPA) axis activation in response to stressors. In this way, the neuroendocrine response to stress maintains the homeostasis in the body (Anisman and Merali, 1999) and is of importance for normal brain development and function (Gutman and Nemeroff, 2002, Walker et al., 2002, de Kloet et al., 2005). However, during PND 4-14, a drastic reduction in corticosterone release is observed due to reduced HPA axis response to stressors. Thus, this period is called the stress hyposensitive period (Sapolsky and Meaney, 1986, Levine et al., 2000) and is suggested to play a protective role during normal development (Sapolsky and Meaney, 1986, Rosenfeld et al., 1992). Pups subjected to short MS are found to have an enhanced ability to cope to stressful stimuli by activation of the HPA axis as adults. On the other hand, the prolonged MS induces changes that are often the opposite (Anisman et al., 1998, Anand and Scalzo, 2000, Ladd et al., 2000, Lehmann and Feldon, 2000, Huot et al., 2001, Levine, 2002, Newport et al., 2002, Cirulli et al., 2003, Pryce and Feldon, 2003).

A large number of reports have described neurobiological differences in animals subjected to MS. Previous studies have, for instance, shown MS-
induced effects in glutamatergic (Szyf et al., 2005, Pickering et al., 2006), GABAergic (Caldji et al., 1998, Caldji et al., 2000), dopaminergic (Ploj et al., 2003a, Brake et al., 2004, Arborelius and Eklund, 2007), noradrenergic (Liu et al., 2000, Matthews et al., 2001, Arborelius and Eklund, 2007) networks, and in stress-related networks (Plotsky and Meaney, 1993, Arborelius et al., 1999, Ladd et al., 2000, Pryce et al., 2005). In addition, early-life experience induces further differences in endogenous peptides, such as the enkephalin (Kalinichev et al., 2002a, Ploj, 2002, Gustafsson, 2007), dynorphin (Kalinichev et al., 2002a, Ploj, 2002, Gustafsson, 2007), oxytocin (OT) (Veenema et al., 2007, Lukas et al., 2009, Todeschin et al., 2009), arginin vasopressin (AVP) (Veenema et al., 2006, Veenema et al., 2007, Lukas et al., 2009, Todeschin et al., 2009, Veenema and Neumann, 2009) and the nociceptin/orphanin FQ (Ploj, 2002) systems.

Sex differences in rats subjected to MS have been shown in behavioural tests (McIntosh et al., 1999, Wigger and Neumann, 1999, Kalinichev et al., 2002b), in the response to different stressors (Papaioannou et al., 2002, Park et al., 2003, Roman et al., 2004), as well as in neurobiology of for example the endogenous opioid (Kalinichev et al., 2001, Gustafsson et al., 2005), dopamine (DA) and 5-hydroxytryptamine (5-HT; serotonin) (Papaioannou et al., 2002) systems.

In a series of experiments, the early environmental impact on voluntary ethanol consumption has been studied using a protocol with daily repeated litter-wise MS for 15 min (MS15) and 360 min (MS360) (Roman and Nylander, 2005). Based on the results from these studies it was suggested that MS15 represents a protective and MS360 a risk environment. The MS15 rats have low ethanol consumption and prefer low concentrations of ethanol in adulthood (Ploj et al., 2003a, Gustafsson and Nylander, 2006). Furthermore, MS15 results in a slow delayed acquisition of excessive ethanol intake in rats with an inherent high ethanol preference and consumption (Roman et al., 2003, Roman et al., 2005). On the other hand, MS360 rats are characterized by higher propensity for high ethanol intake, preference for high ethanol concentrations and increased ethanol intake in ethanol-preferring rats (Ploj et al., 2003a, Roman and Nylander, 2005, Gustafsson and Nylander, 2006). In addition the MS360 rats exhibit a somewhat different behavioural profile including increased risk taking (Roman et al., 2006). Finally, different neurobiological profiles have been described after MS15 and MS360 (Ploj, 2002, Gustafsson, 2007) and this is further described in the section on central dynorphin and enkephalin systems.

The central dopamine systems

In the 1940s, Raab and co-workers (Raab, 1948, Raab and Gigee, 1951) isolated a catechol compound from the brain of different mammalian species
and called it encephalin. Almost a decade later, this substance was chemically characterized and denoted as DA (Weil-Malherbe and Bone, 1957, Carlsson et al., 1958, Bertler and Rosengren, 1959, Sano et al., 1959). The neostriatum contains approximately 70-80% of whole brain DA contents and additionally high concentrations are found in the substantia nigra (Bertler and Rosengren, 1959). The DA systems comprise several dopaminergic pathways but the four major ones are the mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular dopaminergic pathways. The mesocorticolimbic pathways originating from the ventral tegmental area (VTA) innervates, limbic structures, basal ganglia, frontal cortex and other cortical areas (Björklund and Lindvall, 1984), and dorsal raphe nucleus (DRN) (Afifi and Kaelber, 1965, Pasquier et al., 1977, Sakai et al., 1977, Lee and Geyer, 1984, Kalen et al., 1988). The mesocorticolimbic DA pathway is involved in the reinforcing effects of drugs and is further discussed in the section on ethanol (Koob and Le Moal, 2001). The nigrostriatal pathway originating from the substantia nigra projects to the caudate putamen (Björklund and Lindvall, 1984) and is involved in, for example, Parkinson’s disease (Gibb, 1992). Furthermore, the tuberoinfundibular pathway originating from hypothalamus terminates in the pituitary gland (Björklund and Lindvall, 1984), where it modulates prolactin release (Freeman et al., 2000).

DA is synthesised in the terminals of DAergic neurons from the amino acid tyrosine, which is transported across the blood-brain-barrier by an active process. The rate-limiting step in the synthesis of DA is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA). The reaction is catalysed by the enzyme tyrosine hydroxylase. L-DOPA is rapidly converted to DA by L-amino acid decarbyxylase (AAAD). The newly synthesised DA is then packaged in synaptic vesicles and upon depolarisation of the neuron and entrance of Ca\(^{2+}\) in the presynaptic terminal, DA is released into the synaptic cleft. Here, DA acts on the postsynaptic D\(_1\), D\(_3\), D\(_4\), and D\(_5\) receptors and the presynaptic D\(_2\) autoreceptor (Cooper et al., 2003a).

The DA receptors are all seven-transmembrane-region receptor proteins and belong to the superfamily of G protein-coupled receptors (GPCRs). D\(_1\) (Dearry et al., 1990, Monsma et al., 1990, Sunahara et al., 1990, Zhou et al., 1990) and D\(_5\) receptors (Grandy et al., 1991, Sunahara et al., 1991, Tiberi et al., 1991, Weinshank et al., 1991) activate adenyl cyclase, while the D\(_2\), D\(_3\) and D\(_4\) receptors inhibit the same enzyme and also the Ca\(^{2+}\) and K\(^+\) currents. DA signalling is terminated in the synaptic cleft by catechol-O-methyltransferase (COMT) forming 3-methoxy-4-hydroxyphenylacetic acid (HVA; homovanillic acid). In addition, DA may also be reuptaken via the membrane-bound DA transporter and then metabolised in the terminal in a two-step process by the monoamine oxidase (MAO) and aldehyde dehydrogenase enzymes which lead to the metabolite 3,2-dihydroxyphenylacetic acid (DOPAC) (Cooper et al., 2003a).
DA is detectable in the rat brain already at embryonic day 15. The concentration increases substantially up to the third postnatal week and reaches adult levels at about the age of 8-9 weeks. However, there are variations in the developmental timeline of the different DAergic cell groups. For example, the DAergic cell bodies and the completion of their projecting terminals differ. Hence, the rapid increase between embryonic day 15 and the third postnatal week is primarily due to an expansion of cell groups in the midbrain. In addition, the increase in DAergic innervations seen during postnatal week four occurs mainly in the striatum. In the hypothalamus, however, DA levels rise slowly and reaches adult levels as late as week 14 after birth (Kalsbeek et al., 1992).

The DA system has been shown to be sensitive to early-life environment. Brake and colleagues (Brake et al., 2004) reported that prolonged litter-wise MS have lower DA transporter density in the mesolimbic DA pathway in adult male rats. On the other hand, a reduction in D$_3$ receptor binding and gene transcript levels is observed in males exposed to short litter-wise. In our earlier study, we have seen a decrease in D$_2$-like receptor binding in the prolonged litter-wise MS rats as compared to the short litter-wise MS rats in the VTA (Ploj et al., 2003a). In addition, prolonged litter-wise MS in females induce higher HVA levels in the NAcc as compared to the AFR rats (Arborelius and Eklund, 2007). Altered DA levels and DA ratios is further suggested following prolonged litter-wise MS and compared to short litter-wise MS (Matthews et al., 2001).

The central 5-hydroxytryptamine systems

In the 1930s a substance called enteramine was isolated from the gut and in the 1940s, a substance referred as serotonin was discovered in the circulation. Later on, another research group found that enteramin and serotonin covered the same entity, i.e., 5-hydroxytryptamine (Hannon and Hoyer, 2008). 5-HT is abundant in the central nervous system and immunohistochemical data have revealed that there exist nine nuclei of 5-HT-containing cell bodies lying in or adjacent to the midline raphe regions of the pons and upper brain stem (Dahlstroem and Fuxe, 1964). The neurons originating from the caudal raphe nuclei mainly project within the brainstem and spinal cord, whereas the rostral part innervates the forebrain regions. The rostral raphe nuclei include the median raphe nucleus and the DRN. Neurons originating from the median raphe nucleus and DRN project to the limbic areas, basal ganglia, hypothalamus and several nuclei in the brainstem (Steinbusch, 1981, Consolazione et al., 1984). However, 5-HT neurons projecting from the DRN predominantly project to the caudate putamen, VTA, frontal cortex and other cortical areas (Molliver, 1987).
5-HT is synthesised by the two-step pathway from the essential amino acid tryptophan. This amino acid is converted to 5-hydroxytryptophan by the rate-limiting enzyme tryptophan hydroxylase. The L-5-hydroxytryptophan is further decarboxylated by AAAD to form 5-HT. Both tryptophan hydroxylase and the availability of dietary tryptophan are rate limiting for the biosynthesis of 5-HT. As for DA, the newly synthesised 5-HT is either metabolised mainly in a two-step process by the intracellular MAO and aldehyde dehydrogenase enzymes to form 5-hydroxyindoleacetic acid (5-HIAA) or packaged in synaptic vesicles. Upon depolarisation of the neuron, the vesicles fuse with the cell membrane and 5-HT is released into the extracellular synaptic fluid. Most of the 5-HT is taken up into the presynaptic terminal by the membrane bound 5-HTT (Cooper et al., 2003b).

Upon release into the synaptic cleft 5-HT acts by binding to 5-HT receptors. There are currently 14 distinct mammalian 5-HT receptor subtypes and these are divided into seven different classes denoted as 5-HT1-5-HT7. These subtypes exhibit different distributions within the central nervous system and also mediate distinct behavioural effects. The 5-HT3 receptor is the only ligand-gated ion channel while the rest are GPCRs. The 5-HT1 receptor family consists of five isoforms (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F) and inhibit adenyl cyclase or regulate K+ and Ca2+ channels upon activation (Hannon and Hoyer, 2008). In the DRN, the 5-HT1A receptors are abundantly expressed (Sotelo et al., 1990, Pompeiano et al., 1992) and function as somatodendritic autoreceptors, thus, inhibiting raphe cell firing when activated. The 5-HT2 receptor family includes the 5-HT2A, 5-HT2B and 5-HT2C. These receptors are linked to activate phospholipase C (Hannon and Hoyer, 2008). The 5-HT2A and 5-HT2C subtypes are abundantly expressed in the forebrain regions, but are also found in different nuclei in the brainstem (Fay and Kubin, 2000) and are involved in anxiety-like behaviour (Salchner and Singewald, 2006). The 5-HT3 receptors are expressed in the area postrema and nucleus of the solitary tract (Laporte et al., 1992), where they enhance Na+ and K+ currents and are assembled in a pentameric configuration (Lovinger, 1999). The brainstem 5-HT3 receptors are involved in emesis, cardiovascular responses and feeding behaviour (Costall and Naylor, 1992, Merahi et al., 1992, Himmi et al., 1996). Moreover, ethanol interferes with 5-HT actions on 5-HT3 receptors. A growing literature indicates the involvement of 5-HT in ethanol seeking and intoxication behaviour as well as reward and reinforcement (Lovinger, 1999). 5-HT4 receptors activate adenyl cyclase (Hegde and Eglen, 1996) and are expressed in the nigrostriatal and limbic pathways (Barnes and Sharp, 1999). The 5-HT5A, 5-HT5B, 5-HT6 and 5-HT7 receptors have recently been cloned. The two latter receptors are linked to the activation of adenyl cyclase, whereas 5-HT5A receptor inhibits this enzyme (Hannon and Hoyer, 2008). However, further studies are needed to establish their functions and functional couplings.
The 5-HT systems in rodents are not completely developed at birth and therefore undergo substantial alterations during the postnatal period (Hedner and Lundborg, 1980, Lidov and Molliver, 1982a, b, Morilak and Ciaranello, 1993). Therefore, influences during this time period may alter the development of the brain and induce changes in central 5-HT functioning later in life (Vogel et al., 1990).

The first postnatal weeks represent a sensitive period when 5-HT networks mature. Influence during this time period may change the course of development and induce long-term changes in central 5-HT functioning (Vogel et al., 1990). Accordingly, MS induces changes in the 5-HT networks in various brain regions. Prolonged litter-wise MS alters the sensitivity of 5-HT receptors and transporter in brain regions related to mood disorders in male rats (Gartside et al., 2003, Arborelius et al., 2004). Additionally, rats subjected to prolonged litter-wise MS exhibit reduction in 5-HT levels in the frontal cortex and hippocampus (Matthews et al., 2001). The short litter-wise MS alters 5-HTT and 5-HT1A receptor densities in the amygdala as compared to NH and AFR rats (Vicentic et al., 2006).

The central dynorphin and enkephalin systems
During the 1970s, several major discoveries led to the identification of the endogenous opioid system. First, the existence of opioid binding sites was reported (Pert and Snyder, 1973, Simon et al., 1973, Terenius, 1973) and this provided evidence for endogenous opioid receptors. Shortly after, the first reports of endogenous morphine-like molecules were published (Hughes et al., 1975, Pasternak et al., 1975, Terenius and Wahlström, 1975, Bradbury et al., 1976, Goldstein et al., 1979). Currently, a number of endogenous opioids have been identified in three major subfamilies; enkephalins, dynorphins and endorphins (Terenius, 2000). The enkephalins are widely spread throughout the central nervous system and are present in both interneurons and long neuronal projections. The dynorphins exhibit a more restricted distribution pattern and are present in well-defined pathways such as the striato-nigral pathway (Christensson-Nylander et al., 1986).

The synthesis of neuropeptides differs from those of classical transmitters. In the cell body, the prohormones, prodynorphin and proenkephalin, are synthesized and then transported by axonal transport to the nerve terminal. The opioid prohormones are further processed enzymatically and several biologically active peptides are generated from each prohormone (Morita, 1992).

Proenkephalin is, for instance, cleaved into Leu-enkephalin, Met-enkephalin and Met-enkephalin-Arg6-Phe7, while processing of prodynorphin generates, for instance, dynorphin A and dynorphin B (Khachaturian et al., 1985, Akil et al., 1998, van Ree et al., 1999). The peptides are stored in
vesicles and are released into the synaptic cleft upon cell stimulation. After release and action on the opioid receptors, the opioid peptides are degraded by aminopeptidases into biologically active or inactive peptide fragments. Three opioid receptors have been identified: The mu-opioid peptide (MOP), delta-opioid peptide (DOP) and the kappa-opioid peptide (KOP) receptor. The enkephalin peptides preferentially bind to the DOP receptors, while the dynorphin peptides have high affinity for the KOP receptors (Akil et al., 1998, van Ree et al., 1999, Terenius, 2000, Gutstein and Akil, 2001). The opioid receptors belong to the GPCR family and inhibit neural transmission upon activation by inhibiting the enzyme adenylyl cyclase (Mansour et al., 1988, Knapp et al., 1995, Akil et al., 1998, Gutstein and Akil, 2001). The opioid peptides are involved in various physiological functions, such as pain, thermoregulation, endocrine modulation, learning and memory, stress and reward and reinforcement (Akil et al., 1998), as well as social behaviour (Panksepp et al., 1980, Panksepp et al., 1994, Nelson and Panksepp, 1998). Interestingly, opioids tonically regulate the transmission in mesocorticolimbic and mesostriatal DA pathways (Nylander and Terenius, 1987, Zapata and Shippenberg, 2006). For example, dynorphins and enkephalins have opposite actions on the mesocorticolimbic neurons. Enkephalins activate these neurons while dynorphins inhibit DA release in the nucleus accumbens (Spanagel et al., 1992, Zapata and Shippenberg, 2006).

The endogenous opioids are present during the embryonic period and the opioid networks continue to mature during the postnatal period (McDowell and Kitchen, 1987, Leslie and Loughlin, 1993). The KOP receptors are present during embryogenesis, while the DOP receptors are expressed after birth. During the first postnatal weeks, the KOP receptors increase considerably before declining to adult levels. During the second postnatal week, the DOP receptors reach their highest expression level before declining to adult levels (Spain et al., 1985, Petrillo et al., 1987).

The opioid system is sensitive to early-life experiences. It has been shown that litter-wise MS15 reduces endopeptidase activity in the amygdala (Irazusta et al., 1999). Furthermore, DOP receptor binding in the basomedial amygdala was increased in adult males subjected to individual MS15, while no changes in KOP receptors were seen (Ploj et al., 1999). Long-term effects of MS were observed in the dynorphin and enkephalin systems in different brain regions related to stress, reward and reinforcement (Ploj, 2002, Gustafsson, 2007). Moreover, the changes were greater in the enkephalin system than the dynorphin system. Since the enkephalin system matures later than the dynorphin system, the enkephalin network may be more sensitive to postnatal manipulations. In addition, sex-specific changes were observed. The endogenous peptides were less affected by MS in female rats as compared to male rats (Gustafsson et al., 2005).
The central oxytocin and vasopressin systems

Oxytocin (OT) and arginine vasopressin (AVP) are cyclic nonapeptides containing nine amino acids. These peptides are structurally similar to each other and differ only by two amino acids. The cell bodies are located in two specific hypothalamic nuclei, i.e., the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). The neurons project to various brain regions, such as the anterior (AL) and neurointermediate lobe of the pituitary (NIL), amygdala, hypothalamus, DRN and locus coeruleus (Gimpl and Fahrenholz, 2001).

The OT and AVP genes are located on the same chromosome in rats, but are transcribed in opposite directions yielding the OT and AVP prepropeptides. The prepropeptides are further cleaved during their transportation down the axon to the nerve terminal and the mature peptides are released to the extracellular fluid when neural inputs elicit their release (Gimpl and Fahrenholz, 2001). Like the opioid peptides, OT and AVP are metabolised by aminopeptidases in the extracellular fluid, which in turn produces potent active peptides (Argiolas and Gessa, 1991).

OT acts via the GPCR and when stimulated activates phospholipase C to produce the second messenger inositol 1,4,5-triphosphate (Gimpl and Fahrenholz, 2001). AVP acts through the V$_{1A}$, V$_{1B}$ and V$_2$ receptors (Lolait et al., 1992, Morel et al., 1992, Sugimoto et al., 1994). When an agonist binds to the V$_1$ receptor, phospholipase C is activated (Thibonnier et al., 1993), while V$_2$ receptors mediate the activation of adenylyl cyclase (Snyder et al., 1992). OT and AVP are related to social recognition memory, aggression and to maternal behaviour (Insel and Shapiro, 1992, Nelson and Panksepp, 1998, Insel, 2003, Carter et al., 2008), but also addiction (Russell et al., 1995, Kovacs et al., 1998, Sarnyai, 1998). Moreover, OT is widely known to also affect maternal behaviour (Carter, 2003).

OT and AVP are found early in development. On gestational day 16 in rats, the gene for OT and AVP are transcribed. However, the OT levels are low during gestation and the synthesis of OT is first detected on the second postnatal day. The AVP networks are fully matured at weaning, while the OT networks reach their maturity during puberty (Buijs, 1992, Carter, 2003, Kramer et al., 2003).

Few studies have examined the effects of MS on the OT and AVP systems. Recently, however, MS-induced effects have been described in studies showing that postnatal MS affected the OT and AVP circuits in rats (Veenema et al., 2006, Veenema et al., 2007, Lukas et al., 2009, Todeschin et al., 2009). Increased OT and V$_{1A}$ receptor binding was seen in prolonged separated male rats in distinct brain areas and at different ages (Lukas et al., 2009). MS for 1 min decreased OT neurons in the PVN in both adult male and female rats. In addition, the adult males exhibited increased numbers of AVP neurons in the same region (Todeschin et al., 2009).
Ethanol

The distillation of fermented solutions, e.g., alcohols was first developed by the Arabs in A.D. 800. The word actually means “something subtle” in Arabic. In the present thesis, ‘alcohol’ and ‘ethanol’ are interchangeably used. Upon consumption, alcohol is rapidly absorbed from the stomach and small intestine to the bloodstream and is distributed throughout the body. When alcohol is taken orally, it undergoes metabolism in the stomach and liver, where alcohol is converted to acetaldehyde by alcohol dehydrogenase. This product is then further oxidised to acetate by aldehyde dehydrogenase in the liver and subsequently secreted through the kidney. Ethanol has both peripheral and central effects and in the present thesis the focus was on the effects in the brain. Ethanol acts on several targets in the central nervous system, such as DA, gamma-aminobutyric acid (GABA), glutamate, opioids, 5-HT, corticotrophin releasing hormone, acetylcholine and many more (Dick and Foroud, 2003). The initial rewarding effects of ethanol comprise the activation of the mesocorticolumbic DA pathway, also called the reward pathway, where DA neurons projecting from the VTA terminate in the forebrain area including the NAcc, amygdala and the frontal cortices (Di Chiara, 1999, Berridge and Robinson, 2003, Everitt and Robbins, 2005, Koob, 2006, Wise, 2008). In response to ethanol, DA may be released in the NAcc. However, this circuit is linked to other systems that may play a crucial role in modulating the rewarding and reinforcing effects of ethanol (Koob and Le Moal, 2001). In the VTA, the DA neurons are under the control of the inhibitory GABA interneurons. These neurons are in turn modulated by the enkephalins and endorphins, which modulate the DA-dependent reward circuit. In other words, a release of these opioids blocks the inhibitory actions of GABA and DA will be released in the NAcc. On the other hand, the dynorphins have the opposite effect on DA and block the release. Furthermore, a dysregulation in the opioid systems may lead to vulnerability for addiction (Nylander and Silberring, 1998, LaForge et al., 2000, Van Ree et al., 2000, Gianoulakis, 2004, Oswald and Wand, 2004). Of course, the rewarding effects are dependent on the ethanol dose ingested (Di Chiara and Imperato, 1985). However, long-term repeated administration of ethanol causes adaptation of the brain. Voluntary consumption of ethanol may then lead to a habitual or even a compulsive use of the drug. During this period, the involvement of different brain structures shifts from one brain area to another (Everitt et al., 2008, Belin et al., 2009), but the mechanisms behind the different phases between the initial intake of ethanol and addiction and dependence are not entirely clear and need further investigation.

The 5-HT system is involved in substance abuse. A number of animal and clinical studies have generated evidence supporting a role for 5-HT function in the neurobiological basis for predisposition for alcohol addiction (McBride and Li, 1998, Barr et al., 2003, Oreland, 2004). A dysfunction in
central 5-HT neurotransmission has been implicated in the pathogenesis of alcohol addiction (Johnson, 2004, Lesch, 2005). Several studies also support the involvement of 5-HT in ethanol-induced effects even though the exact mechanisms remain unclear, especially after voluntary ethanol consumption (Yoshimoto et al., 1992, LeMarquand et al., 1994, Grant, 1995, Boehm et al., 2005, Heilig and Egli, 2006).

In 2005, an interesting study in humans suggested an interaction between gene and early-life environment and excessive alcohol drinking during adolescence (Nilsson et al., 2005). In fact, some years earlier, another study but in non-human primates found that an allelic variation in the 5-HTT gene and early-life environment affects the sensitivity to ethanol intake (Barr et al., 2003). Studies of effects of ethanol on the monoaminergic systems in rodents subjected to different early environments are limited (Ploj et al., 2003a, Vicentic et al., 2006, Advani et al., 2007). MS in rodents have generated further evidence for the role of early environmental influence on later response to ethanol (Ploj et al., 2003a, Roman et al., 2003, Gustafsson and Nylander, 2006), but also other drugs of abuse, such as psychostimulants (Matthews et al., 1999) and opiates (Kalinichev et al., 2002a). In addition, sex-specific differences of MS were reported in ethanol consumption (Gustafsson et al., 2005, Roman et al., 2005).

Ethanol also acts on the HPA and hypothalamic-pituitary-gonadal (HPG) axis. Acute challenge to ethanol releases corticotrophin-releasing hormone and consequently increases plasma corticosterone levels (Fadda and Rossetti, 1998). During chronic ethanol administration, the HPA axis is dysregulated (Koob et al., 1998a, Weiss et al., 2001, O'Brien et al., 2006). Furthermore, adrenalectomised rats reduce their ethanol consumption and treatment with corticosterone reverses these effects (Fahlke and Eriksson, 2000). Chronic ethanol administration also suppresses the HPG axis and thereby affects the male reproductive system dysregulating testosterone biosynthesis and release (Rasmussen et al., 2003).

Ethanol administration models

To study the effect of ethanol, a number of ethanol administration models can be used. Ethanol can be given passively, i.e. administered by the experimenter, by several routes of administration. Intravenous, intracranial, intracerebroventricular and intragastric administration can, for instance, be used (Meisch and Lemaire, 1993, Koob et al., 2003, Spanagel, 2003). These routes of administration are used to examine the acute and chronic effects of ethanol. However, passive administration involves handling, injection procedures etc. and can induce neurobiological and behavioural effects other than those seen after self-administration of a drug. Therefore, to examine the effects of voluntary ethanol self-administration either operant or non-operant
techniques are used. In operant models, the rats are trained to press a lever to receive the drug. The delivered drug may consist of a certain dose given, for example, through an intravenous catheter or may appear as drug solution for oral intake. Thus, the rat can control the rate of the delivery and thereby the dosage of the drug. However, ethanol can also be self-administered through non-operant techniques.

Such drinking models are used to, for instance, examine the propensity for excessive ethanol consumption. In the 1940s, the two-bottle free-choice paradigm was introduced (Richter and Campbell, 1940). Here, the animals can choose between a bottle containing ethanol or water. But, also multiple free-choice paradigms can be used. In addition, the animals may be given continuous or limited access to ethanol. Blood ethanol concentration levels are suitable to determine when using the limited access paradigm, since ethanol is only present at certain limited time periods. Furthermore, higher ethanol intake can be induced using the limited access paradigm, but also by adding sweeteners to the ethanol of solution. To study effects of dependence, withdrawal and/or relapse, vapour chambers and alcohol deprivation models are used (Spanagel, 2003).

When rats have continuous access to ethanol, the solution is preferably gradually increased, since the traditional laboratory rat normally does not voluntarily start to consume 6% of ethanol (Richter and Campbell, 1940, Hansen et al., 1994). The two-bottle free-choice paradigm is herein used to study acquisition and maintenance of ethanol consumption.

**Neurochemical models**

**HPLC with electrochemical detection**

High performance liquid chromatography (HPLC) with electrochemical detection is a two-step process in which the compounds in the sample are first separated and then detected. This method was used for detection of monoamines in Paper I. To separate the non-polar analytes, a reversed phase C18 column was used. The mobile phase consisted of aqueous buffer and organic solvents to elute the analytes from the column. In the present thesis, the elution was performed with an isocratic pump, meaning that the mobile phase composition was not altered during the separation process. Monoamines and their metabolites have electroactive properties, thus, they can be detected with high selectivity and sensitivity using an electrochemical detector. The analyte is oxidised by the potential that is applied across the detector cell. The observed current is proportional to the concentration of the analyte. The HPLC method coupled to an electrochemical detector is an established and robust method for analyses of tissue levels of monoamines and their metabolites (Mefford, 1981).
In Paper II, the quantitative polymerase chain reaction (qPCR; real-time PCR) was used to measure the gene transcript levels of 5-HT receptor and transporter. The introduction of qPCR has made it possible to quantify small amounts of starting materials of the nucleic acids. In addition, qPCR is highly sensitive, reproducible and fast (Vandesompele et al., 2002, Bengtsson et al., 2003).

The general idea regarding qPCR is to quantify the products as they accumulate, i.e. in real time. The qPCR is performed as traditional PCRs, however, a fluorescent reporter is also added. The fluorescent reporter binds to the product and reports its presence by fluorescence. One of the most commonly used fluorophore is SYBR Green I (Bengtsson et al., 2003). This fluorophore binds to unspecific double-stranded DNA products and fluoresces only when it is bound. During the amplification, the fluorescent increases proportionally to the amount of the specific product being generated (van der Velden et al., 2003). During the first amplification cycles, the fluorescent signal is weak due to background noise, but as more products are being produced the signal increases exponentially. A sigmoidal response curve is observed reflecting the accumulated product. The separation of the curves, in the growth phase, mirrors the difference in initial concentration of template present in the samples. The difference is quantified by comparing the number of amplification cycles needed for the sample’s response curve to reach a certain threshold fluorescence signal level. The number of cycles required to reach threshold is called the Ct value, which reflects the transcriptional activity of the gene of interest in the sample (Kubista et al., 2006). Melting curve analysis is always performed to be certain that a specific product has been produced. When comparing the samples, a normalisation is required to compensate for differences in the amount of biological material tested in the sample. The aim is to remove quantitative and qualitative variability and thereby identifying real gene-specific variations and not artefacts. By using housekeeping gene a normalisation can be performed and these gene are also used as internal standards (Huggett et al., 2005). To determine which housekeeping gene is the most stable the software GeNorm can be used (Vandesompele et al., 2002). The variation between these genes should be as small as possible (Kubista et al., 2006).

Radioimmunoassay

Radioimmunoassay (RIA) is used in Papers III and IV. RIA is based on the method where unlabelled antigen competes with the corresponding radioactive labelled antigen for a limited number of antibody binding sites. After achieving equilibrium, some of the antigen and antibody has built complexes with each other, while some will be free. After separating these two frac-
tions, the bound fraction is measured in a radioactivity counter, herein a gamma counter, since $^{125}$I-labelled antigen was used. RIA is a highly sensitive and specific method for analysing immunoreactive (ir) levels of peptides and proteins (Yalow and Berson, 1960).
Aims

In both human and non-human primates and rodents, extensive research has shown that early-life experience has a profound impact on adult physiology and behaviour. Different environmental experiences at an early age might contribute to the vulnerability for psychopathology such as depression and addiction. However, the neurobiological mechanisms mediating these effects are not understood.

The general aim of the present thesis was to evaluate how different early-life events in rats affect voluntary ethanol consumption and the DA, 5-HT, MEAP, DYNB, OT and AVP systems. These brain circuits are known to be important for social interaction early in life and are involved in the mechanisms of ethanol reward and reinforcement.

The specific objectives of this thesis were:

- To examine the voluntary ethanol consumption in male rats subjected to different postnatal rearing conditions.
- To examine whether long-term voluntary ethanol consumption induce different effects on DA, 5-HT and metabolite levels in male rats subjected to different postnatal rearing conditions.
- To examine the short- and long-term consequences of different postnatal maternal separation conditions on 5-HT receptors and transporter in male and female rats.
- To examine possible sex differences in the effects of different postnatal maternal separation conditions on serotonin transporter and receptors.
- To examine the short- and long-term consequences of different postnatal maternal separation conditions on endogenous opioid peptides in male rats.
- To examine the short- and long-term consequences of different postnatal maternal separation conditions on OT and AVP peptides in male rats.
Materials and methods

Animals

Time-mated pregnant Wistar rats (Scanbur BK AB, Sollentuna, Sweden) arrived at the animal facility on gestational days 14-15. The dams were singly housed in standard macrolon cages (59x38x20 cm) containing wood chip bedding and nesting material, and were maintained on food pellets (R36 Labfor, Lactamin AB, Vadstena, Sweden) and water ad libitum. All animals were housed in temperature- (22±1ºC) and humidity- (55±10%) controlled cabinets maintained on a 12 h light/dark cycle with lights on at 06:00 AM. All animal experiments were performed according to a protocol approved by the Uppsala ethics committee and in accordance with the Swedish Animal Protection Legislation.

Maternal separation

On the day of birth (postnatal day, PND 0), the pups were cross-fostered to avoid biological littermates. Each litter comprised nine pups (males, n = 5; females, n = 4) and the litters were randomly selected into different experimental groups. In Study I, the litters were assigned to one of three rearing environments, i.e. 15 (MS15) or 360 (MS360) min of individual (i) maternal separation (MS), or to normal animal facility rearing (AFR). In Papers II-IV two additional groups were included consisting of 15 and 360 min of litter-wise MS, that is, MS15/ and MS360/, respectively. MS was performed once daily during PND 1-21 with start at 09:00 AM (MS15) and 09:35 AM (MS360). First the dam and then the pups were removed from the home cage. During the separations, the pups either exposed to litter-wise or individual MS were placed in adjacent room in macrolon cages (26x20x14 cm) containing wood chip bedding material. The MS15/ and MS360/ pups were kept together as litters during separation from the dam and were placed in animal cabinets at 25°C (Paper II-IV). The MS15i and MS360i pups were separated from each other by insertion of a metal cross and were then placed at a higher temperature (31.5°C) to avoid hypothermia (Blumberg and Sokoloff, 1998, Ruedi-Bettschen et al., 2004). The dams in the MS15 groups were moved to other cages during the separations and the litters were returned to the home cages before the dams. In the MS360 groups, however,
the dams were returned to the home cages during the separations, and moved again just before the litters were returned.

The home cages were changed twice during the MS period. MS15 and MS360 pups were weighed as whole litters on PND 4, 7, 10, 13, 16 and 19. The AFR litters were weighed only when the cages were changed on PND 7 and 16. In Study I, all litters were weighed PND 0 and on the day of weaning, PND 22. In Papers II-IV, all litters were weighed at PND 1 and the last day of MS, PND 21. After weaning, the litters were divided by sex, group-housed and kept under normal animal facility care. In Paper I, only males were used. The ethanol consumption was assessed by using a two-bottle free-choice ethanol-drinking paradigm and after seven weeks of voluntary ethanol consumption, the animals were decapitated for dissection and subsequent neurobiological analyses. In Paper II, the neurobiological analysis included male and female rats, whereas in Paper III and IV only males were analysed. See Figure 1 for a general outline of the experimental designs in the current thesis. Note that the same animals are used in Papers II-IV for the different neurobiological measurements (Figure 1).

![Figure 1](image)

**Figure 1.** A schematic overview of the experimental set-up for the different studies presented herein.

**Ethanol consumption (Paper I)**

The consequences of repeated individual MS on long-term voluntary ethanol consumption were examined in adult male rats using the two-bottle free-choice drinking paradigm. The rat pups were subjected to the MS15i, MS360i and AFR conditions until weaning and then left undisturbed except for conventional animal care. At 10 weeks of age, the male rats were divided into water-consuming (MS15W, MS360W and AFW) and ethanol-consuming (MS15E, MS360E and AFRE) groups. The water-consuming rats had two bottles of water and the ethanol-consuming rats were given unlimited access to two bottles containing ethanol and water, respectively, for seven weeks. The positions of the bottles were randomised daily to avoid place preference and were weighed daily to measure the fluid consumption.
over 24 hours. The ethanol concentration was increased gradually from 2-8% (v/v) ethanol during a two-week period. Thereafter, rats were given five weeks of free access to 8% ethanol. The food intake was measured throughout the drinking period.

Blood collection
The male rats used in Paper I were sacrificed by decapitation after seven weeks of ethanol and/or water consumption and trunk blood was collected from each animal. The blood was first kept in room temperature for 20-30 minutes and then kept cold until centrifugation. Serum was separated from the cells by centrifugation at 1 640xg at 4°C for 10 minutes. The serum was stored in aliquots in -80°C until further use.

Brain dissection (Paper I-IV)
In Paper I, the male rats were decapitated after seven weeks of ethanol and/or water consumption, whereas in Papers II-IV, the rats were decapitated at two time points, either at weaning or as adults, i.e. at three and ten weeks of age, respectively. In Paper II, male rats as well as female rats were dissected. After decapitation the brains (Paper I-IV) and the pituitary gland (Paper III and IV) were immediately taken out. The pituitary gland was divided into the NIL and anterior AL lobe in the adult rats. The brains were manually dissected according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1997) with pre-cooled razor blades in coronal sections (2 mm slots, Paper I; 1 mm slots, Paper II-IV). In Paper I, frontal cortex, cingulate cortex, NAcc, caudate putamen, hippocampus, amygdala, VTA and DRN were dissected. In Paper II, the brainstem was used. In Paper III, besides the pituitary gland, also the frontal cortex, medial prefrontal cortex, NAcc, caudate putamen, hippocampus, amygdala, substantia nigra, VTA, periaqueductal grey and hypothalamus were dissected. The hypothalamus was removed before slicing the brains. In Paper IV, besides the pituitary gland, also the hypothalamus and amygdala were analysed. The tissues were immediately frozen on dry ice and stored in -80°C until further analysis.

Monoamine and metabolite extraction (Paper I)
The brain tissues were homogenized with an ultrasonic homogenizer (Bandelin Sonopuls, Bandelin Electronic, Berlin, Germany) in 0.1 M perchloric acid containing 5 mM sodium bisulphate and 0.4 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA) to avoid oxidation. The
homogenates were then centrifuged for 10 min at 17 000xg in 4°C. The supernatants were stored in -80°C until further use.

**HPLC with electrochemical detection (Paper I)**

Brain DA and 5-HT and their respective metabolite levels were measured using reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection. 10 μL of the supernatant obtained from the homogenate and containing the monoamines and metabolites were separated on a Luna C18(2) column (150x2 mm; 5 μm). The system consisted of a pump set to isocratic conditions (Hewlett Packard HP 1100 Series), a temperature-regulated autosampler and column compartment. The mobile phase was applied at a column temperature of 30°C and contained 0.05 M sodium citrate buffer at pH 3.7; 0.02 mM EDTA; 1 mM potassium chloride (KCl); 1 mM sodium octylsulphonate and 5.6% acetonitrile. An electrochemical detector (HP 1049, Agilent, Waldbronn, Germany) with glassy carbon electrode with a potential of +700 mV versus the Ag/AgCl reference electrode was used. The detection limit was 0.05 - 0.10 pmol/mg tissue depending on the analyte at a signal to noise ratio of 3.

**RNA extraction and cDNA synthesis (Paper II)**

In Study II, the brain tissues were homogenized in cell lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM Dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.9. For extraction of total ribonucleic acid (RNA), 200 μL of the homogenates were transferred to other tubes and RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA was eluted from the RNeasy Mini kit columns with 50 μL of RNase free water and the concentrations were determined using a Nano Drop® ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA).

Synthesis of complementary deoxyribonucleic acid (cDNA) was performed using the Ready-to-Go (RTG) You-Prime First-Strand Beads kit (Amersham Pharmacia Biotech and GE Healthcare, Uppsala, Sweden). The RNA samples were diluted in water to a concentration of 1 000 ng/μL and mixed with 0.25 μg/μL of random hexamer (pdN₆) and 10 mM of dNTPs. The solution was then added to the RTG tubes and incubated at 65°C for 10 minutes followed by 1 h of incubation at 37°C for a complete synthesis of cDNA. The samples were finally diluted to 5 ng/μL and stored at -20°C until further use.
**qPCR (Paper II)**

In Study II, qPCR was used to measure the gene transcript levels of serotonin receptors (5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2c}$ and 5-HT$_{3}$), and transporter (5-HTT). Each well of a 96-well plate (total volume of 20 μL) consisted of 2 μL 10 x PCR buffer, 50 mM MgCl$_2$, 20 mM dNTP, 100 pmol/μL forward and reverse primers, 1:50 000 SYBR Green I, 0.08 μL (5 U/μL) Taq DNA polymerase and 10.62 μL RNase-free water mixed with 5 μL (5 ng/μL) cDNA template. The primer pairs were designed with Beacon Designer v4.0 (Premier Biosoft, USA) and the specificity was verified using the Basic local alignment search tool (BLAST) against the rat genome. The primers were optimized to run at 95°C for 15 s, annealing at 60°C for 30 s followed by extension at 72°C for 30 s. For each primer pair, 50 PCR cycles were run. Melting curves were included after the cycling was completed to confirm that a single product was produced by the PCR reaction. The data provided was analysed in iCycler™ iQ, Optical System Software, version 3.0a (Bio-Rad Laboratories, Sweden). (Bengtsson et al., 2003) A normalization factor was calculated using four housekeeping genes as reference genes (Vandesompele et al., 2002). The GeNorm method (Vandesompele et al., 2002) was applied to evaluate which set of housekeeping genes was the most stable in terms of a gene-stability measure for a given set of samples for each particular sex and age. To calculate primer efficiency the assumption-free analysis method of Ramakers and co-workers (Ramakers et al., 2003) was used. The LinRegPCR method was then used to calculate corrected Ct values. To calculate the expression value, the corrected Ct value was divided by the normalization factor obtained for that tissue from GeNorm. Each value was normalized to the mean of the AFR group (100 %) such that all values are expressed as % of AFR.

**Peptide extraction (Paper II-IV)**

The peptides dynorphin B (DYNB), Met-enkephalin-Arg$_6$-Phe$_7$ (MEAP), OT and AVP were extracted from the brain according to a protocol established by Christensson-Nylander and co-workers (Christensson-Nylander et al., 1985). The tissue extracts were applied onto an ion exchange column containing SP Sephadex C-25 gel (Pharmacia Diagnostics, Uppsala, Sweden) and the peptides were eluted in separate fractions using buffers containing a mixture of purified pyridine and formic acid with increasing ionic strength. Fraction III contained MEAP and OT, while DYNB and AVP was eluted in Fraction V (Christensson-Nylander et al., 1985). These fractions were dried in a vacuum centrifuge and stored in -20°C for further analysis.
Radioimmunoassay (Paper II-IV)

In Paper III, the ir MEAP and DYNB levels were analysed using a well-established RIA previously described in detail (Nylander et al., 1997). The tissue samples or synthetic standard peptides were dissolved in methanol/hydrochloric acid (1:1). The samples were then incubated with antiserum and labelled $^{125}$I-peptide, both diluted in gelatine buffer. The $^{125}$I-peptides were labelled with the chloramines T method and purified with reversed phase HPLC prior to use. The MEAP (90:3D II) and DYNB (113+) antisera were generated in rabbits (Christensson-Nylander et al., 1985). To separate free peptide and antibody-bound peptide in the samples, a charcoal suspension was used in the MEAP assay, whereas a double-antibody technique with sheep-antirabbit antiserum (Pharmacia Decanting Suspension 3, Pharmacia Diagnostics, Uppsala, Sweden) was used in the DYNB assay.

In Paper IV, the ir OT and AVP levels were analyzed using a commercial RIA kit (S-2033, OT; S-2196, AVP; Bachem, USA). In this protocol, the tissue samples were pre-incubated with a primary antibody and then further incubated with labelled $^{125}$I-peptide. The free and antibody-bound peptides were then separated by adding a secondary antibody (goat anti-rabbit IgG serum).

Ir testosterone and corticosterone serum levels in the ethanol and/or water-consuming rats were measured using a commercial RIA kit (Coat-A-Count Total Testosterone and the Coat-A-Count Rat Corticosterone, respectively, DPC, Bad Nauheim, Germany), according to the manufacturer’s protocol. The detection limit of ir testosterone was 0.14 nmol/L and ir corticosterone was 16.45 nmol/L.

For all RIA assays, the radioactivity of the bound fraction, i.e. the supernatant for the MEAP and the pellet for the OT, AVP and DYNB assays, was measured in a gamma counter. Standard curves with known concentrations of the ir peptides were used for quantitative analysis. Cross-reactivity for the MEAP antiserum was less than 0.1% for Met-enkephalin, Met-enkephalin-Arg$^6$, Met-enkephalin-Arg$^6$Gly$^7$Leu$^8$, Leu-enkephalin or DYNA (1-6). Cross-reactivity for DYNB antiserum was 1% for DYNB 29 and 100% for DYN 32. The DYNB antiserum did not cross-react with DYNA.

Statistical analyses

Statistical differences in body weight during the MS period between rats exposed to the different rearing environments was analysed using the one-way analysis of variance (ANOVA) followed by Fisher’s Protected Least Significant Difference (PLSD) post-hoc test (Paper I and IV). In Paper II and III, the Bonferroni-Holm correction was used. Body weight in water- and ethanol-drinking adult male rats was analysed with two-way ANOVA fol-
ollowed by Fisher’s post-hoc test (Paper I). To compare the levels of ir peptides and 5-HT receptors and transporter gene transcripts in the five rearing environments, i.e. MS15, MS15i, MS360, MS360i and AFR groups, one-way ANOVA was used followed by Fisher’s post-hoc test in Paper II and the Bonferroni-Holm correction in Paper II and IV. In addition, the two-way ANOVA followed by Fisher’s post-hoc test was applied to examine the effect of rearing condition (MS15i versus MS360i), the effect of separation condition (individual versus litter-wise separation) and the effect on interaction between rearing condition and separation condition (Paper II-IV). The same statistical test was performed to examine the effects of rearing condition (MS15i, MS360i versus AFRi), fluid intake (water versus ethanol) and the interaction between rearing condition and fluid intake on adult body weight and DA and 5-HT and their metabolite levels, respectively (Paper I).

In Paper I, the non-parametric test was used when analysing fluid and food intake, since the data set was not normally distributed. The Kruskal-Wallis analysis followed by the Mann-Whitney U-test was assessed to compare differences in fluid intake between the groups. The individual changes in fluid intake between the different weeks with access to ethanol and water were analyzed using the Wilcoxon signed rank test. Since the ethanol consuming rats had a large variation in their intake, these groups were divided into two subgroups, i.e. those drinking >1 g/kg/day (high ethanol consuming, HEC) and those drinking <1 g/kg/day (low ethanol consuming, LEC). The statistical analyses were performed separately in these groups.

Statistical analyses were conducted with StatView v5.0.1 (SAS Institute Inc., Cary, NC) for Macintosh computers. Differences were considered significant at $p < 0.05$.

In Paper II-IV the traditional statistical analyses were further supplemented by use of multivariate data analysis. The principal component analysis (PCA) (Jackson, 2003, Eriksson et al., 2006) was performed in order to illustrate the relationship between the five experimental groups. This was performed to see patterns in the neurochemical results within the same animal. The programme creates a score and a loading plot. The score plot shows the summary of the relationship among the individuals, while the loading plot identifies variables important for creating these relationships. SIMCA-P+ software version 12.0 (Umetrics AB, Umeå, Sweden) was used.
Results and discussion

In the present thesis, MS was performed between postnatal days 1-21. The rat pups were separated either 15 or 360 minutes from their mother. In previous studies using this model, the consequences of litter-wise and individual MS on basal opioid peptide levels (Ploj et al., 2000, Ploj et al., 2002, Ploj and Nylander, 2003, Ploj et al., 2003b), on voluntary ethanol consumption (Ploj et al., 2003a, Roman et al., 2003) and ethanol-induced effects on opioid levels (Gustafsson et al., 2007) in adult rats have been reported. In the present thesis the impact of individual MS were studied and also compared with the effects of litter-wise MS in young as well as adult rats. In Paper I, the ethanol consumption and ethanol-induced effects on monoamines were analysed in MS15i and MS360i rats. In Paper II-IV the effects of individual and litter-wise MS, respectively, were examined at two time points and the effects on 5-HTergic gene expression (Paper II), opioid peptides (Paper III) and OT and AVP peptides (Paper IV) were reported. Animal facility reared rats, AFR, served as controls in all studies.

Effects on body weight (Papers I-IV)

During the first three postnatal weeks, the body weights were taken for the entire litters. The body weight is thus given as mean pup weight for each litter in the different experimental groups. In Paper I, using MS15i, MS360i and AFR groups, lower body weight was observed in the MS360i group as compared to the other experimental groups (Figure 2A). In Papers II-IV, the experimental groups included five rearing environments, i.e. MS15l, MS360l, MS15i, MS360i and AFR groups. Lower body weights were seen in the MS360l and MS360i litters as compared to the AFR litters (Figure 2B). In addition, an effect of the rearing environment (MS15 versus MS360) was detected with lower body weights in the MS360 groups as compared to the MS15 groups, whereas no effect on separation condition (litter-wise versus individual) was seen. These results strongly indicate that the differences observed are mainly due to the duration of separation and that the separation condition, that is tactile contact with littermates or not, had minor influence. Furthermore, the results in the litter-separated pups are also congruent with our previous studies. During the MS period, the MS360l/ pups had lower body weight than MS15l and/or AFR pups (Roman et al., 2005, Gustafsson
and Nylander, 2006, Gustafsson et al., 2007). The differences in body weight in the young MS360 rats may be affected by numerous factors, such as the housing temperature during separations, feeding pattern and maternal behaviour. The normal nest temperature is reported to be approximately 34°C (Kuhn and Schanberg, 1998, Zimmerberg and Brown, 1998), which is higher than the temperatures used in the present thesis (25°C in litter-wise MS and 32°C in the individual MS). Thus, this condition may have activated processes that demand increased energy utilization and thereby affect body weight (Blumberg and Sokoloff, 1998, Ruedi-Bettschen et al., 2004). Moreover, since the duration of the prolonged MS is 360 min, it might be speculated that feeding behaviour was altered in the pups. However, a visible inspection after the separations upon reunion with their mother reveals that the pups have milk left in their stomach. This can be noticed until the pups develop fur. In a review article, Macrì and Würbel highlighted that the mothers compensate by frequent feeding bouts when mother and pups are together (Macrì and Würbel, 2006). These results indicate that the rat pups are not starving although the feeding pattern is disrupted. In addition, the reduced weight gain in MS360 rats may be a result of altered maternal behaviour of the dam (Boccia et al., 2007). This situation could for example affect the nursing behaviour of the dam or affect the physiology and thereby changing the milk composition to less nutritious milk.

Figure 2. The body weights in the experimental groups during the first three postnatal weeks, i.e. during the MS period. The values represent the mean rat pup weight/litter. A) Illustrates the body weight increase in MS15\(i\), MS360\(i\), and AFR rats. B) Illustrates the body weight in MS15\(i\), MS360\(i\), MS15\(l\), MS360\(l\) and AFR rats. *MS360\(i\), as compared to AFR; #MS360\(i\), as compared to AFR; §MS360\(i\), as compared MS15\(l\); °MS360\(i\), as compared to MS15\(l\).

In the adult male rats at 10 weeks of age, lower body weight in the MS360\(i\) rats was seen as compared to MS15\(i\) and AFR rats (Paper I). Thus, a persistent effect on body weight is found after individual MS, and that has not been reported in studies using litter-wise MS where no differences are observed between the adult MS15, MS360 and AFR rats (Gustafsson and Nylander, 2006, Gustafsson et al., 2007). In addition, in Paper I it is shown that after seven weeks of voluntary ethanol consumption, these differences
persisted. However, no differences were noted between the ethanol- and water-consuming males. The food intake during the ethanol and/or water access period was initially lower in the ethanol-consuming rats than in the water-consuming rats, but did not differ between the ethanol-consuming groups. Otherwise no differences in food intake were observed.

Taken together, the MS360 rats have significantly lower body weight than other rats during the first postnatal weeks and the differences persist into adulthood for the MS360i males. In MS studies, any effect on parameters such as body weight may be an obstacle and in that respect the individual MS paradigm are suggested to be less suitable than the litter-wise MS. However, it is less possible that this might have affected the neurobiology and fluid intake behaviour studied in the present thesis. The changes seen when analysing the neurobiology were not in accordance with the changes observed in body weight.

**Voluntary ethanol consumption**

In Paper I, the male rats were subjected to repeated individual MS during PND 1-21 and then seven weeks of conventional group housing in the animal facility. From the age of ten weeks they were given access to ethanol or water using the two-bottle free-choice drinking paradigm during seven weeks. The AFR rats had a faster acquisition of 8% ethanol than the other rats. However, no statistically significant differences in ethanol intake were seen between MS15i, MS360i and AFR rats. In addition, a large variation in ethanol consumption was detected within all three experimental groups. The ethanol consumption and variability in the MS360i and AFR groups are in agreement with previous findings after MS360l and AFR. However, the MS15i rats differed from previous reports of ethanol consumption in MS15l rats (Roman and Nylander, 2005, Gustafsson and Nylander, 2006, Moffett et al., 2007). They had higher ethanol intake and a larger variability within the group.

Prolonged periods of MS thus result in the same adult voluntary ethanol intake irrespective of separation condition. The supposedly more stressful individual separation did not result in higher ethanol consumption. Based on the previous findings of low ethanol intake and low preference for high ethanol concentrations in MS15l rats it has been suggested that this rearing condition represents a protective environment (Roman and Nylander, 2005, Gustafsson and Nylander, 2006, Moffett et al., 2007) The results presented in the present study indicated that this was not the case for MS15i. Thus, with the MSi condition where rat pups were separated not only from the mother but also from littermates, the repeated short separations were not related to a protective environment in terms of adult ethanol consumption.

Previous reports of individual variability after MS360l have led to the proposition of subgroups within the MS360 group, i.e. some rats were considered
more sensitive to early environmental influence as evidenced by higher voluntary ethanol intake and preference for high ethanol concentrations in adulthood (Ploj et al., 2003a, Gustafsson and Nylander, 2006). In the present study, the ethanol intake and individual differences were similar regardless of rearing condition. These results indicate the presence of individual differences in response to environmental experiences in all groups. A closer analysis of the individual ethanol intake the last week before decapitation revealed that approximately half the number of the rats within each experimental group had an ethanol consumption $>1g/kg/day$. The rats consuming $>1g/kg/day$ ethanol the last week were designated as the high-ethanol consuming (HEC) rats, while those consuming $<1g/kg/day$ were assigned as low-ethanol consuming (LEC) rats. The statistical analyses showed that the MS15-LEC group consumed less ethanol as compared to the MS360-LEC rats, whereas similar ethanol consumption was found in the MS15-HEC, MS360-HEC and AFR-HEC rats. Thus, it is possible that when using the repeated individual MS paradigm to predict differences in ethanol intake between the different early environments longer exposure to ethanol than seven weeks is needed.

Monoamines and metabolites

Repeated administration of ethanol alters the neurobiology and may in turn lead to the progression from voluntary intake of ethanol to habitual and compulsive consumption (Everitt et al., 2008, Belin et al., 2009). Nevertheless, the neurobiological mechanisms behind this shift in behaviour are not yet understood. Therefore, in Paper I, we analysed DA and 5-HT measurements in brain areas related to addiction in male rats subjected to repeated individual MS and at ten-weeks of age given access to either ethanol/water or water/water in a two-bottle free-choice paradigm. All adult rats including the water- (MS15W, MS360W and AFRW) and ethanol-consuming rats (MS15-HEC, MS360-HEC, AFR-HEC, MS15-LEC, MS360-LEC and AFR-LEC) were individually housed during the seven weeks of free choice drinking.

Basal effects after individual MS

The analysis of monoamines and metabolites in water-drinking rats were considered basal levels in individually housed rats and used as controls for the levels in the respective ethanol-drinking rats. In the 17-weeks old water-drinking male rats, changes in DA and 5-HT levels were found in the frontal cortex, NAcc, amygdala and VTA, i.e., in the mesocorticolimbic areas involved in the acquisition and maintenance of ethanol consumption (Koob et al., 1998b, Everitt et al., 2008). In the frontal cortex (Figure 3A), differences in the DA system was detected, while in the NAcc (Figure 3B), amygdala (Figure 3C) and VTA (Figure 3D) changes were seen in the 5-HT systems suggesting that MS induces different long-term changes in different brain
regions and transmitter systems. The DA projections to cortical areas are sensitive to various stressors (Deutch and Roth, 1990, Horger and Roth, 1996, Finlay and Zigmond, 1997, Tonissaar et al., 2004). In addition, mesocortical DA may be involved in adaptation processes to environmental challenges, for instance, stress-related changes in the environment (Morrow et al., 1999) but also to chronic drug intake (Jentsch and Taylor, 1999). Furthermore, lower levels of 5-HT were seen in the NAcc and VTA in the MS360W rats indicating environmentally induced adaptations in this system that might lead to changes in adult behaviour. 5-HT dysfunction has, for instance, been linked with increased vulnerability for psychopathology including ethanol reward and reinforcement (McBride and Li, 1998, Barr et al., 2003, Oreland, 2004). In the amygdala, the 5-HT ratio was higher in the MS360W rats. In addition, a trend towards lower 5-HT levels was seen in the same group which may therefore support the notion of reduced 5-HT innervations to the amygdala following maternal deprivation (Andersen et al., 1999). A dysfunction in the 5-HT system has

\[ \text{Figure 3.} \] The tissue levels of monoamines and their metabolite/transmitter ratios in water-drinking MS15W, MS360W and AFRW rats in the A) frontal cortex, B) nucleus accumbens (NAcc), and C) amygdala. \(*\ast p < 0.01, \#p < 0.05, \#\# p < 0.01\), as compared to MS15W; \#p < 0.05, \#\# p < 0.01, as compared to AFRW.

been linked with increased vulnerability for instance to high ethanol intake (Higley and Bennett, 1999, Barr et al., 2003, Oreland, 2004, Nilsson et al., 2005). The present results are in line with previous studies showing that early environmental factors can cause alterations in monoamine circuits (Liu et al., 2000, Matthews et al., 2001, Caspi et al., 2002, Caspi et al., 2003, Gartside et al., 2003, Ploj et al., 2003a, Arborelius et al., 2004, Brake et al., 2004, Vicentic et al., 2006, Arborelius and Eklund, 2007). The results provide evidence for MS360i being more affected relative to the other groups. A deranged monoamine system in rats subjected to this putative stressful environment may contribute to an altered response to ethanol that indeed was seen in the rats that were drinking ethanol.
Ethanol-induced effects after individual MS

Voluntary ethanol consumption for seven weeks induced region- and transmitter-specific alterations depending on the ingested ethanol dose. In addition, some ethanol-induced effects were similar in all experimental groups, i.e. independent of early rearing environment, whereas other effects differed indicating that the neurobiological responses are dependent on early environmental factors. As mentioned before, the rats were divided into HEC (consuming >1g/kg/day ethanol) and LEC (consuming <1g/kg/day ethanol) groups due to a large variation in ethanol consumption the last week before decapitation.

In the HEC rats, voluntary ethanol intake for seven weeks produced changes in the DA system in the caudate putamen regardless of early rearing environment. It was noted that no effects of long-term voluntary drinking were observed in the NAcc. These findings provide further evidence to the notion that tolerance develops to the initial rewarding effects of ethanol mediated by the mesocorticolimbic DA system (Koob et al., 1998a, Wise, 2008). Nevertheless, the higher tissue levels of DA and DOPAC seen in the caudate putamen of the HEC rats as compared to the water-drinking rats may indicate the putative role of this brain area in mechanisms underlying the progression from voluntary to habitual intake of ethanol (Everitt et al., 2008, Belin et al., 2009).

Interestingly, opposite effects were detected in the VTA and DRN after long-term ethanol consumption in the MS360-HEC and AFR-HEC rats. However, the MS15-HEC rats were seemingly unresponsive to ethanol (Figure 4). Ethanol intake increased the VTA-5-HIAA levels (Figure 4A), with the same trend seen for 5-HT, in the MS360-HEC rats indicating an up-regulated 5-HT system. The results in the VTA somewhat reflected the outcome in the DRN, where the MS360-HEC rats had higher 5-HIAA levels as compared to the AFR-HEC rats (Figure 4B). The lower 5-HIAA levels seen in the AFR-HEC rats may be due to inhibitory effects of ethanol consumption. Furthermore, the DRN-DA levels (Figure 4C) induced opposite effects of ethanol in the MS360-HEC and AFR-HEC rats. However, in contrast to 5-HT, the DA system seems to be down regulated, since lower levels of DA with the same trend for the metabolites are seen. Interaction between 5-HT and DA networks in the VTA and DRN are well established (Di Giovanni et al., 2008), hence the opposing effects in the 5-HT and DA systems seen in the present thesis are interesting. The DAergic projections originating from the VTA and terminating in the DRN seems to have excitatory effects. On the hand, the DRN-5-HT neurons tonically inhibit DA neurons in the VTA and thus suppress the reward circuits (Liu and Ikemoto, 2007, Nakamura et al., 2008). The findings in the MS360-HEC and AFR-HEC rats suggest that prolonged activation of the mesocorticolimbic DA reward pathways may induce different compensatory effects in the DRN.
Figure 4. Neurochemical measurements in HEC rats consuming >1g/kg/day the week prior to decapitation. The figure illustrates 5-HIAA levels in the ventral tegmental area and DA and 5-HIAA levels, respectively, in the dorsal raphe nucleus. I = interaction effect between rearing environment and fluid intake. *p < 0.01, **p < 0.01.

The LEC rats consumed 0.7 and 0.9g/kg/day ethanol the last week before decapitation. Although, the amount of ethanol was low, it was sufficient enough to induce changes in the monoamine measurements. In fact, acute doses as low as 0.5g/kg/day have been reported to accomplish changes in monoamines (Di Chiara and Imperato, 1985, Tizabi et al., 2002, Apter and Eriksson, 2006). In the present study, seven weeks of voluntary ethanol intake produced changes in the 5-HT system in the cingulate cortex and VTA regardless of early rearing environment as compared to the water-drinking rats. In both areas, an increase in 5-HT levels was found. In addition, 5-HIAA levels were increased in the VTA after ethanol intake, but only in the MS360-HEC rats. It is widely accepted that 5-HT networks are affected by
ethanol (LeMarquand et al., 1994, Spanagel, 2009) and chronic ethanol administration has been shown to produce transient increases in 5-HT levels and functioning (LeMarquand et al., 1994).

Also in the LEC rats, chronic intake differentially affected monoamines in the MS360 and AFR rats in certain brain areas whereas MS15 rats were again unresponsive to ethanol. In the frontal cortex decreased DA levels was seen in MS360-LEC rats and decreased DA turnover in AFR-LEC rats (Figure 5A). Higher cortical DA levels were found in the MS360W as compared to the other groups. Hence, it appears that voluntary consumption of low doses of ethanol for seven weeks normalized the levels in this group, which was not the case for the MS360-HEC rats. In the hippocampus, low ethanol intake induced opposite effects in MS360-LEC and AFR-LEC rats on 5-HT turnover. The MS360-LEC rats had an increased whereas the AFR-LEC rats had a decreased 5-HT turnover (Figure 5B). This brain region has been suggested to process contextual drug associations, which contribute to context-evoked craving and drug-seeking behaviour (Gould, 2006). Hence, the changes seen in 5-HT neurotransmission may affect such behaviour in adulthood and thereby influence drinking habits differentially depending on the early environmental experiences.

Long-term drinking of low doses of ethanol produced different effects in the amygdala depending on the early environmental rearing condition. The MS360-LEC rats retained higher 5-HT levels and corresponding lower ratio, whereas the contrary was observed in the AFR-LEC rats with regard to the 5-HT levels (Figure 5C). It is well established that chronic ethanol intake

![Figure 5](image)

**Figure 5.** Neurochemical measurements in LEC rats consuming <1g/kg/day the week prior to decapitation. The figure illustrates A) DA levels and DOPAC/DA ratio in the frontal cortex, B) 5-HT levels and 5-HIAA/5-HT ratio in the hippocampus, C) 5-HT levels and 5-HIAA/5-HT ratio in the amygdala. I = interaction effect between rearing environment and fluid intake. *p < 0.01, **p < 0.01, ***p < 0.001.
leads to adaptations of several transmitter systems in the amygdala (Koob, 2003, Everitt et al., 2008). Furthermore, early-life experiences affect ethanol-induced effects on opioid peptides differentially in the amygdala (Gustafsson et al., 2007). The present study thus provides further evidence suggesting a key role for the amygdala in individual differences in drug-induced effects caused by early environmental experiences.

Taken together, albeit minor differences in ethanol intake were seen, the long-term ethanol consumption indeed induced brain region- and transmitter-specific changes in the adult male rats subjected to different early-life experiences. The changes depended furthermore on the dose of ethanol ingested. Often, opposite effects were found in the ethanol drinking MS360 and AFR rats, while ethanol did not induce changes in the MS15 rats. Interestingly, the lower doses of ethanol affected different 5-HT pathways when compared to the higher doses. It is suggested that the LEC rats have low intake because they preferentially seek effects related to the low doses of ethanol. On the other hand, they may have low ethanol consumption as a result of such effects. In this case the effects observed in LEC rats would relate to protection against excessive consumption whereas the effects in HEC rats would relate to vulnerability to progress into high consumption.

Serum corticosterone and testosterone after individual MS

The consequence of repeated individual MS (MS15W, MS360W and AFRW) and long-term voluntary ethanol consumption (MS15E, MS360E and AFRE) on ir corticosterone and testosterone serum levels was examined in adult male rats.

Corticosterone exhibits a well-established diurnal variation (Windle et al., 1998, Lightman et al., 2000). However, there were no differences in diurnal variation between rats subjected to different early rearing. The serum ir corticosterone levels in ethanol- and water-consuming rats exposed to different early rearing environment are shown in Table 1. The HPA-axis is a target for early environmental factors and several studies have reported effects on corticosterone levels in rats subjected to repeated litter-wise MS (Lehmann and Feldon, 2000, Pryce and Feldon, 2003). In line with other studies (Lehmann and Feldon, 2000, Pryce and Feldon, 2003, Roman et al., 2006), the present study supports the notion that basal corticosterone levels are not affected in rats subjected to MS using up to six hours of individual MS. Furthermore, no significant effect of solution was found between the water- and ethanol-drinking rats, indicating that voluntary drinking for seven weeks had no effect. In addition, no interaction between rearing condition (MS15 versus MS360 versus AFR) and fluid intake (water versus ethanol) was observed. Reports of ethanol-induced alterations on HPA-axis function are numerous but not easy to interpret since the effects of ethanol are dependent on, for
instance, doses, administration routes and addiction phase (Dai et al., 2007). Findings in the current study are in line with previous studies showing that low doses of ethanol do not alter the concentration of this hormone (Eriksson et al., 1983). We can conclude that long-term voluntary ethanol consumption had no effects on circulating corticosterone and that the rearing condition had no effect on the response to ethanol.

<table>
<thead>
<tr>
<th>Rearing environment</th>
<th>A.M.***</th>
<th>P.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>70 ± 12</td>
<td>393 ± 88</td>
</tr>
<tr>
<td>Ethanol</td>
<td>142 ± 33</td>
<td>372 ± 45</td>
</tr>
<tr>
<td>MS360</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>170 ± 45</td>
<td>351 ± 43</td>
</tr>
<tr>
<td>Ethanol</td>
<td>124 ± 42</td>
<td>274 ± 45</td>
</tr>
<tr>
<td>AFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>100 ± 30</td>
<td>299 ± 58</td>
</tr>
<tr>
<td>Ethanol</td>
<td>120 ± 18</td>
<td>252 ± 50</td>
</tr>
</tbody>
</table>

Table 1. Ir corticosterone serum levels (nmol/l; mean ± SEM) in MS15, MS360 and AFR rats having access to water or ethanol solution and sacrificed either A.M. or P.M. ***p < 0.001 as compared to P.M.

The serum ir testosterone levels in the ethanol- and water-consuming rats subjected to different early rearing environment are illustrated in Figure 6. One research group has reported MS-induced alterations in female gonadal hormones (Gomes et al., 2005), but whether this is true for testosterone in male rats has, to our knowledge, not previously been studied. In the present study, basal testosterone levels were the same in the MS15W, MS360W and AFRW groups showing that neither handling procedures nor separation length affected circulating ir testosterone levels. It has been suggested that shown that ethanol suppresses the HPG-axis (van Thiel et al., 1979, Cicero, 1981, 1982, Emanuele and Emanuele, 2001, Kim et al., 2003, Dissen et al., 2004), which thereby reduces the serum testosterone levels after chronic ethanol exposure (van Thiel et al., 1979). However, in the present study no ethanol-induced effects on ir testosterone levels were found after seven weeks of voluntary ethanol drinking. Although, it is worth mentioning that the role of testosterone in ethanol drinking is complex. On one hand, testosterone stimulates the development of ethanol preference (Lakoza and Barkov, 1980), while on the other hand, testosterone itself is affected after chronic ethanol administration (Little et al., 1992, Jimenez et al., 2004). However, these animals received higher concentration of ethanol as compared to the current study. Unaltered levels have been shown after low doses of ethanol (Apter and Eriksson, 2006) and the ethanol ingested in a free-choice drinking paradigm may not be enough to alter circulating testosterone. Many reports show that circulating testosterone is subjected to large variations, such as individual, diurnal and seasonal variations (Mock et al., 1975, Kalra and Kalra, 1977, Heywood, 1980, Leal and Moreira, 1997). Interestingly, in the present study, a diurnal variation was detected in the
MS360E group. The MS360E males sacrificed *ante meridiem* (A.M.) had higher testosterone levels than MS360E males sacrificed *post meridiem* (P.M). Taken together, even though no differences were observed in MS- or ethanol-induced levels, the rearing condition altered the cyclic pattern of testosterone, a finding that needs to be further investigated in terms of possible behavioural consequences.

**Figure 6.** Serum ir testosterone levels (nmol/l; mean ± SEM) in A) MS15W, MS360W, AFRW, and B) MS15E, MS360E and AFRE. The black bars represent hormone levels in rats sacrificed A.M., while the grey bars represent hormone levels in rats sacrificed P.M. *p* < 0.05, MS360E A.M. as compared to MS360E P.M.

### Effects of individual and litter-wise MS (Papers II-IV)

Various MS procedures complicate the interpretation of the impact of MS on endocrinology, neurobiology and behaviour (Lehmann and Feldon, 2000, Pryce and Feldon, 2003, Roman and Nylander, 2005, Macrì and Würbel, 2006, Moffett et al., 2007). Parameters, such as duration (short vs. prolonged) and condition (litter-wise vs. individual) of MS combined with the evaluation at different time points have, to our knowledge, not previously been evaluated in the same experimental setting. It was also of interest to examine the differences between different MS groups with the conventional laboratory control group, AFR.
The 5-HT transporter and receptors (Paper II)

Short- and long-term effects of MS on the gene expression levels of 5-HT transporter and receptors related to anxiety-like behaviour, reward and reinforcement (Lovinger, 1999, Salchner and Singewald, 2006) were measured in the brain stem of three- and ten-week old male and female rats subjected to MS15l, MS360l, MS15i, MS360i and AFR.

The impact of handling (AFR vs. MS)

The 5-HT receptors and transporter mRNA levels in young and adult male and female rats reared in different postnatal environments is shown in Figure 7. Herein, the 5-HT 1A, 5-HT 2A, 5-HT 2C and 5-HT 3 receptors and the 5-HTT gene transcripts were examined. In the male rats, all genes except the 5-HT 3 receptor gene were affected by MS. Short-term MS-induced effects were found in the 5-HT 1A, 5-HT 2A and 5-HT 2C receptors mRNA levels. The changes in the 5-HT 1A, and 5-HT 2C persisted into adult age. In addition, as adults, differences between the experimental groups were also seen in the gene transcript level of the 5-HTT gene. Interestingly, whenever differences were observed, only the male MS15l group differed from all experimental groups with lower gene transcript levels. Also note that the putative most stressful MS360 male rats had similar mRNA levels as the commonly used laboratory AFR rats in all genes measured in the current study. In the female rats, MS induced changes in the 5-HT 2C receptor gene in the adult rats and the 5-HT 3 receptor gene in the young rats. In contrast to the male rats, higher mRNA levels were found in the MS15l females as compared to some of the other rearing environments, such as the MS360l and AFR conditions.

The DRN contains the highest density of 5-HTT (Hrdina et al., 1990). Therefore, the lower expression of 5-HTT gene transcripts seen in the adult MS15l males may be reflecting a decrease seen in DRN. These changes could increase the 5-HT transmission, if more 5-HT is released into the extracellular fluid, affecting developmental processes and resulting in axonal sprouting of the developing neurons (Hansen and Mikkelsen, 1998). Indirectly, this might increase the 5-HTT mRNA expression, as would be the case for the putative more stressful rats, i.e. the MS15i, MS360l, MS360i and AFR rats, in this study. On the other hand, the differences seen in this gene could also be secondary MS-induced effects due to changes in the 5-HT 1A, 5-HT 2A and 5-HT 2C receptor genes. It might be speculated that changes in expression of any, or all of these receptors in young rats could lead to a compensatory change in 5-HTT gene expression in adulthood. The finding regarding the lower expression of 5-HT 1A receptors in the MS15l males most likely represents a decreased expression of somatodendritic 5-HT 1A autoreceptors in this region (Laaris et al., 1997). A decreased expression of soma-
Figure 7. The gene expression of A) 5-HT₁A receptor, B) 5-HT₂A receptor, C) 5-HT₂C receptor, D) 5-HT₃ receptor and E) 5-HTT in young and adult male and female rats, respectively. The values are expressed as % of AFR. *p < 0.05, **p < 0.01, ***p < 0.001. The statistically significant effects observed from the two-way ANOVA analysis are shown in the graphs as E (rearing environment), C (separation condition) and I (interaction between E and C). In each bar, the number of rats in each experimental group and gene is shown.
todendritic 5-HT₁₅ autoreceptors results in less feedback inhibition of DRN-5-HT neurons located in the brainstem and thereby an altered sensitivity and/or a higher activity in the MS15₁ males. This would be in line with results from a previous study showing that the inhibitory effect on 5-HT neuronal firing of the selective 5-HTT inhibitor citalopram, which is mediated by an increased stimulation of these autoreceptors, is lower in adult MS15₁ male rats as compared to male rats subjected to prolonged 180 minutes of litter-wise MS (Arborelius et al., 2004). The long-term changes in 5-HT₁₅ and 5-HTT mRNA levels found in the present study indicate an MS-induced altered 5-HT function and possibly an altered vulnerability for later challenges and thereby contribute to the previously described differences in voluntary ethanol consumption in MS15₁ and MS360₁ rats (Ploj et al., 2003a, Gustafsson and Nylander, 2006). The lower expression of both 5-HT₂A and 5-HT₂C receptors in the MS15₁ males as compared to the other experimental groups may influence a multitude of behavioural and physiological responses. The brain stem 5-HT₂C receptor is involved in the mediation of anxiety-like behaviour (Salchner and Singewald, 2006). A lower gene expression of 5-HT₂C receptors was seen in adult male MS15₁ rats but a higher gene expression in the female MS15₁ rats. Some studies have reported reduced anxiety-like behaviour in male MS15₁ rats in comparison to non-handled rats (Bodnoff et al., 1987, Nunez et al., 1995), whereas other studies reported no effects on anxiety-like behaviour (Eklund and Arborelius, 2006). The brain stem 5-HT₃ receptor is involved in, for example, in feeding behaviour (Himmi et al., 1996). However, further MS investigations of brain stem 5-HT transmission are needed to understand the consequences of an altered expression of 5-HT₂C receptors in the short MS15₁ rats.

**Influence of duration and condition on MS effects**

MS15₁ males often differed from the MS15ᵢ males, whereas no differences in expression pattern between the MS360ᵢ and MS360ᵢi males were seen. Hence, the separation condition during MS seems to exert a large influence in rats separated for 15 min. The mother is absent both during the MS15₁ and MS15ᵢ procedure but during the individual separation, the rat pups were also deprived of tactile contact from their littermates although they could hear and smell the other pups. Postnatal tactile stimuli is of importance for normal development (Panksepp et al., 1997, Chatterjee et al., 2007) and the results presented here clearly show that daily loss of tactile contact with littermates for 15 minutes is enough to alter gene expression. After prolonged separation, on the other hand, the gene expression was the same regardless of separation condition. These results indicate that deprivation of tactile stimuli has no impact on the consequences of longer periods of MS on the gene expression of 5-HTT or the 5-HT receptors studied.
**Sex-specific effects**

Another interesting finding in Paper II was the sex differences in the gene transcript levels observed. Generally, fewer 5-HT-related genes were affected in female than in male rats and whenever differences between the rearing groups were seen the effects were opposite to those seen in the male rats. Studies examining the psychiatric conditions in relation to the 5-HT systems have shown differences between the sexes (Oreland et al., 1981, Kessler et al., 1993, Zhang et al., 1999, Gorman, 2006, Weiss et al., 2006, Cosgrove et al., 2007, Jovanovic et al., 2008) and especially with respect to ethanol consumption in non-human primates and rodents (Higley et al., 1991b, Vazquez et al., 2002, Barr et al., 2003). Furthermore, additional evidence for the sex-specific consequences of MS has been shown in several previous studies (Lehmann et al., 1999, McIntosh et al., 1999, Wigger and Neumann, 1999, Eklund and Arborelius, 2006). Fewer MS-induced effects in females than in males in terms of expression of 5-HT related genes may support the notion that males are more prone to be affected by the early environmental factors and as regard to higher ethanol consumption than females (Roman et al., 2005).

Taken together, a pronounced effect on gene expression levels of 5-HT receptors and transporter in the brainstem was found in the young and adult MS15/l male rats compared to the rats subjected to MS360/l, MS15/i, MS360/i and AFR conditions. Additionally, the differences between the litter-wise and individual MS15 group seen herein suggests that the individually short separated MS15/i group does not represent a protective environment as shown in litter-wise MS. The MS-induced effects were either transient or persistent and further pronounced impact of sex was observed.

**The opioid peptides (Paper III)**

Short- and long-term effects of MS on ir levels of MEAP and DYNB were measured in nine different brain structures related to addiction processes in three- and ten-week old male rats subjected to MS15/l, MS360/l, MS15/i, MS360/i and AFR. DYNB is exclusively derived from prodynorphin and MEAP from proenkephalin and therefore these peptides were measured as the markers for these two opioid peptide systems (Nylander et al., 1994, Ploj et al., 2000).

**Impact of handling (AFR vs. MS)**

The ir MEAP and DYNB levels in the young and adult male rats reared in different postnatal environments are shown in Table 2 and 3. A general finding in the present study was that the differences in ir MEAP levels were more pronounced, while minor changes were seen in the ir DYNB levels. Since, the enkephalin system is still quite immature as compared to the
dynorphin system during the postnatal period (McDowell and Kitchen, 1987, Leslie and Loughlin, 1993), the differences in development may explain this outcome. The present study also found that the MS15 groups had higher ir peptide levels as compared to the AFR group. Higher ir levels were observed in the pituitary gland, hypothalamus and caudate putamen in the young rats. However, in the NAcc, the ir DYNB levels were lower while the ir MEAP levels were higher, which interestingly supports the notion of the opposite action of these peptides on the mesocorticolimbic dopaminergic neurons.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>MS15/</th>
<th>MS360/</th>
<th>AFR</th>
<th>MS15/</th>
<th>MS360/</th>
</tr>
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<td><strong>Three-week old rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>1.39 ± 0.15**</td>
<td>1.02 ± 0.14#</td>
<td>0.86 ± 0.07</td>
<td>1.64 ± 0.23***</td>
<td>0.76 ± 0.10$$§§§</td>
</tr>
<tr>
<td>HT</td>
<td>50.0 ± 1.81**</td>
<td>47.0 ± 1.52</td>
<td>41.9 ± 2.14</td>
<td>53.3 ± 2.00***</td>
<td>45.0 ± 1.85$$</td>
</tr>
<tr>
<td>FCx</td>
<td>7.23 ± 1.22</td>
<td>8.78 ± 1.18</td>
<td>7.50 ± 0.90</td>
<td>9.03 ± 0.81</td>
<td>12.5 ± 1.02$$§§§</td>
</tr>
<tr>
<td>MPFC</td>
<td>2.66 ± 0.26</td>
<td>2.07 ± 0.23</td>
<td>2.22 ± 0.23</td>
<td>2.55 ± 0.25</td>
<td>2.53 ± 0.24</td>
</tr>
<tr>
<td>NAcc</td>
<td>57.9 ± 5.17</td>
<td>53.8 ± 3.85</td>
<td>49.5 ± 3.60</td>
<td>62.8 ± 4.28*</td>
<td>69.9 ± 4.88**</td>
</tr>
<tr>
<td>VTA</td>
<td>8.72 ± 1.20</td>
<td>9.39 ± 1.27</td>
<td>7.49 ± 1.13</td>
<td>10.1 ± 1.41</td>
<td>9.87 ± 0.92</td>
</tr>
<tr>
<td>CPu</td>
<td>57.2 ± 3.10</td>
<td>59.8 ± 2.97</td>
<td>51.5 ± 3.48</td>
<td>71.3 ± 5.16**</td>
<td>53.4 ± 2.30$$</td>
</tr>
<tr>
<td>SN</td>
<td>6.21 ± 0.97</td>
<td>6.80 ± 0.76</td>
<td>4.51 ± 0.43</td>
<td>5.00 ± 0.59</td>
<td>7.08 ± 0.73**§§</td>
</tr>
<tr>
<td>AMY</td>
<td>14.9 ± 1.56</td>
<td>19.4 ± 2.54</td>
<td>19.3 ± 1.67</td>
<td>15.8 ± 1.30</td>
<td>25.8 ± 3.05$$§§§</td>
</tr>
<tr>
<td>PAG</td>
<td>35.8 ± 1.68</td>
<td>32.6 ± 2.93</td>
<td>33.8 ± 1.65</td>
<td>38.2 ± 3.15</td>
<td>41.2 ± 2.06</td>
</tr>
<tr>
<td>HC</td>
<td>3.85 ± 0.14</td>
<td>4.36 ± 0.20</td>
<td>4.15 ± 0.21</td>
<td>4.36 ± 0.25</td>
<td>4.12 ± 0.26</td>
</tr>
<tr>
<td><strong>Ten-week old rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>0.86 ± 0.14</td>
<td>0.86 ± 0.07</td>
<td>0.95 ± 0.17</td>
<td>1.26 ± 0.18</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>HT</td>
<td>73.1 ± 3.41</td>
<td>71.6 ± 5.11</td>
<td>71.1 ± 2.69</td>
<td>77.4 ± 3.51</td>
<td>68.5 ± 1.84</td>
</tr>
<tr>
<td>FCx</td>
<td>7.77 ± 1.21</td>
<td>10.5 ± 0.62</td>
<td>7.78 ± 0.69</td>
<td>10.2 ± 1.06</td>
<td>9.37 ± 0.86</td>
</tr>
<tr>
<td>MPFC</td>
<td>4.12 ± 0.33**</td>
<td>3.64 ± 0.18*</td>
<td>2.82 ± 0.26</td>
<td>4.07 ± 0.29**</td>
<td>4.04 ± 0.35**</td>
</tr>
<tr>
<td>NAcc</td>
<td>47.8 ± 4.13</td>
<td>48.1 ± 4.06</td>
<td>54.2 ± 3.17</td>
<td>58.9 ± 4.74</td>
<td>51.3 ± 4.34</td>
</tr>
<tr>
<td>VTA</td>
<td>12.4 ± 1.55**</td>
<td>6.33 ± 1.15##</td>
<td>7.23 ± 0.97</td>
<td>11.2 ± 1.35</td>
<td>9.17 ± 1.16</td>
</tr>
<tr>
<td>CPu</td>
<td>72.8 ± 3.40</td>
<td>56.8 ± 5.10#</td>
<td>65.8 ± 4.09</td>
<td>73.3 ± 3.60</td>
<td>70.6 ± 2.76</td>
</tr>
<tr>
<td>SN</td>
<td>6.40 ± 0.72</td>
<td>4.19 ± 0.34*#</td>
<td>6.08 ± 0.75</td>
<td>5.50 ± 0.56</td>
<td>5.10 ± 0.58</td>
</tr>
<tr>
<td>AMY</td>
<td>31.7 ± 2.46***</td>
<td>22.8 ± 1.64##</td>
<td>18.0 ± 1.31</td>
<td>26.1 ± 2.73**</td>
<td>17.2 ± 1.29$$</td>
</tr>
<tr>
<td>PAG</td>
<td>39.1 ± 3.52</td>
<td>37.9 ± 1.95</td>
<td>49.9 ± 5.16</td>
<td>38.1 ± 2.69</td>
<td>45.6 ± 3.67</td>
</tr>
<tr>
<td>HC</td>
<td>4.55 ± 0.14</td>
<td>4.85 ± 0.19</td>
<td>4.97 ± 0.22</td>
<td>5.45 ± 0.24</td>
<td>5.76 ± 0.19*</td>
</tr>
</tbody>
</table>

Table 2. The ir MEAP levels in brain tissue extracts in three- and ten-week-old male rats reared in different environmental conditions. The levels represent mean ± SEM values (fmol/mg tissue) detected with radioimmunoassay. N = 9-10. *p < 0.05, **p < 0.01, ***p < 0.001, compared with AFR; #p < 0.05, ##p < 0.01, compared with MS15; and $p < 0.05, $$$p < 0.01, compared with MS15 (one-way ANOVA followed by Fisher’s PLSD post-hoc test). AMY; amygdala, FCx; frontal cortex, CPu; caudate putamen, HT; hypothalamus, MPFC; medial prefrontal cortex, NAcc; nucleus accumbens, PAG; periaqueductal gray, PG; pituitary gland, SN; substantia nigra. VTA; ventral tegmental area.
(Spanagel et al., 1992). In the adults, ir MEAP levels were again higher in the MS15 groups in the medial prefrontal cortex and amygdala. It is notable that in several areas the levels in MS15 rats were not only higher as compared to the AFR rats but also as compared to the MS360 rats. In other words, the MS360 rats resemble the AFR rats, while the MS15 rats differed.

**Influence of duration and condition on MS effects**

The differences between MS15 and MS360 were more pronounced in ir MEAP levels than in the ir DYNB levels. Furthermore, the time point for evaluation is important as evidenced by different results in young and adult rats, respectively. For example, individual MS resulted in region-specific short-term effects whereas the effects of litter-wise MS were detected in other areas and not until adulthood. In addition, fewer MS-induced effects were seen in the adult rats than in the young rats. However, the pituitary gland and amygdala appear to be important areas for the impact of duration rather than condition of MS. In the pituitary gland, a lower level of ir MEAP was seen in the young MS360 rats. In the amygdala, the levels were higher in the young MS360, but lower in the adult MS360 as compared to the MS15 rats at their corresponding age. The amygdala is related to HPA-axis responsiveness to stress and this axis is a target for early environmental factors, but the literature examining the responsiveness of this axis is not consistent (Macri and Würbel, 2006).

The finding of lower ir MEAP levels in the adult MS360/ rats in several brain areas in the mesocorticolimbic reward- and reinforcement-related networks (Berridge and Robinson, 2003, Everitt and Robbins, 2005, Koob, 2006) may contribute to the higher propensity for high ethanol intake in these rats as compared to MS15/ rats (Gustafsson et al., 2007). The opioid-dependent DA release in the NAcc (Spanagel et al., 1992) is closely associated to drug-induced reward (Di Chiara and Bassareo, 2007). Thus, the findings in the MS360 rats are in agreement with the hypothesis stating that an opioid hypofunction and/or a hypersensitivity to ethanol may lead to an initiation and/or maintenance of drug dependence (Gianoulakis and de Waele, 1994, Nylander et al., 1994, Nylander and Silberring, 1998, van Ree et al., 1999, Ploj et al., 2000, Ploj et al., 2003b, Oswald and Wand, 2004, Vazquez et al., 2005, Gustafsson et al., 2007).

Taken together, the MS-induced changes were more pronounced in ir MEAP levels. These results were especially seen in the young individually separated rats and in the adult litter-wise separated rats. The alterations in ir opioid peptide levels were found in brain areas related to stress, drug reward and reinforcement. Moreover, the dysfunction in the enkephalin networks seen in the adult MS360/ rats may consequently lead to the vulnerability for drug abuse later in life.
### Table 3.
The ir DYNB levels in brain tissue extracts in three- and ten-week-old male rats reared in different environmental conditions. The levels represent mean ± SEM values (fmol/mg tissue) detected with radioimmunoassay. \( N = 9-10 \). *\( p < 0.05 \), **\( p < 0.01 \), compared with AFR (one-way ANOVA followed by Fisher’s PLSD post-hoc test). AMY; amygdala, FCx; frontal cortex, CPu; caudate putamen, HT; hypothalamus, MPFC; medial prefrontal cortex, NAcc; nucleus accumbens, PAG; periaqueductal gray, PG; pituitary gland, SN; substantia nigra. VTA; ventral tegmental area.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>MS15/</th>
<th>MS360/</th>
<th>AFR</th>
<th>MS15i</th>
<th>MS360i</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Three-week old rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>105 ± 6.31*</td>
<td>85.5 ± 6.94</td>
<td>80.0 ± 7.28</td>
<td>98.3 ± 3.92</td>
<td>83.3 ± 6.57</td>
</tr>
<tr>
<td>HT</td>
<td>21.6 ± 0.71</td>
<td>22.6 ± 0.77</td>
<td>22.2 ± 1.73</td>
<td>21.1 ± 1.08</td>
<td>19.1 ± 0.72</td>
</tr>
<tr>
<td>FCx</td>
<td>3.59 ± 0.45</td>
<td>2.72 ± 0.68</td>
<td>1.96 ± 0.44</td>
<td>1.66 ± 0.35</td>
<td>2.09 ± 0.29</td>
</tr>
<tr>
<td>MPFC</td>
<td>1.07 ± 0.11</td>
<td>0.95 ± 0.09</td>
<td>1.00 ± 0.15</td>
<td>1.14 ± 0.10</td>
<td>1.10 ± 0.09</td>
</tr>
<tr>
<td>NAcc</td>
<td>15.0 ± 1.67**</td>
<td>14.3 ± 0.97**</td>
<td>21.9 ± 1.75</td>
<td>14.9 ± 1.46**</td>
<td>17.2 ± 1.29*</td>
</tr>
<tr>
<td>VTA</td>
<td>1.59 ± 0.19</td>
<td>2.24 ± 0.31</td>
<td>1.60 ± 0.21</td>
<td>2.09 ± 0.26</td>
<td>1.95 ± 0.26</td>
</tr>
<tr>
<td>CPu</td>
<td>10.8 ± 0.74</td>
<td>11.8 ± 0.83</td>
<td>9.57 ± 0.75</td>
<td>12.2 ± 0.86*</td>
<td>13.1 ± 0.63**</td>
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<tr>
<td>SN</td>
<td>37.7 ± 6.16</td>
<td>42.8 ± 3.97</td>
<td>41.9 ± 3.74</td>
<td>48.8 ± 7.68</td>
<td>40.2 ± 5.03</td>
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<td>AMY</td>
<td>2.91 ± 0.34</td>
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<td>3.75 ± 0.43</td>
<td>3.69 ± 0.32</td>
<td>4.82 ± 0.62</td>
</tr>
<tr>
<td>PAG</td>
<td>10.6 ± 1.20</td>
<td>10.6 ± 1.25</td>
<td>10.2 ± 0.67</td>
<td>12.0 ± 1.30</td>
<td>11.1 ± 0.72</td>
</tr>
<tr>
<td>HC</td>
<td>5.08 ± 0.35</td>
<td>4.67 ± 0.24</td>
<td>5.46 ± 0.32</td>
<td>4.23 ± 0.24</td>
<td>5.28 ± 0.68</td>
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</table>

<table>
<thead>
<tr>
<th><strong>Ten-week old rats</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>83.6 ± 5.65</td>
<td>87.4 ± 6.74</td>
<td>71.1 ± 6.26</td>
<td>85.7 ± 6.04</td>
<td>70.2 ± 3.63</td>
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<tr>
<td>HT</td>
<td>34.9 ± 0.80</td>
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<td>32.6 ± 1.56</td>
<td>35.9 ± 1.58</td>
<td>33.2 ± 1.17</td>
</tr>
<tr>
<td>FCx</td>
<td>2.28 ± 0.42</td>
<td>1.64 ± 0.34</td>
<td>1.69 ± 0.34</td>
<td>1.97 ± 0.31</td>
<td>2.08 ± 0.34</td>
</tr>
<tr>
<td>MPFC</td>
<td>0.89 ± 0.10</td>
<td>0.77 ± 0.06**</td>
<td>1.10 ± 0.06</td>
<td>1.06 ± 0.06</td>
<td>1.29 ± 0.10</td>
</tr>
<tr>
<td>NAcc</td>
<td>19.4 ± 1.80</td>
<td>20.6 ± 1.04</td>
<td>20.3 ± 1.83</td>
<td>22.3 ± 1.70</td>
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</tr>
<tr>
<td>VTA</td>
<td>1.90 ± 0.27</td>
<td>1.45 ± 0.16</td>
<td>1.37 ± 0.20</td>
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<td>2.23 ± 0.25*</td>
</tr>
<tr>
<td>CPu</td>
<td>11.6 ± 1.05</td>
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<td>15.1 ± 1.17</td>
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<tr>
<td>SN</td>
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<td>AMY</td>
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<tr>
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<td>HC</td>
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<td>8.77 ± 0.56</td>
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</tr>
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</table>

The oxytocin and vasopressin peptides (Paper IV)

Short- and long-term effects of MS on ir levels of OT and AVP were measured in the pituitary gland (the young rats) and NIL (the adults), hypothalamus and amygdala in three- and ten-week old male rats subjected to MS15/1, MS360/1, MS15i, MS360i and AFR. The involvement of OT and AVP in males is limited. In the current study, novel information regarding the peptide levels in rats subjected to different early-life events is presented in young and adult rats.
Impact of handling (AFR vs. MS)

The ir OT levels in the young and adult rats are illustrated in Figure 8 and 9. In the young rats, the MS15l, MS360l and MS15i groups differed in ir OT levels with regard to the AFR group. In the pituitary gland, higher levels of ir OT were found in these MS groups, while in the hypothalamus and amygdala, lower levels were seen. In addition, lower levels were found in the MS360i rats as compared to the AFR rats in the hypothalamus. Lukas and co-workers (Lukas et al., 2009) found an increase in hypothalamic OT receptor binding in eight- and 16-week old rats subjected to prolonged litter-wise MS180 rats as compared to the AFR rats. Therefore, we speculate that the changes could be compensatory due to the low peptide levels seen in the

![Figure 8](image)

**Figure 8.** The ir OT levels (pg/mg tissue) in tissue extracts of A) pituitary gland, B) hypothalamus and C) amygdala in three-week-old male rats reared in different environmental conditions. The levels represent mean ± SEM values detected with radioimmunoassay. MS15l, n = 9-10; MS15i, n = 9-10; MS360l, n = 9-10; MS360i, n = 9-10 and or AFR, n = 10. *p < 0.05, **p < 0.01, ***p < 0.001, compared with AFR and #p < 0.05, ##p < 0.01, ###p < 0.001. The statistically significant effects observed from the two-way ANOVA analysis are shown in the graphs as E (rearing environment) and C (separation condition).

In contrast to the apparent MS-induced effects on ir OT levels only minor changes were seen in the ir AVP peptide levels (Table 4). No long-term MS-induced effects were seen in the areas measured, which is in concordance with the previously published papers reporting no differences between the MS180l rats and AFR rats in AVP receptor binding and AVP mRNA and ir
Figure 9. The ir OT levels (pg/mg tissue) in tissue extracts of A) neurointermediate lobe, B) hypothalamus and C) amygdala in ten-week old male rats reared in different environmental conditions. The levels represent mean ± SEM values detected with radioimmunoassay in 10-week-old male rats. The rats were subjected to MS15l (n = 10), MS15i (n = 9-10), MS360l (n = 9-10), MS360i (n = 10) or AFR (n = 10) during the first three weeks of life. * p < 0.05, compared with AFR and # p < 0.05, ## p < 0.01. The statistically significant effects observed from the two-way ANOVA analysis are shown in the graphs as E (rearing environment).

peptide levels in the hypothalamus (Veenema et al., 2006, Lukas et al., 2009). In addition, no changes in AVP receptor binding between the experimental groups in the amygdala were detected (Lukas et al., 2009). Furthermore, male rats subjected to 3 minutes of individual MS had similar AVP mRNA levels in the amygdala and PVN as non-handled adult rats (Gabriel et al., 2005). This is in agreement with the present study, where no differences in ir AVP levels in the hypothalamus and amygdala between the adult MS15 and AFR groups were found. Nevertheless, in the three-week old MS360i rats, higher ir AVP levels as compared to both MS15i and AFR were found in the amygdala. OT and AVP in the amygdala have been suggested to be important regulators of the social response to various social stimuli (Albers and Bamshad, 1998, Bielsky and Young, 2004), such as facial cues (Thompson et al., 2004, Kosfeld et al., 2005, Thompson et al., 2006, Domes et al., 2007) and aggression (Ferris, 1992, Coccaro et al., 1998). The early environment has caused alterations in the amygdala, which might have consequences for social behaviour later in life.

Influence of duration and condition on MS effects
The duration of MS, i.e. 15 or 360 minutes induced changes in ir OT levels in the youngsters. In the pituitary gland, lower levels of ir OT were observed in the MS360 rats than in the MS15 rats. The opposite relation was found in the hypothalamus and amygdala. The differences in the amygdala were in fact persistent, suggesting long-term changes in this brain area. The young and adult MS15 and MS360 males had similar ir AVP levels in all areas measured. The condition of MS, i.e. litter-wise or individual separation, induced changes in the amygdala-OT levels of young rats. No additional influence of separation condition on other brain areas was noticed. The condition had an impact only in the amygdala. The differences did, however, not persist into adulthood. Here, higher levels were seen in the MSi groups as compared to the MSl groups. The total absence of tactile contact from dam and littermates
Three-week old rats

<table>
<thead>
<tr>
<th>Brain area</th>
<th>MS15/</th>
<th>MS360/</th>
<th>AFR</th>
<th>MS15i</th>
<th>MS360i</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>21.1±1.70</td>
<td>20.9±1.98</td>
<td>21.1±1.94</td>
<td>21.3±1.66</td>
<td>20.6±1.72</td>
</tr>
<tr>
<td>HT</td>
<td>138±13.1</td>
<td>150±11.8</td>
<td>134±8.60</td>
<td>146±12.9</td>
<td>137±10.0</td>
</tr>
<tr>
<td>AMY</td>
<td>1.24±0.29</td>
<td>1.20±0.10</td>
<td>1.31±0.12</td>
<td>1.05±0.11</td>
<td>2.30±0.58*§</td>
</tr>
</tbody>
</table>

Ten-week old rats

<table>
<thead>
<tr>
<th>Brain area</th>
<th>NIL</th>
<th>HT</th>
<th>AMY</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIL</td>
<td>104±6.82</td>
<td>113±11.0</td>
<td>122±12.2</td>
</tr>
<tr>
<td>HT</td>
<td>501±43.5</td>
<td>455±59.3</td>
<td>507±24.8</td>
</tr>
<tr>
<td>AMY</td>
<td>1.83±0.16</td>
<td>1.93±0.14</td>
<td>2.01±0.14</td>
</tr>
</tbody>
</table>

Table 4. The ir AVP levels (pg/mg tissue) in brain tissue extracts in three- and ten-week-old male rats reared in different environmental conditions. The levels represent mean ± SEM values detected with radioimmunoassay. MS15/l, n = 8-10; MS15/i, n = 8-10; MS360/l, n = 8-10; MS360/i, n = 9-10 and or AFR, n = 10. The values are expressed as ng/mg in the pituitary gland (PG) and pg/mg in the neurointermediate pituitary lobe (NIL), hypothalamus (HT) and amygdala (AMY) and represent mean ± SEM. *p<0.05, as compared to AFR. §p<0.05, as compared to MS15/i.

during the separation time in the individually separated pups seems to affect the ir OT levels not only in the prolonged separated rats, but also in the short separated rats. The role OT plays in tactile contact and bonding is well established (Nelson and Panksepp, 1998, Carter et al., 2008). Ir AVP levels did not differ between MSi and MS/l at any age.

The higher levels seen in the hypothalamus and/or amygdala in the MS360 as compared to MS15 and MS/i as compared to MS/l may relate to the attenuated stress-response (Insel and Shapiro, 1992, Nelson and Panksepp, 1998, Insel, 2003, Carter et al., 2008) seen in MS360/l rats. Previous studies have shown that these rats are more active, more risk taking behaviour and have a blunted corticosterone response when subjected to stress challenges (Roman et al., 2006).

Taken together, minor differences were observed in the ir AVP levels. However, a pronounced impact of MS was found in ir OT levels in the pituitary gland, hypothalamus and the amygdala in young male rats. In the amygdala the changes in ir OT levels persisted into adulthood. The amygdala is involved in stress-related behaviour. Hence, this area is of significance for
HPA-axis function, drug-seeking behaviour and neuroadaptive mechanisms related to drug dependence (Weiss et al., 2001, Koob, 2006). The duration of maternal absence had a larger impact on ir OT levels then the separation condition had. The changes seen in the prolonged MS rats may have consequences for behaviour in adulthood.

The principal component analysis (Paper II-IV)

In the current thesis, rats reared in different early environments have provided extensive information regarding the 5-HT, opioid, OT and AVP systems from various brain regions within the same individual. The male rats were subjected to different early-life experiences and sacrificed at two different time points, i.e. three- and ten-weeks of age. In Paper II, the mRNA levels of 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{3} receptors and transporter were measured in the brain stem. In Paper III, the ir levels of MEAP and DYNB in the pituitary gland and nine different brain areas, including the hypothalamus and amygdala, were measured. In Paper IV, the ir levels of OT and AVP in the pituitary gland, hypothalamus and the amygdala were analysed. The abundant data enabled assessment by the use of PCA (Jackson, 2003, Eriksson et al., 2006), since data exists from one and the same animal. The PCA is useful in obtaining an overview of the data, e.g. groups of observations, trends and outliers, and also to uncover the relationships between observations and variables, and among the variables themselves. The PCA creates a score plot showing a summary of the relationship between the individuals, and a loading plot identifying variables important for creating these relationships, i.e. neurobiological data. The direction of the score plot corresponds to the direction in the loading plot. Here, the purpose with the PCA was to find patterns within the experimental set up and between the different experimental groups.

Interestingly, clear groupings emerged in the young rats (Figure 10). Herein, the MS_{15l} rats are separated from the other groups. On the opposite side from the MS_{15l}, the AFR and the MS_{360i} groups were found. This is in agreement with the statistical data presented in the present thesis where the rats subjected to conventional rearing conditions, i.e. the AFR rats, have similar neurochemistry as the putatively most stressful rats, i.e. the MS_{360i} rats. Hence, these results may further enlighten the discussion regarding which experimental group would be more suitable serving as the control group (Lehmann and Feldon, 2000, Pryce and Feldon, 2003, Roman and Nylander, 2005, Macri and Würbel, 2006, Moffett et al., 2007). However, in the adult rats (Figure 11), the pattern disappeared and also supports the findings from the traditional statistical analysis performed in Papers II-IV. Although the basal MS-induced effects appear to be restored in adulthood, an altered susceptibility for psychopathological conditions and/or response to later challenges may still persist depending on the individual interaction between the early-life environment and genes. The loading plot shows the
variables important for the separation of groups at three-weeks of age. Interestingly, a number of parameters seem to be associated and this will be a topic of investigation in future studies.

**Figure 10.** PCA charts showing the score plot (A) and the corresponding loading plot (B) in three-week old male rats subjected to different early-life experiences. Abbreviations: 5-HT1A; 5-hydroxytryptamine 1A receptor, 5-HT2A; 5-hydroxytryptamine 2A receptor, 5-HT2C; 5-hydroxytryptamine 2C receptor, 5-HT3; 5-hydroxytryptamine 3 receptor, 5-HTT; 5-hydroxytryptamine transporter, AFR; animal facility rearing, AMY; amygdala, AVP; arginine vasopressin, CPU; caudate putamen, DYNB; dynorphin B, FCx; frontal cortex, HT; hypothalamus, MEAP; Met-enkephalin-Arg6-Phe7, MPFC; medial prefrontal cortex, MS15i; 15 min of litter-wise maternal separation, MS15i; 15 min of individual maternal separation, MS360i; 360 min of litter-wise maternal separation, MS360i; 360 min of individual maternal separation, NAcc; nucleus accumbens, NIL; neurointermediate lobe of the pituitary, OT; oxytocin, PAG; periaqueductal gray, PG; pituitary gland, SN; substantia nigra. VTA; ventral tegmental area.
Figure 11 PCA chart showing the score plot in ten-week old male rats subjected to different early-life experiences. Abbreviations: MS15l; 15 min of litter-wise maternal separation, MS15i; 15 min of individual maternal separation, MS360l; 360 min of litter-wise maternal separation, MS360i; 360 min of individual maternal separation and AFR; animal facility rearing.

General discussion

In the present thesis, the experimental rodent model MS was used to simulate different early environmental conditions during the first postnatal weeks. The MS model offers a possibility to simulate various early environmental settings using controlled conditions and then examine the short and long-term consequences.

Individual MS

Based on previous findings it has been suggested that short periods of repeated litter-wise MS represents a rearing environment with similarities to natural conditions and thereby safe and protective, whereas prolonged separations from the mother has been hypothesized to be an environment associated with emotional stress due to disturbed early social interactions (Ladd et al., 2000, Lehmann and Feldon, Boccia and Pedersen, 2001, Pryce et al., 2005). Previous studies of ours have confirmed this hypothesis by showing evidence that rats subjected to MS15l had low ethanol intake in adulthood whereas MS360l had enhanced propensity for high ethanol consumption (Ploj et al., 2003a, Roman et al., 2003). In the present thesis, using individual MS, it was hypothesized that individual MS would cause even more disturbance in the early environment and thereby more pronounced effects. However, minor differences in ethanol consumption were observed between
the MS15i and MS360i male rats. Both the MS15i and the MS360i rats had similar ethanol consumption as previously described for MS360i rats. Furthermore it was shown that a large individual variability within the experimental groups was detected not only in the MS1360i and AFR groups but also in the MS15i group. The only difference between studies was the separation condition. Thus, with the MSi condition where rat pups were separated not only from their mother but also from their littermates, it was difficult to distinguish between a protective and risk environment, respectively.

In addition to analysis of voluntary ethanol consumption it was of interest to study ethanol-induced effects in rats subjected to individual MS. Ethanol-induced effects on central monoamines are well described in the literature. However, the majority of these studies describe effects of passive acute and chronic administration or during forced drinking schedules. There is less information about the effects during the early phases of voluntary drinking. The early response to ethanol is a critical factor for later ethanol consumption behaviour (Schuckit et al., 2004, Kuperman et al., 2005) and is therefore of interest to study further. Furthermore, previous studies of ours have provided evidence for different responses in the opioid peptides after voluntary ethanol consumption in rats subjected to different rearing environment (Gustafsson et al., 2005, Gustafsson et al., 2007). Indeed, also in the present study evidence for different responses was presented. Although minor differences in ethanol consumption were seen between MS15i, MS360i and AFR rats, diverse results were found in these groups regarding DA and 5-HT measurements after long-term voluntary ethanol intake. All HEC rats had increased DA levels in the caudate putamen confirming the role of this area in habitual drinking (Everitt et al., 2008, Belin et al., 2009). Interestingly, opposite effects were found in the VTA and DRN in the MS360-HEC and AFR-HEC rats, whereas the ethanol-drinking MS15-HEC rats were remarkably unresponsive to ethanol. These results indicate different adaptive processes in voluntary drinking animals depending on the early environment that in turn may affect their continued ethanol consumption behaviour. Even though the ethanol consumption was low, neurobiological changes were seen also in the LEC rats. Mainly 5-HT measurements were affected and in other brain areas than those observed for HEC rats. The pharmacological relevance for these effects remains unclear. It is unlikely that 0.7-0.9 g/kg/day induce reward unless the dose is ingested within a small time period. Nevertheless, the LEC rats may preferentially seek other effects related to the low doses of ethanol. On the other hand, it may also be speculated that these rats may consume low doses as a result of the effects seen in the monoamine systems and may, therefore, represent a protection against excessive ethanol intake.
Individual MS versus litter-wise MS

To examine the neurobiological consequences of separation condition, i.e. MS/ versus MSi, and the duration of MS, i.e. MS15 versus MS360, two MS protocols were used. The rat pups were subjected to MS15l, MS360l, MS15i or MS360i during the first three postnatal weeks. In addition, both short- and long-term effects were assessed. At three- and ten-weeks of age the rats were sacrificed and the expression levels of 5-HT receptors and transporter genes and the immunoreactive (ir) levels of endogenous neuropeptides, i.e. the opioid, oxytocin and vasopressin peptides, were measured. The neurobiological measurements were performed in the same animals, which permitted correlation analysis between them. The alterations in the gene transcripts and ir peptide levels at the different ages of animals subjected to MS show that postnatal environmental factors can disturb the normal brain development of these neural circuits. With regard to OT, the duration of MS and not the condition of MS appeared to be the most important feature. In other words, pronounced differences were observed between in rats experiencing either 15 or 360 min absence of the dam. Considering the important role of OT for early social interactions and attachment to the caregiver (Nelson and Panksepp, 1998, Carter et al., 2008) these results were not surprising. However, the MS condition was not influencing the outcome showing that the presence or absence of littermates was less significant. On the other hand, the MS condition had a greater influence on the opioid peptides with regard to the duration of MS. In the young males, the MS-induced effects on the peptides were most pronounced in the individually separated rats, while in the adult rats more pronounced differences were found after litter-wise MS. That is, both MSi and MS/ affected the opioid peptide levels but MSi-induced effects were present immediately after the MS-period and then normalised during adolescence whereas the MS/-induced effects appeared later in life. Interestingly, both the duration and the condition of MS were important for the 5-HTergic genes. The remarkably low mRNA levels in the male MS15/ rats as compared to the MS360/ and also MS15i rats show that the 5-HT system is sensitive to separation conditions during MS. The presence or absence of littermates during MS had a large impact on the outcome.

The effects of MS, individual or litter-wise, are usually compared with an additional group of rats acting as controls for the handling procedure. The choice of proper control is a matter of discussion in the MS field (Lehmann and Feldon, 2000, Pryce and Feldon, 2003, Roman and Nylander, 2005, Macrì and Würbel, 2006, Moffett et al., 2007). For instance, various parameters in rats subjected to prolonged MS have been compared to rats separated short periods of time, to non-handled rats, to handled but not separated rats or to AFR rats. The results from different studies are therefore at times difficult to compare and hence to interpret. In the present thesis rats AFR rats served as controls. The AFR rats were reared as conventional laboratory
animals, i.e. like a “normal” control rat. Interestingly, as compared to the different MS procedures the AFR conditions often resulted in similar outcome in the neurobiological evaluations as the putative most stressful condition, i.e. the MS360. For example, in the males similar 5-HTergic mRNA levels were observed between the MSi and AFR groups. The results presented in this thesis thus strongly confirm the notion that AFR do not represent appropriate controls. This finding was further supported by the PCA, and was especially evident in young rats. The effects induced by prolonged separations may also be compared to those in the MS15l group. Grota and Ader (Grota and Ader, 1969) studied the natural behaviour of rats and found that the mothers leave the pups for a short period of time during the postnatal period to, for instance, forage food. Therefore, the MS15l rearing condition resembles this natural situation in the rats. Consequently, MS15l could be considered as a normal control in MS studies. With that in mind it is interesting to note that the AFR clearly differs from the MS15l rats. Moreover, the AFR rats also appear to be further separated from the MS15l rats when performing the multivariate analysis.

The findings in the present thesis provide further evidences for sex-specific outcome in rats reared in different early rearing environment. Minor effects were seen in 5-HTergic gene expression levels in the females, thus suggesting that the females are less sensitive to early-life experiences than males. In addition, whenever changes between the experimental groups were observed, these were in the opposite direction in female than in male rats. These results are in agreement with previous studies showing sex-specific effects in MS rats on ethanol consumption and ethanol-induced opioid peptide levels (Roman et al., 2004, Gustafsson et al., 2005, Roman et al., 2005). In both humans and non-human primates it has previously been reported sex-specific effects depending on the early environmental factors, especially with regard to the 5-HT system (Barr et al., 2004, Sjoberg et al., 2006), thus, supporting the notion of a sexual dichotomy of early-life environment on the 5-HT system.

Taken together, the present thesis describes impact of early-life experiences on monoamines after long-term voluntary ethanol consumption in adult male rats. Furthermore, by the use of the animal experimental model maternal separation simulating safe and adverse early-life environments, short- and long-term profound effects on 5-HT, opioid, OT and AVP systems were found. These systems are important for the establishment of a normal social behaviour and derangements in these systems may result in far-reaching consequences. The findings in the present thesis suggest that the neurobiological changes seen in rats subjected to the adverse early-rearing environment may alter the susceptibility for psychopathological conditions later in life.
Conclusions

Male rats subjected to MS15\textit{i}, MS360\textit{i} and AFR had no differences in adult ethanol consumption and a large individual variation within all groups was seen. The results show less clear differences between the individually separated MS15 and MS360 groups in comparison to previous results using litter-wise MS. The MS15\textit{i} condition does not relate to a protective environment and the MS360\textit{i} rats do not drink more as a consequence of lack of tactile contact with littermates in addition to the maternal absence.

Voluntary ethanol drinking for seven weeks induced different effects on brain DA, 5-HT and their metabolites in rats subjected to different early environmental conditions. Ethanol-induced effects were seen in both HEC and LEC rats. However, different brain areas were affected in these subgroups. The opposite ethanol-induced effects in MS360-HEC and AFR-HEC rats and the lack of effects in the MS15-HEC rats indicate different adaptive processes during voluntary ethanol drinking depending on the early environmental experiences.

MS induced short- and long-term changes in gene expression levels of brainstem 5-HT transporter and receptors. Both the MS condition, i.e. litter-wise or individual MS, and the duration of MS, i.e. MS15 or MS360 were found to have significant influences on the outcome. In addition, the changes were primarily seen in the MS15/ rats relative to the other rats. Interestingly, sex-specific consequences of MS were seen with fewer effects in the females. Moreover, the results in the females were often opposite than those seen in the males. These findings confirm the notion of sex-dependent MS effects and of less difference between MS15\textit{i} and MS360\textit{i} rats and a large difference between MS15/ and MS360/ rats.

MS-induced alterations in the male rats were observed in the ir opioid peptide levels in brain areas related to stress and drug reward and reinforcement. More pronounced changes were seen in ir MEAP levels than ir DYNB levels. The short-term effects were primarily found in rats exposed to individual MS and in areas related to stress reactivity. Furthermore, the results indicated low levels of enkephalin in adult MS360/ rats. Considering the association of opioid dysfunction and vulnerability for excessive ethanol intake these results may offer an explanation to the propensity for high voluntary ethanol consumption in these rats.
Pronounced differences between young MS15 and MS360 rats were observed in ir OT levels in the pituitary gland, hypothalamus and amygdala. In adult rats altered ir OT levels in the amygdala persisted into adulthood. Since dysfunction in OT transmission has been implicated in pathophysiological mechanisms it was suggested that an environmentally induced change in OT circuits might contribute to mechanisms underlying vulnerability for psychopathology.

In summary, evidence was provided for a poor distinction between a safe and stressful environment using the individual MS paradigm. Whereas pronounced differences were observed between MS15/ and MS360/ rats in the neurobiological analyses, the MS15i rats were more similar to the MS360i rats. These results may contribute to differences in voluntary ethanol consumption using the two paradigms. In addition, the most putative stressful environment, MS360i, often resulted in similar neurobiological outcome as AFR. Furthermore, neuropeptides and monoamines were shown to be targets for early environmental factors and should be considered in studies of individual differences in susceptibility for psychopathological conditions.
Samspelet mellan arv och miljö bestämmer individens särskiljande karaktärstika. Betydande individuella skillnader finns i fysiologiska funktioner och även i sårbarhet för olika sjukdomstillstånd. Flera studier har visat att i synnerhet den tidiga uppväxtmiljön kan orsaka förändringar i hjärnan, vilket i sin tur kan leda till sårbarhet för psykisk sjukdom. Flertalet kliniska studier har visat att traumatiska händelser såsom förlust av förälder, sexuella övergrepp och misshandel är relaterade till en ökad riskbenägenhet för t ex depression, antisocialt beteende och alkoholmissbruk. Trots detta är de bakomliggande mekanismerna i hjärnan ännu inte klarlagda. Av etiska skäl är det inte alltid möjligt att genomföra studier på människor, därför har en djurexperiment modell, *maternal separation* (MS), utvecklats för att studera uppväxtmiljöns betydelse för hjärnans funktion och för beteende senare i livet. I denna modell separeras ungarna en kort stund från sin mamma, vilket ska efterlikna en trygg miljö eller en längre period för att simulera en emotionell stressrelaterad miljö.

I de ingående studierna har råttungar separerats från sin mamma antingen 15 minuter (MS15) eller 360 minuter (MS360) varje dag under de första tre levnadsveckorna. MS15 och MS360 har vidare delats in i grupper där ungarne separerats tillsammans med sina kullsyskon (*k*) eller också individuellt (*i*). Som kontrollgrupp användes AFR (animal facility rearing) djur, vilka fick konventionell djurskötsel och hanterades enbart då burarna byttes.

I studie I, fick vuxna MS15*i*, MS360*i* och AFR hanråttor fri tillgång till alkohol Hälften av råtorna hade fritt val mellan etanol och vatten. Den andra hälften fick två flaskor med bara vatten och agerade därmed kontroller för de alkoholkonsumerande råtorna. Efter sju veckor fann vi inga större skillnader mellan MS15*i*, MS360*i* och AFR. Detta var lite överraskande eftersom vi i tidigare studier såg att MS15 djuren hade ett lägre etanolintag. En förklaring till detta kan vara att djuren separerades tillsammans med sina kullsyskon i de tidigare studierna, vilket inte var fallet i denna studie. Ett intressant fynd var dock att MS15*i*, MS360*i* samt AFR djurens råttor svarade neurobiologiskt olika på alkoholintaget beroende på vilken uppväxtmiljö de har haft. Alkohol påverkade på olika sätt dopamin och serotonin vilka är involverade i bl. a. drogrelaterade mekaniser. Hos de alkoholdrickande MS15*i* råttorna, sågs inga effekter av alkohol på dopamin eller serotonin i jämförelse med sina motsvarande vattenkontroller. Däremot sågs alkoholeffekter i de andra gruppena men effekterna på dopamin och serotonin i MS360*i* och AFR råttorna.
gick i motsatt riktning. Individuella skillnader i alkoholeffekter i början på ett frivilligt intag kan styra individens fortsatta alkoholintag och en skillnad i alkoholeffekter beroende på uppväxtmiljö är således viktigt att notera. Det är möjligt att en dysfunktion i hjärnbanor innehållande dopamin och serotonin kan ge inverkan på fortsatt intag av alkohol, som övergår från ett frivilligt drickande till kompulsiv drickande.

I studie II-IV, studerades olika bansystem (serotonin, enkefalin, dynorfin, oxytocin samt vasopressin) i hjärnan relaterade till t.ex. socialt beteende och drogberöende hos hanråttor. I dessa studier ingick MS15k, MS360k, MS15i, MS360i och AFR råttor. Neurobiologiska effekter studerades vid tre veckors ålder, dvs. precis efter att MS hade avslutats och innan de kom in i puberten, och vid tio veckors ålder, dvs. i unga vuxna. Det tydliga fyndet var att i de minst emotionell stressade djuren MS15k skilde sig nästan samtliga av dessa bansystem jämfört med andra grupper. Däremot sågs oftast mindre skillnader mellan den grupp som upplevt den minst trygga uppväxtmiljön, MS360i och kontroll gruppen, dvs. AFR. I MS litteraturen diskuteras om AFR djuren verkligen representerar den bästa kontrollgruppen eftersom mamman hela tiden är närvarande. Uti naturen måste mamman lämna sina ungar för en kortare tid för att hämta mat och dessa korta separationer påminner mer om MS15k. De stora skillnaderna mellan AFR och MS15k i denna avhandling tyder på att MS15 djuren är bäst lämpade för att agera kontrollgrupp. I studie II, studerades serotonin systemet och här studerades även könsskillnader. Det intressanta var att vi fann tydliga könsspecifika skillnader i serotonerga genuttryck.

Generellt fanns mindre skillnader mellan grupperna vid vuxenålder jämfört med då de var unga. Även om neurobiologin tycks ha normaliserats så kan en sårbarhet ändå föreligga, vilken då kan leda till att ett trauma eller en stressrelaterad utmaning senare i livet ge upphov till diverse psykologiska problem, såsom ångest, depression eller drog missbruk.

Sammanfattningsvis, trots att alkoholintaget inte skilde sig signifikant mellan MS15i, MS360i samt AFR uppvisade dessa grupper ändå skillnader i det neurobiologiska svaret beroende på uppväxtmiljö. Vidare fann vi könsskillnader i serotonin systemet, vilket stämde överens med vad tidigare studier har sett. Beroende på uppväxtmiljön fann vi stora skillnader mellan grupperna med avseende på serotonin, enkefalin, dynorfin, oxytocin och vasopressin system hos hanar. Resultaten visar att neuropeptider såsom morfinlika peptider och oxytocin samt serotonin systemet är viktiga mål för olika effekter i uppväxtmiljön. En väl fungerande social kontakt under uppväxtperioden är oerhört viktig för en normal utveckling av hjärnan och därmed för individens beteende. Det är väl känt att sårbarhet för psykiska sjukdomar kan vara ett resultat av dysfunktionell uppväxt. Vidare studier behövs därför i detta område.
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