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Maternal Separation in Rats

An Experimental Model for Long-Term Effects of Early Life Experiences on Neurochemistry, Voluntary Ethanol Intake and Exploration and Risk Assessment Behavior

BY

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Abstract
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The period of early life is important for the development of individual brain function and behavior. Human studies have shown altered vulnerability to develop psychopathology and/or excessive drug intake, possibly leading to dependence, as a consequence of early life experiences. In the present thesis, maternal separation (MS), an experimental model for studies of early environmental influences, was used to investigate long-term effects on neurochemistry, voluntary ethanol intake and exploration and risk assessment behavior in rats. Rat pups were assigned to one of three different rearing conditions: daily 15 min (MS15) or 360 min (MS360) of MS and normal animal facility rearing (AFR) during the first three weeks of life. Measurements of adult endogenous opioid peptide levels, opioid- and dopamine receptor density revealed minor MS-induced effects on the opioid system whereas interesting alterations were found in dopamine receptor density. Long-term effects on voluntary ethanol intake showed distinct MS-induced alterations in male Wistar and ethanol-preferring AA (Alko, Alcohol) rats. Female Wistar rats were unaffected, indicating sex differences in the effects of MS on ethanol intake. Male MS15 rats generally had a slower acquisition phase and a low subsequent ethanol intake whereas male MS360 rats had a high ethanol intake. MS15 is therefore suggested to protect against a high voluntary ethanol intake in male rats whereas MS360 may serve as a risk factor. The recently established concentric square field test indicated alterations in risk assessment as well as an increased exploratory drive and somewhat higher risk-taking behavior in adult MS360 rats, while minor effects were seen in MS15 rats. Altogether, these results demonstrate that environmental influences during the period of early life can have long-term effects on neurochemistry and behavior. Of special interest is the finding that MS altered the inherited high ethanol intake in adult ethanol-preferring AA rats.

Keywords: Handling, Maternal Deprivation, Environment, Opioids, Dopamine, Alcohol, Stress, Concentric Square Field, Open Field, Elevated Plus-maze

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List of Papers

This thesis is based on the papers listed below, which are referred to by their Roman numerals I-VI.

I. Ploj K, Roman E, Nylander I. Long-term effects of short and long periods of maternal separation on brain opioid peptide levels in male Wistar rats. *Neuropeptides* 37, 149-156, 2003.

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

III. Roman E, Ploj K, Nylander I. Maternal separation has no effect on voluntary ethanol intake in female Wistar rats. *Alcohol*. In press.

IV. Roman E, Hyytiä P, Nylander I. Maternal separation alters acquisition of ethanol intake in male ethanol-preferring AA rats. *Alcoholism: Clinical and Experimental Research* 27, 31-37, 2003.

V. Roman E, Gustafsson L, Hyytiä P, Nylander I. Short and prolonged periods of maternal separation on voluntary ethanol intake. I. Findings in male ethanol-preferring AA and ethanol-avoiding ANA rats. Manuscript submitted for publication.

VI. Roman E, Gustafsson L, Berg M, Meyerson BJ, Nylander I. Exploration and risk assessment after short and prolonged periods of maternal separation in male Wistar rats. Manuscript.

Abbreviations

AA rats	Alko, Alcohol rats
ADH	Alcohol dehydrogenase
AFR	Animal facility rearing
Ala	Alanine
ALDH	Aldehyde dehydrogenase
ANA rats	Alko, Non-Alcohol rats
ANOVA	Analyses of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
BRG	Bridge
CNS	Central nervous system
CORR	Corridor
CSF	Concentric square field
CTRCI	Central circle
DCR	Dark corner room
DIP	Head dipping
DIST	Distance
DOR	Delta opioid receptor
DUR	Duration
DYNA	Dynorphin A
DYNB	Dynorphin B
EPM	Elevated plus-maze
Fisher's PLSD test	Fisher's protected least significant difference test
FREQ	Frequency
GABA	Gamma-aminobutyric acid
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
HAD rats	High alcohol-drinking rats
His	Histidine
Ile	Isoleucine
ir	Immunoreactive
KOR	Kappa opioid receptor
LAD rats	Low alcohol-drinking rats
LAT	Latency
Leu	Leucine
Lys	Lysine
MAD	Median absolute deviation
MEAP	Met-enkephalin-Arg^{6}Phe^{7}
Met	Methionine
MOR	Mu opioid receptor
mRNA	Messenger ribonucleic acid
MS	Maternal separation
MS15	15 min of maternal separation during postnatal day 1-21
MS360	360 min of maternal separation during postnatal day 1-21
NAD^{+}	Nicotinamide adenine dinucleotide (oxidized)
NMDA	*N*-methyl-D-aspartate
NP rats	Non-preferring rats
NPY	Neuropeptide Y
N-terminal	Amino-terminal
OF	Open field
P rats	Preferring rats
PCA	Principal component analysis
Phe	Phenylalanine
PND	Postnatal day
POMC	Proopiomelanocortin
Pro	Proline
RIA	Radioimmunoassay
SAP	Stretched attended posture
SEM	Standrard error of the mean
Ser	Serine
sNP rats	Sardinian non-preferring rats
sP rats	Sardinian preferring rats
Thr	Threonine
TOT	Total
Trp	Tryptophan
Tyr	Tyrosine
USV	Ultrasonic vocalization
v/v	Volume/volume
Val	Valine
VTA	Ventral tegmental area

Contents

Introduction

Genetic and environmental factors in psychopathology

Genes, interacting with environmental influences, guide the individual during development and both genetic and environmental factors can thus have positive and negative consequences on this formation. The role of early life events in the development of neurobiobehavioral mechanisms and the subsequent establishment of mental functions has been studied extensively. However, the neurobiological mechanisms of this process are not fully understood. In high intake of drugs of abuse and the vulnerability to develop drug dependence, it is well acknowledged that genetic and environmental influences, drug availability, sex, personality, history of drug use and other life events affect and contribute to the final output. Although many people experiment with drugs of abuse and start to occasionally use the drug, especially during the teenage period, only a limited number acquire dependence i.e. a compulsive drug intake beyond control (e.g. Bohman et al., 1984; Chambers et al., 2003; Lende and Smith, 2002; Vanyukov et al., 2003). Recent studies have shown that genotypes could moderate children's sensitivity to early life experiences. Maltreated children with a specific functional polymorphism in the neurotransmitter-metabolizing enzyme monoamine oxidase A were less likely to develop later antisocial behavior. The effect of childhood maltreatment on antisocial behavior was weaker among those with a high enzymatic activity that among those with a low enzymatic activity (Caspi et al., 2002). Furthermore, children experiencing stressful events early in life were less likely to develop depression if they had a particular genotype that confers efficient transport of serotonin (Caspi et al., 2003). Therefore, risk factors as well as protective factors play an important role for the individual vulnerability to develop psychopathology.

Genetic factors

It is generally acknowledged that most types of behavior, including not only normal variations but also disorders, depend on a complex interplay between environmental factors and multiple genes. Twin and adoption studies have been used to evaluate whether traits that appear repeatedly in families were a result from shared genes, shared environments or a combination of both. Twin studies are particularly useful in demonstrating that genetic contributions exist to disorders as well as measures

of normal behavior. Monozygotic twins share all genes in common while dizygotic twins share half of their chromosomal genes. Adoption studies provide insight into the separation of the effects of genes and environment and have been an important tool providing evidence for hereditary components in various disorders (e.g. Tandon and McGuffin, 2002). Recently, insights into mammalian evolution have greatly improved with the elucidation of the rat, human and mouse genome (Gibbs et al., 2004; Lander et al., 2001; Waterston et al., 2002), however, the genetic differences underlying many disorders remain elusive.

Genes play an important role in the development of alcohol (the terms alcohol and ethanol are used interchangeably herein) dependence. Alcoholism has a heritability of 50-70% depending on diagnostic criteria, population characteristics and sex (Tyndale, 2003). Genetic influences have been well described using both twin and adoption studies. Early studies were mainly focused on males, but it is now known that the genetic contribution is similar in females (e.g. Bohman et al., 1984; Cadoret et al., 1994; Goodwin, 1979; Heath et al., 1997; Kendler et al., 1994; Schuckit et al., 2000). The genetic susceptibility for one of two defined variants of alcoholism, Type I and Type II, was compared in males. Type I alcoholism was characterized by relatively mild abuse, minimal criminality and passive-dependent personality, whereas the Type II was characterized by early onset, violence, criminality, impulsivity and an active drug-seeking behavior (Cloninger, 1987). Type II alcoholism was also found to have a strong genetic component (Cloninger et al., 1981). The Type II criteria for alcoholism have also been confirmed in females with severe alcoholism (Hallman et al., 2001). Several genes possibly involved in alcohol dependence have been identified such as those involved in function of the endogenous opioid system, gamma-aminobutyric acid (GABA), dopamine and serotonin and genetic variations in neuropeptide Y (NPY) and *N*-methyl-D-aspartate (NMDA) receptors may also be of importance (e.g. Dick and Foroud, 2003; Kreek et al., 2004; LaForge et al., 2000; Tyndale, 2003).

The significance of genetic factors in ethanol self-administration and the development of ethanol dependence has been strengthened by findings in experimental animals (e.g. Crabbe and Phillips, 2004; Schumann et al., 2003). Animal models are useful tools in identifying heritable biological characteristics that are associated with a high intake of drugs of abuse and the efficacy of genetic, behavioral as well as pharmacological agents can be studied using these models (McBride and Li, 1998; Spanagel, 2003; Tabakoff and Hoffman, 2000). Inbred rodent strains represent populations of genetically similar animals that have been produced by more than 20 generations of inbreeding and such strains have been used in studies of intake of drugs of abuse. The Lewis strain of rat shows a higher rate of operant responding for several drugs of abuse and has a higher ethanol intake than the Fischer 344 strain (Suzuki et al., 1988). Similarly, C57BL/6J mice have a higher voluntary ethanol intake than the DBA/2J strain (Belknap et al., 1993). However, because inbreeding also results in fixation of genes not involved in a specific disease or trait, inbred strains may not always be the best models for examining genetic factors underlying excessive ethanol intake.

Selective breeding for a specific purpose, e.g. high and low voluntary ethanol intake and preference, has been used in order to generate lines of rats that could serve as models for assessing genetic predispositions to the disparate extremes of alcohol use found in humans. Selectively bred lines are presumed to possess a high frequency of genes that influence a desired phenotype. To date, a number of selectively bred sets of alcohol-preferring and non-preferring lines of rats have been developed, e.g. the Alko, Alcohol/Non-Alcohol (AA/ANA) lines (Eriksson, 1968; Hilakivi et al., 1984), the Sardinian alcohol-preferring/non-preferring (sP/sNP) lines (Colombo, 1997), the Indiana alcohol-preferring/non-preferring (P/NP) lines and the Indiana high/low alcohol-drinking (HAD/LAD) lines (Li TK et al., 1993). These animals have been used in numerous studies in order to determine genetic differences in a number of neurotransmitter systems and behavioral parameters underlying ethanol intake, both under basal conditions and during ethanol intake and withdrawal.

Further tools utilized to determine how genes may influence the development of drug addiction in humans are the use of genetically modified animals. In genetically modified animals, a foreign gene can be inserted into the genetic material of an animal or targeted genes can be either rendered nonfunctional or over-expressed. These animal models permit studies of the effects of an altered gene expression or function in the entire animal (e.g. Bowers, 2000). However, there are also problems with genetically manipulated animals, for instance problems with the genetic background (Crusio, 2004). The use of inbred rodent strains, selectively bred lines and genetic animal models in studies of ethanol self-administration has recently been reviewed (Crabbe and Phillips, 2004).

Environmental factors

Emotional attachment, e.g. mother-infant bonds is essential for survival and normal development in mammals. Bowlby (Bowlby, 1954), who established the attachment theory, has studied the importance of the capacity to make relationships in humans. It could be shown a warm and lasting relationship between infant and parent is important for the development of a psychologically healthy adult and that adverse childhood experiences profoundly affected later development (Bowlby, 1982). This field of research has gained increasing interest. In humans, adverse experiences early in life, such as parental loss, neglect, distant parent-child relationships and physical and/or sexual abuse can result in neurobiological events with a potential to cause changes in brain development (e.g. Teicher et al., 2003) and enhance the vulnerability to develop adult psychopathology including depression, anxiety disorders and posttraumatic stress disorder (e.g. Canetti et al., 1997; Carlson and Earls, 1997; Gilmer and McKinney, 2003; Langeland et al., 2004; Nemeroff, 1998). It has further been tested if maternal expressed emotion is an environmental risk factor for children's antisocial behavior problems by investigating monozygotic twin pairs. This determines the environmental influences on children with similar genetic background. It was found that within monozygotic pairs, the twin receiving less warmth and more maternal negativity had more antisocial behavior problems

(Caspi et al., 2004). Psychiatric disorders are often co-morbid with substance abuse disorders (Deas and Thomas, 2002; Petrakis et al., 2002) and early life experiences have been shown to affect intake of drugs of abuse later in life (e.g. Gordon, 2002; Langbehn et al., 2003; Langeland and Hartgers, 1998; Spak et al., 2001). However, questions have been raised regarding the association between psychiatric disorders and drug intake and the cause and consequence in this relationship (Allan, 1995). It should also be remembered that not all children experiencing difficulties or adversities during childhood develop problems later in life. It has been shown that warm, nurturing families tend to promote resistance to adversities and to diminish vulnerability for later problems (Kim-Cohen et al., 2004; Smith and Prior, 1995).

Findings in humans have been further supported by data obtained from studies in non-human primates. The impact of early life experiences on later behavior in non-human primates is well established (e.g. Harlow, 1962; Ruppenthal et al., 1976). It has for instance been shown that mothers that were able to cope with environmental demands could more efficiently prepare their infants to various environmental changes, resulting in more "secure" infants. In contrast, mothers that experienced an unpredictable environment (varied and unpredictable access to food resources) were less able to maintain sufficient coping mechanisms and stable attachment relationship toward their infants. These infants showed behavioral alterations during development, such as less playing behavior and more depression-like behavior, and as young adults they revealed disturbances including reduced sociability and timidity (Rosenblum and Andrews, 1994). Studies have further elucidated the long-term effects of absence of contact with the mother on neurochemistry, behavior and voluntary ethanol intake (e.g. Fahlke et al., 2000; Higley et al., 1991a; Higley et al., 1991b). In a recent study in non-human primates, it was demonstrated that a specific genetic profile could affect ethanol intake as a consequence of environmental manipulations (Barr et al., 2003). These findings strengthen the evidence for an association between genetic and environmental factors and the vulnerability for high ethanol intake.

Experimental animal models

Studies that control for environmental stimuli are very difficult, if not impossible to conduct in humans, especially from an ethical perspective, and longitudinal studies of development take decades to complete. Furthermore, neurochemical analyses are sometimes difficult to perform in humans. Therefore, controlled animal studies are essential in which it is possible to control for environmental influences, at least to a certain extent (Crabbe et al., 1999; Wahlsten et al., 2003), in the study of behaviors of interest in combination with neurochemical analyses of brain function.

Research in experimental animals has generated basic biological understanding, ranging over physiology, genetics and behavior. Much of the current knowledge on human biology originates to an important extent from research in experimental animals. This has also been an important contribution to the understanding

of pathogenesis and specific pharmacological therapies. One animal often used in studies to model human behavior and development is the rat. As a laboratory animal, the rat has many advantages. Maybe the most important advantage is that the rat is similar to humans in terms of its genome, physiology and brain functions. Furthermore, it has a relatively short life span compared to primates and it is also easy to work with.

This thesis deals with the influence of early life influences on adult neurochemistry and certain forms of behavior. The time period from the, in this context, critical neonatal period to adultness is approximately 2-3 months.

Development in the rat

The early relationship between mother (or primary care giver) and offspring is an important environmental factor during early development in mammals. Newborn mammals are dependent on their mothers not only for survival but also for normal development.

In the rat, parturition occurs most often during the light (inactive) period of the light/dark cycle on day 21-23 of pregnancy. Litter sizes vary among different strains of rats. Rat pups, like most other rodents, are born blind, naked and with their ear canals closed. Furthermore, they are unable to urinate or defecate properly and unable to maintain their own body temperature (Krinke, 2000). The mother-infant relation regulates physiological responses such as heart rate, sleep/wake cycle, thermoregulation, gastrointestinal activity and growth hormone production in the infant (Hofer, 1994).

After birth, the dam initiates maternal behavior towards the pups. Females are attracted to pups in part by their odors and identify their own pups by their odors. Maternal behavior is initially hormonally dependent but changes soon after parturition to be elicited by stimulation from the young. Maternal care occurs in bouts. A nesting bout begins when the mother approaches the litter, gathers the pups in the nest by retrieving pups found outside the nest and crouches over the pups to make her nipples available for nursing, also referred to as arched-back nursing. The mother then systemically licks the pups, particularly the head and the ano-genital region in order to stimulate elimination (Elwood, 1983; Rosenblatt, 1989). Nest bouts also result in a rise in maternal temperature and it has been suggested that the rate of temperature rise determines the duration of each bout (Leon et al., 1978). Another important component of maternal behavior is to protect the litter from conspecific and other intruders (Elwood, 1983; Rosenblatt, 1989). At the beginning of the lactating period the mother spends approximately 80-85% of the time with her litter. This time slowly declines along with the development of the pups to around 30% on postnatal day (PND) 21 (Grota and Ader, 1969; Leon et al., 1978).

Female rats display normal variations in the extent of maternal behavior, i.e. a high and low amount, respectively, of licking/grooming and arched-back nursing, toward their pups (Caldji et al., 1998; Champagne et al., 2003; Liu et al., 1997). An early study showed that dams interacted differently with male and female offspring

(Moore and Morelli, 1979). Although, a recent study indicated no such differences (Champagne et al., 2003). Interestingly, maternal behavior, i.e. licking/grooming and arched-back nursing, is transmitted through generations. Cross-fostering studies have shown that offspring adopts such behaviors from the nursing mother and not the biological mother (Champagne and Meaney, 2001).

Pups communicate by emitting ultrasonic vocalizations (USVs), which are whistle-like sounds characterized by frequencies ranging between 30 and 90 kHz (e.g. Branchi et al., 2001; Hofer, 1996). The ontogenetic development of Wistar rat pup USVs displays a characteristic time course starting around PND 2-3, reaching a plateau level around PND 4-6 and then gradually decreases (Johansson-Wallsten, 1993). It is believed that the purpose of USVs is to elicit maternal care and subsequently the important tactile stimulation, maintenance of body temperature and it might also reflect a general emotional distress caused by the absence of the mother (e.g. Branchi et al., 2001; Hofer, 1996).

In the course of normal mother-pup interaction in the wild, the dam shows an intense caregiving behavior as described above, but regularly leaves the nest and the pups for short periods of time (Fleming and Rosenblatt, 1974; Grota and Ader, 1969; Jans and Woodside, 1990). However, during semi-naturalistic conditions, subordinate females have been shown to be forced to locate nests at some distance from food and water sources, resulting in more prolonged periods of separations (Calhoun, 1962).

In the beginning of the second week of life, the rat pups develop fur and, as a consequence, begin self-thermoregulation. By two weeks of age they open their eyes and ears, locomotor functions become more efficient and the pups begin to show increased physiological autonomy. As soon as pups can see, hear and move they start to explore their environment more frequently and by weaning, on PND 21-25, they are able to live independently (Elwood, 1983). Weaning occurs because the young lose interest in suckling from the mother and because the mother makes it more difficult for the pups to do so (Rosenblatt, 1989).

Puberty in male rats is hormonally dependent and occurs around PND 30-40. In the female rat, puberty is associated with the vaginal opening and the first proestrus and the timing is apparently dependent more on body weight than on age. Vaginal opening occurs approximately on PND 33-42 with the body weight just above 100 g. About one week after the vaginal opening, when the body weight reaches approximately 120 g, the female starts to show regular estrous cycles (Krinke, 2000).

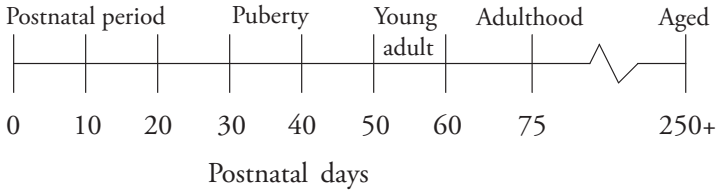


Figure 1. The development from infant into aged rat.

In general, stages of brain development are divided into phases of intense cell proliferation, migration, axonal outgrowth and the process of dendritic maturation. The point of time for these phases may be different for various brain areas. During the prenatal (before birth) and postnatal (the period between birth and weaning) periods, the maturation process is important, that is the establishment of the neural transmission including appropriate biosynthesis, storage, release and elimination of transmitters and the efficacy of receptors. Besides the genomic programming, environmental factors contribute to the development of the brain and influence the establishment of mental functions in the adult individual (Andersen, 2003). From a developmental perspective, the postnatal period in the rat can be compared to the last trimmest of the human pregnancy. In relation to the rat, the human brain is relatively mature at birth whereas the rat brain is immature and continues to develop during the postnatal period. A more detailed discussion of the ontogeny of the dopamine system and endogenous opioid system will follow in the respective chapters.

The maternal separation model

During the postnatal period, the infant is dependent on the mother not only for nursing and protection but also for normal brain development, as described previously. Exploring environmental influences during this period, which is important for the establishment of normal neural functions (Andersen, 2003), can help us to gain a greater understanding of early life influences on the development of aberrant adult behaviors.

The impact of early life experiences in animals can be studied using the maternal separation (MS) model. Separation of rat pups from their dams during the postnatal period results in a variety of physiological changes, the nature of which are dependent on the specifics of the separation experience, the environmental conditions and the duration of the separation (Lehmann and Feldon, 2000). Research on early environmental manipulations and its effects on development, physiology and behavior originate from early studies in rodents and primates (e.g. Denenberg and Smith, 1963; Harlow, 1962; Levine, 1957; Weininger, 1954). For example, it was found that laboratory rats that had been held in the experimenter's hand and been gently stroked (gentled) for a few minutes developed differently than non-handled rats (Weininger, 1954). It was further discovered that it was not necessary to hold the pup in the hand stroking it, as similar effects could be achieved by only separating the pups from the mother (handling) (Denenberg and Smith, 1963; Levine, 1957). The results of this short maternal separation (MS), with or without stroking, was found to have profound long-term effects including altered corticosterone response, decreased emotional reactivity and enhanced learning performance and attention abilities (e.g. Levine, 2002). Along with these findings, the search began for an animal model with opposite effects suitable to mimic human symptoms of depression and anxiety disorders. For this purpose, more prolonged periods of MS were used (Hall, 1998; Kuhn and Schanberg, 1998).

In rats, a sensitive period for MS procedures is the period lasting from PND 4-14, which has been identified as the stress hypo-responsive period. During this period the developing rat does not respond to mild stressors due to low levels of circulating glucocorticoids. One explanation for this reduced responsiveness is that different brain pathways may mature at different time points (Levine et al., 2000; Sapolsky and Meaney, 1986). Several different protocols for MS are currently in practice in which the pups are separated from the dam either in litters or isolated from the littermates. MS can be performed at a single occasion, at several time periods or repeatedly during parts of or the entire stress hypo-responsive period as well as during the entire postnatal period. Control groups for comparisons are either non-handled or housed under normal animal facility rearing (AFR) conditions (Lehmann and Feldon, 2000; Pryce and Feldon, 2003). Non-handled pups are left completely undisturbed and never handled whereas AFR pups have experienced human contact during cage cleaning. The question has been raised whether rat pups require a minimal amount of stimulation in order to develop into adult rats with a behavioral profile typical for laboratory rats and that non-handling of pups might be below this limit (Pryce and Feldon, 2003). However, the use of AFR as a control is not without problems since different animal facilities might vary in animal husbandry (Crabbe et al., 1999; Levine, 2002).

Common experimental protocols for MS involve short periods of MS (3-15 min), also referred to as handling, as well as more prolonged periods of MS (> 1 h). A variety of long-term neurochemical, hormonal as well as behavioral changes have been observed after short and prolonged periods of MS. For instance, animals subjected to short periods of MS show an increased ability to cope with and adapt to stressful stimuli as adults. Interestingly, prolonged periods of MS result in effects that sometimes are opposite to those observed after short periods of MS (Anand and Scalzo, 2000; Anisman et al., 1998; Cirulli et al., 2003; Ladd et al., 2000; Lehmann and Feldon, 2000; Levine, 2002; Newport et al., 2002; Pryce and Feldon, 2003). However, results among studies that utilized prolonged periods of MS are sometimes contradictory. These conflicting results appear to be due to large variations in the experimental protocols used, such as frequency and duration of separation, individual or litter separation, temperature and light/dark cycle as well as different control groups used for comparisons (Lehmann and Feldon, 2000; Pryce and Feldon, 2003). Results from studies on early life experiences on development and effects in adult rats are further supported by findings in mice (Clausing et al., 2000; Romeo et al., 2003; Schmidt et al., 2004; Zaharia et al., 1996) and primates (Coplan et al., 1996; Levine and Mody, 2003; Sanchez et al., 2001; Suomi, 1997).

The underlying mechanisms for the effects of short and prolonged periods of MS are not fully understood but are believed to be partly mediated by changes in maternal behavior. Short periods of MS can be viewed as mimicking the behavior of wild rats where the mother has to leave her pups to collect food. Upon reunion, after short periods of MS, the mother shows an increased maternal behavior of licking and grooming her pups (Liu et al., 1997; Pryce et al., 2001). On the other hand, prolonged periods of MS may result in disturbances in maternal behavior and

mother-pup interactions. Upon reunion these mothers show a decrease in the amount of licking and grooming towards the pups (Huot et al., 2000). Furthermore, possible effects caused by normal variations in mother-infant behavior (Caldji et al., 1998; Champagne et al., 2003) could be further affected by MS since it has been shown that short periods of MS can influence the normal interactions between mother and pup (Liu et al., 1997; Pryce et al., 2001). Additional evidence for the impact of maternal influences comes from studies showing differences between dams of pups subjected to either short or prolonged periods of MS (Eklund and Arborelius, 2003; Kalinichev et al., 2000; 2003). For instance, AFR dams and dams that where separated from their pups for 240 min on 8 random days during PND 1-14, showed a decrease in behaviors normally considered as anxiety-like compared to dams that were separated from their pups for 15 min and females that were mated but did not become pregnant (Eklund and Arborelius, 2003). However, the impact of maternal behavior on later MS-induced effects is also questioned. Contradictory findings on maternal care during prolonged periods of separation have been reported (Pryce et al., 2001). Studies in other species add further to the discussion about the influence of maternal behavior on MS-induced effects. As described above, short periods of MS often results in a decreased emotional reactivity in rats. In contrast to rats and mice, the maternal behavior in rabbits is very different. The doe nurses the pups for 3-10 min per day and shows no licking and grooming behavior. Rabbits that were handled during infancy showed similar decreased emotional reactivity in adulthood, as rats, also under conditions where mother-infant interaction was limited to nursing or was not present at all (Denenberg, 1999). It is therefore difficult to interpret the impact of mother-infant interactions as a mediator for MS-induced effects even though an effect cannot be excluded.

The endogenous opioid system

The milky juice of the seed case of the opium poppy, *Papaver somniferum*, contains compounds, which have been used for their medicinal properties for millennia. Opium, the Greek word for juice, contains more than 20 distinct alkaloids. Morphine, named after Morpheus, the Greek god of dreams, was the first pure substance isolated from opium by Sertürner in 1806. “Opiates” are drugs derived from opium, such as morphine and codeine. These have long been used as analgesics, antitussives and antidiarrheals. The term “opioid” is more inclusive and refers to the endogenous opioid system and to all agonists with morphine-like activity as well as to naturally occurring and synthetic opioid peptides (Gutstein and Akil, 2001).

Three separate research groups simultaneously discovered the opioid receptors in 1973 (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). These receptors were later classified into three types, i.e. mu opioid receptors (MORs), delta opioid receptors (DORs) and kappa opioid receptors (KORs). MORs are widely distributed with a high density of binding sites in the caudate putamen, thalamus, neocortex, nucleus accumbens, hippocampus and the amygdala. The

highest DOR density is found in the olfactory bulb, neocortex, caudate putamen and the nucleus accumbens. The number of KOR binding sites is relatively low in the rat brain relative to MOR and DOR binding sites. The number of rat brain KOR binding sites is lower also compared to humans. The highest KOR density in the rat is found in the nucleus accumbens, dorsal endopiriform nucleus, claustrum and the interpeduncular nucleus. The distinct distribution patterns indicate that the respective receptor classes contribute differently to the opioid function (e.g. Dhawan et al., 1996; Mansour et al., 1988).

After the identification of the opioid receptors, the search began for the endogenous ligands for these receptors. The classical endogenous opioid peptides are the enkephalins, beta-endorphin and the dynorphins (Goldstein et al., 1979; Hughes et al., 1975; Lord et al., 1977; Terenius and Wahlström, 1975), all of which share the N-terminal amino acid sequence Tyr-Gly-Gly-Phe, designated the "opioid motif". More recently, it has been shown that also other peptides have opioid-like activity and affinity for the opioid receptors such as the endogenous peptides endomorphin-1 and endomorphin-2. Those are non-classical opioid peptides that represent potent endogenous ligands for the MOR (Zadina et al., 1997). The respective receptor preference(s) for the endogenous opioid peptides and the endomorphins is shown in Table 1.

Peptide	**Amino acid sequence**	**Receptor preference**
Leu-enkephalin	*Tyr-Gly-Gly-Phe*-Leu	DOR
Met-enkephalin	*Tyr-Gly-Gly-Phe*-Met	DOR
Met-enkephalin-Arg^{6}Phe^{7} (MEAP)	*Tyr-Gly-Gly-Phe*-Met-Arg-Phe	DOR
Dynorphin A	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	KOR
Dynorphin B	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr	KOR
beta-endorphin $_{human}$	*Tyr-Gly-Gly-Phe*-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	MOR/DOR
beta-endorphin $_{rat}$	*Tyr-Gly-Gly-Phe*-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln	MOR/DOR
Endomorphin-1	Tyr-Pro-Trp-Phe	MOR
Endomorphin-2	Tyr-Pro-Phe-Phe	MOR

Table 1. *The amino acid sequences and preferred receptor(s) for the endogenous opioid peptides and the endomorphins.*

The opioid peptides beta-endorphin, enkephalins and dynorphins are processed from three distinct precursor proteins, proopiomelanocortin (POMC), proenkephalin and prodynorphin, respectively (Mansour et al., 1988; Massotte and Kieffer, 1998). The major sites for POMC biosynthesis are the anterior pituitary, the arcuate nucleus

and the nucleus tractus solitaris with widespread projections throughout the brain. Neurons containing proenkephalin are present throughout the brain with both local circuits as well as long projections. Prodynorphin is synthesized in several cell groups in the brain and in the anterior pituitary lobe and neurons containing prodynorphin have both short and long projections (Mansour et al., 1988).

Almost 20 years following the discovery of the opioid receptors, two groups simultaneously reported the first molecular cloning of an opioid receptor (Evans et al., 1992; Kieffer et al., 1992). Following this report of the cloning of the DOR, the clones for MOR (Chen et al., 1993; Fukuda et al., 1993; Thompson et al., 1993; Wang et al., 1993) and KOR (Li S et al., 1993; Meng et al., 1993; Minami et al., 1993; Yasuda et al., 1993) were soon identified based on their homology to the DOR. The cloning of the opioid receptors later on enabled the development of genetically modified animals.

The endogenous opioid system has been implicated in a range of biological mechanisms. It has a central role in drug dependence (Gerrits et al., 2003; Gianoulakis, 2004; Oswald and Wand, 2004) as well as in the endogenous regulation of pain sensitivity (Gutstein and Akil, 2001). Furthermore, the endogenous opioid system is implicated in functions such as learning and memory, psychiatric disorders, immune function, thermoregulation, gastrointestinal motility, cardiovascular and respiratory functions as well as endocrine functions (Akil et al., 1984; Massotte and Kieffer, 1998).

The endogenous opioid system has been shown to also be involved in maternal behavior. Administration of morphine during late gestation was found to delay the onset of maternal behavior (Grimm and Bridges, 1983). Furthermore, especially activation of MOR has been shown to be involved in infant attachment behavior, such as modulation of USV and activation during nursing. The role of MORs in mediating the rewarding properties of mother-related stimuli has, however, not been determined (Nelson and Panksepp, 1998). A recent study showed that mice pups lacking the MOR gene had altered attachment bonds towards their mothers. These MOR knockout pups emitted fewer USVs when removed from their mothers and did not show a preference toward their mothers' cues (Moles et al., 2004). These data suggest that opioid activation is necessary in order for the pups to elicit a normal attachment towards the dam.

The ontogenetic profile of the endogenous opioid system in the rat is complex with developmental patterns of the three precursors occurring independently, and also that the opioid peptide products have distinct profiles. Opioid peptide products are all detectable during gestation but the full expression is not completed until well after birth with the third postnatal week often exhibiting the most marked increases. Differences in ontogenesis are, however, dependent on whether results are expressed per brain area or as total brain concentration. Opioid receptor types show distinct ontogeny. MOR and KOR sites are present at birth while DOR sites are absent until the second postnatal week. The maturation of MOR and DOR is completed during the third and fourth postnatal weeks respectively (McDowell and Kitchen, 1987). There has been less agreement on the ontogeny of KOR sites

in the rat brain. It was first reported that KOR binding sites remained constant until PND 10 and then increased to PND 35 (Barr et al., 1986; Spain et al., 1985). Using a more specific ligand, it was then demonstrated that the major development of KOR sites occurs in the first 10 PNDs (Kitchen et al., 1990). By use of an even more selective ligand it could be demonstrated that KORs are the earliest opioid sites to appear in rat brain and are fully developed at birth (Kitchen et al., 1992). Few studies have investigated peptide and receptor development in parallel and it is therefore unclear whether specific peptide development is directly linked to that of a single receptor site (McDowell and Kitchen, 1987). Even though the peptides and receptors are detectable, the function of the system may not be mature and therefore the system, and the interactions with other transmitter systems, might be affected by various stimuli.

The endogenous opioid system has been shown to be sensitive to postnatal manipulations. MS for 15 min was shown to reduce neural endopeptidase activity, one of the major enzymes that degrade endogenous enkephalin, in the amygdala (Irazusta et al., 1999). Furthermore, 15 min of individual MS during the postnatal period was shown to increase adult DOR density in the amygdala whereas adult KOR density was unaffected (Ploj and Nylander, 2003). Long-term effects of short periods of individual MS on the dynorphin (DYN) system were found in male rats, in terms of higher immunoreactive (ir) tissue levels of DYNA and DYNB in certain brain areas (Ploj et al., 1999). In contrast, less pronounced long-term effects were found in female rats subjected to 15 min of individual MS (Ploj et al., 2001). These results indicate that sex differences exist in the response of short periods of MS. The effects of prolonged periods of MS on the endogenous opioid system need further elucidation.

The dopamine system

Dopamine was first identified as a potential neurotransmitter in the brain by Carlsson (Carlsson, 1959). Dopamine is important in regulating several aspects of basic brain function in the mammalian brain. In the central nervous system (CNS) dopamine is implicated in a variety of functions including locomotor activity, cognition, emotions and endocrine regulation. Furthermore, dopamine is central in the reinforcing and rewarding properties of drugs of abuse. Dopamine is implicated in a number of disorders, for instance drug dependence, Parkinson's disease, schizophrenia and attention deficit hyperactive disorder (e.g. Girault and Greengard, 2004; Vallone et al., 2000).

There are three main dopaminergic pathways in the brain (e.g. Girault and Greengard, 2004; Vallone et al., 2000):

- *The tuberoinfundibular pathway*, with projections from the arcuate and paraventricular nuclei of the hypothalamus into the intermediate pituitary lobe and into the median eminence, functions in endocrine regulation.

- *The nigrostriatal pathway*, with projections from the substantia nigra to the caudate putamen. This pathway is involved in locomotor activity and its degeneration causes Parkinson's disease.
- *The mesocorticolimbic pathway* also referred to as the brain reward system, which arises from the ventral tegmental area (VTA) and innervates a set of interconnected forebrain areas including the nucleus accumbens, the basal forebrain (regions which have been termed the extended amygdala) and regions of the frontal cortex. This pathway is mainly associated with the actions of drugs of abuse but also in aspects of memory and learning.

Five dopamine receptors have been characterized, which are divided into two subfamilies, D1-like and D2-like receptors. The dopamine D1-like receptor classification includes the dopamine D1- and D5-receptor subtypes, while the dopamine D2-, D3- and D4-receptor subtypes belong to the dopamine D2-like subfamily. Dopamine D1-like receptors are exclusively postsynaptic whereas the dopamine D2-like receptors also are located presynapticly and thereby function as autoreceptors. The dopamine D1- and D2-receptors are more widespread in the CNS compared to the distribution of the dopamine D3-, D4- and D5-receptors. Dopamine D1-receptor mRNA is found in the striatum, nucleus accumbens, olfactory tubercle, hypothalamus, thalamus and the limbic system. The dopamine D1-receptor protein is also highly expressed in the entopeduncular nucleus and the substantia nigra pars reticulata. The dopamine D2-receptor is expressed in the striatum, olfactory tubercle and the nucleus accumbens. Dopamine D2-receptor mRNA is found in cortical areas, amygdala, substantia nigra pars compacta and in the VTA. Dopamine D3-receptors are expressed in the islands of Calleja, olfactory tubercle and the ventromedial shell of the nucleus accumbens. mRNA for the dopamine D3-receptor is also found in the substantia nigra pars compacta and in the VTA. The dopamine D4-receptors are expressed in the frontal cortex, amygdala, hippocampus, hypothalamus and mesencephalon. Finally, expression of the dopamine D5-receptor is restricted to the hippocampus, lateral mammillary nucleus and the parafasicular nucleus of the thalamus. Dopamine D5-receptor mRNA has been found in regions including cerebral cortex, lateral thalamus and the striatum (Missale et al., 1998).

In the rat, the dopamine system is not fully developed at birth. Dopamine receptors have similar pharmacological characteristics in newborn and adult rats (Gelbard et al., 1989; Murrin and Zeng, 1986). Dopamine D1- and D2-receptor density increase during the first weeks of life and reaches adult-like density before the end of the first postnatal month even though it has been suggested that dopamine D1-receptors develop earlier than dopamine D2-receptors (Johansson et al., 1997; Rao et al., 1991). In this context, the dopamine system could potentially be affected by various manipulations during the postnatal period.

Different early postnatal rearing conditions have been used in order to study environmental influences on the dopamine system. Adult rats subjected to 15 min or 3 h of MS during the postnatal period were found to have alterations in dopamine

D1-like receptor density and dopamine transporter levels, whereas no differences were detected in dopamine D2-like receptor density (Brake et al., 2004). In an additional study using microdialysis, no differences were detected in basal nucleus accumbens dopamine levels, while there were differences in levels of dopamine metabolites after a K^+ pulse in rats that experienced 6 h of MS during the postnatal period and control rats (Hall et al., 1999). Furthermore, a third study found altered dopamine transporter levels in the nucleus accumbens and caudate putamen, but no differences in dopamine D1- or D2 receptor binding in adult rats subjected to short and prolonged periods of MS during the postnatal period (Meaney et al., 2002). Taken together, these results support evidence that early life events during the sensitive postnatal period can induce persistent effects in the dopamine system and dopaminergic function.

Ethanol

Alcohol, derived from the Arabic word for "something subtle", is a drug that has been strongly associated with human society. Ethanol is the natural product of fruit or cereal fermentation. In many cultures it is widely available, legal and moderate use is socially accepted. Associated with widespread availability and use are the enormous personal and societal costs of its abuse. It is estimated that approximately 10% of alcohol drinkers progress to levels of consumption that are socially and physically detrimental (Fleming et al., 2001). Large population-based studies in the USA have shown that 10-15% of all men and about 5% of all women are alcohol dependent (SBU, 2001). In Sweden, with a population close to 9 millions of people, a large investigation in 2000 showed that 14% of the men and 8% of the women had harmful levels of alcohol consumption (CAN, 2003). The societal costs for drug abuse and dependence (alcohol included) is estimated to be 30-120 billion Swedish crowns per year (SBU, 2001).

Ethanol affects virtually all body organs but is consumed for its effects on the CNS. The initial effects of ethanol are disinhibition, euphoria and sedation and as the dose increases, ethanol produces confusion, incoordination and coma and finally can lead to death. Relatively high doses of ethanol are required to induce a pharmacological effect. The structure of the ethanol molecule is very simple and the complexity and multitude of effects of ethanol have the origins in its simplicity. Unlike many other drugs of abuse, ethanol does not seem to have a specific binding site in the brain and yet affects many different systems. Therefore, the action of ethanol on the brain is complex and multifaceted (Fleming et al., 2001).

The initial effects of ethanol are believed to be due to activation of $GABA_A$ (gamma-aminobutyric acid) receptors and activation of the endogenous opioid system resulting in effects on the dopamine system as well as inhibition of NMDA (*N*-methyl-D-aspartate) glutamate receptors. At higher doses, ethanol inhibits functioning of most ligand- and voltage-gated ion channels. Ethanol furthermore has been found to interact with other brain systems, for instance the serotonin,

glutamate, acetylcholine, corticotropin-releasing factor and neuropeptide Y (NPY) (e.g. Cowen et al., 2004; Fleming et al., 2001; Koob, 2003; Oswald and Wand, 2004).

Ethanol is rapidly absorbed in the body and a large amount is metabolized in the liver by first-pass metabolism. The enzyme alcohol dehydrogenase (ADH) and the co-factor NAD^+ converts ethanol to acetaldehyde which then is enzymatically metabolized to acetic acid by aldehyde dehydrogenase (ALDH). Polymorphisms in the metabolizing enzymes ADH and ALDH have been consistently found to contribute to variations in susceptibility to alcoholism but also shown that some particular variants may be protective (e.g. Dick and Foroud, 2003; Kreek et al., 2004).

Sex differences in the effects of drugs of abuse have gained increasing interest during the past years. Females seem to be more sensitive to the rewarding effects of drugs of abuse than males and estrogen is suggested to be one factor that underlies this difference. However, the subjective effects of ethanol as a function of the menstrual cycle in alcoholic women is not well understood. Women are more sensitive than men to the physiological effects of ethanol and this has been suggested to be partly associated with women containing more body fat than men and partly by pharmacokinetic differences in the metabolism of ethanol between men and women. This results in higher blood alcohol levels in women after consuming the same amount of ethanol. Furthermore, affective and anxiety disorders are higher in women who are ethanol dependent whereas men typically have higher rates of antisocial personality disorder (e.g. Carroll et al., 2004; Lynch et al., 2002). Other evidence suggests sex differences in a number of the CNS actions of ethanol, including responses by or adaptations of $GABA_A$ and NMDA receptors which may involve sex differences in circulating steroids or brain architecture (Devaud et al., 2003).

Figure 2. Voluntary ethanol intake using the two-bottle free choice paradigm.

Experimental animals are useful as models in investigations of voluntary ethanol intake and the mechanisms underlying ethanol effects during acquisition, maintenance, and withdrawal. In experimental animals, ethanol is most commonly

self-administered via oral, intravenous, intracranial or intracerebroventricular routes. Operant-conditioning methods, in which an arbitrary response, such as a lever press, is reinforced by drug delivery, can be used in ethanol self-administration studies (Meisch and Lemaire, 1993). A more simple method is the two-bottle free choice paradigm, which was introduced by Richter and Campbell (Richter and Campbell, 1940). In the two-bottle free choice paradigm, animals have a free choice between a bottle containing an ethanol solution and a water bottle (Figure 2). Both continuous access (24 h) and limited access (shorter periods of access) are employed depending on the particular experimental question involved. Ethanol intake as well as preference (ethanol intake over total fluid intake) can be determined. Using the two-bottle free choice paradigm, it has been shown that female rats have a higher ethanol intake than males, which is in contrast to human studies (Cailhol and Mormede, 2001; Lancaster and Spiegel, 1992). When the ethanol concentration was varied, rats consumed ethanol to a higher extent than water at ethanol concentrations between 2-6% (Richter and Campbell, 1940). The preference for low ethanol concentrations has been attributed to taste of the solutions rather than to its pharmacological effects. Most drug solutions have an aversive taste and when orally consumed, there is a substantial delay between the behavior of drinking and the onset of drug effects. Rats selectively bred for a high ethanol intake and preference can, however, establish an ethanol intake at higher concentrations. One disadvantage with the two-bottle free choice paradigm is that the animals do not, by definition, become dependent or show any signs of motor impairment since they do not consume enough ethanol to become intoxicated and thus, do not show signs of withdrawal if the ethanol is taken away (Meisch and Lemaire, 1993). This method is therefore suitable for studies of acquisition of voluntary ethanol and subsequent ethanol intake, keeping in mind that it is a model for high ethanol consumption and not necessarily a model for ethanol dependence.

The literature on early life experiences and adult intake of drugs of abuse in experimental animals is sparse. Different MS protocols have been shown to alter cocaine self-administration in rats (Kosten et al., 2000; Kosten et al., 2004; Matthews et al., 1999; Zhang et al., 2004). Few studies have investigated the impact of MS on voluntary ethanol intake in adult experimental animals. When comparing rats that were individually separated for 3 min with non-handled controls, a lower ethanol intake was found in adult separated rats (Weinberg, 1987). Using a mixed individual/litter protocol consisting of separations for 15 min and 60 min and non-handled controls, reduced ethanol intake in adulthood was found in rats separated for a short period compared to the other two groups (Hilakivi-Clarke et al., 1991). Another study comparing 15 min or 180 min of MS and AFR rats, found higher ethanol intake in adult 180 min MS rats compared to 15 min MS and AFR animals, while no differences were found between 15 min MS and AFR rats (Huot et al., 2001). A fourth study found no MS-induced long-term effects on ethanol intake when comparing 240 min MS and 5 min MS rats, either in males or in females (Marmendal et al., 2004). Furthermore, different rearing conditions have been shown to alter ethanol intake in primates with peer-reared primates having a higher

ethanol intake than the mother-reared (Fahlke et al., 2000; Higley et al., 1991a). In a recent study in non-human primates, it was demonstrated that an allelic variation of the serotonin transporter gene was associated with decreased ethanol sensitivity, predominantly among those primates that were peer-reared during the early life period (Barr et al., 2003), giving further evidence for the interaction between genetic and environmental factors influencing the vulnerability for high ethanol intake. Taken together, these results are in accordance with findings in humans indicating that early life experiences can affect intake of drugs of abuse later in life (e.g. Brook et al., 2001; Gordon, 2002; Langbehn et al., 2003; Langeland and Hartgers, 1998; Spak et al., 2001).

The action of ethanol on the mesocorticolimbic dopamine and the endogenous opioid systems

Olds and Milner demonstrated that direct electrical stimulation of certain brain areas could be rewarding and act as a reinforcer (Olds and Milner, 1954). Since then, numerous studies have been conducted in order to identify the function of the mesocorticolimbic dopamine system, also referred to as the brain reward system (see The dopamine system). Research through the years has shown that the mesocorticolimbic dopamine system is complex and involves several regions and neurotransmitters. GABA containing pathways connecting the VTA and the nucleus accumbens, glutamatergic inputs to the nucleus accumbens as well as dopamine-innervated areas such as the amygdala, frontal cortex and the hippocampus are today associated with the function of the mesocorticolimbic dopamine system (e.g. Di Chiara, 1995; Gerrits et al., 2003; Nestler, 2001; Oswald and Wand, 2004; Wise, 2004). Furthermore, the influence of the endogenous opioid system on the mesocorticolimbic dopamine system has been demonstrated by administration of selective endogenous opioid substances (Devine et al., 1993; Di Chiara and Imperato, 1988b; Spanagel et al., 1992). These studies formed the general understanding that dopamine release within the nucleus accumbens is regulated by tonically active and functionally opposing opioid systems (Herz, 1998; Shippenberg et al., 1992). There are currently multiple theories regarding dopamine and its role in reward and drug dependence (Di Chiara et al., 1999; Koob et al., 2004; Lende and Smith, 2002; Nestler, 2001; Robinson and Berridge, 2003; Wise, 2004).

The mesocorticolimbic dopamine system is very old in an evolutionary perspective and is involved in the motivational system that regulates responses to natural reinforces such as food, drink, sexual activity and social interaction (e.g. Nestler, 2001; Wise, 2004). However, drugs of abuse can engage this brain reward system often more potently than natural rewards. A number of drugs are rewarding when injected into the VTA or nucleus accumbens and most drugs that are abused by humans are also self-administered by laboratory animals (Di Chiara and Imperato, 1988a). A recent study used positron emission tomography to investigate brain activation in men during ejaculation. The strongest activation was found in the

mesodiencephalic transition zone that, among other areas, comprises the VTA (Holstege et al., 2003). Increased activation of the VTA has also been found during heroin rush (Sell et al., 1999). These findings were therefore suggested to fit with the notion that the VTA is a key area in natural rewards, here exemplified by sexual orgasm, as well as in the effects of heroin (Holstege et al., 2003).

Like most other drugs of abuse, ethanol acts as a positive reinforcer in the mesocorticolimbic dopamine system. Whereas the effects of other drugs of abuse are due to direct (cocaine) or indirect (e.g. opiates) activation, ethanol exerts more unspecific effects. Ethanol causes an increase in the firing of VTA dopaminergic neurons by direct excitatory cellular activation resulting in an increased dopamine release in the nucleus accumbens. Ethanol also interacts with the endogenous opioid system within the mesocorticolimbic dopamine pathway and ethanol increases nucleus accumbens opioid peptide levels. It is also suggested that ethanol affects opioid peptides and receptors within the VTA and thereby may inhibit GABA inhibition. Furthermore, both non-selective opioid antagonists as well as antagonists selective for MORs and DORs block ethanol-induced dopamine release and reduce ethanol intake in rats (e.g. Gianoulakis, 2004; Herz, 1998; Oswald and Wand, 2004). Additionally, the non-selective opioid antagonist naltrexone is used for treatment of ethanol dependence in humans (e.g. Froehlich et al., 2003). Several substances acting on the dopaminergic and the endogenous opioid system, resulting in a decreased intake of drugs of abuse, are currently investigated in search for new pharmacological treatment of drug dependence (Kreek et al., 2002).

It has been demonstrated that ethanol-preferring and non-preferring animals have basal differences within the endogenous opioid system, for example Lewis and Fischer 344 rats (Nylander et al., 1995), C57BL/6 and DBA/2 mice (Jamensky and Gianoulakis, 1997; 1999; Ploj et al., 2000) as well as the ethanol-preferring AA and ethanol-avoiding ANA rats (Gianoulakis et al., 1992; Marinelli et al., 2000; Nylander et al., 1994). The involvement of the endogenous opioid system in the reinforcing effects of ethanol has also been studied using knockout mice. MOR-deficient mice were found to consume less ethanol or not self-administer ethanol at all (Becker et al., 2002; Hall et al., 2001; Roberts et al., 2000) whereas DOR knockout mice exhibited increased ethanol intake (Roberts et al., 2001). However, the DOR knockout mice exhibited an increased anxiety-like behavior (Filliol et al., 2000) and this behavior was reduced after ethanol intake. The authors therefore concluded that a reversal of the anxiety-like behavior might have led to the increased ethanol self-administration in these mice (Gaveriaux-Ruff and Kieffer, 2002). However, no difference between mice lacking preproenkephalin and wildtype mice was found in ethanol consumption (Koenig and Olive, 2002) and an increased ethanol intake was found in mice with decreased beta-endorphin expression and beta-endorphin-deficient mice compared to the respective wildtypes (Grahame et al., 1998; Grisel et al., 1999). The question have been raised, however compensatory mechanisms have occurred in these animals since classical gene knockout techniques have been used, where the targeted gene is deleted in embryonic tissue (Koenig and Olive, 2002). This could then result in findings in animals, that conflict with the

postulated role of the endogenous opioid system in mediating ethanol consumption and reinforcement.

In recent years, progress has been made in identifying polymorphisms in genes that may be responsible for the functional differences in the endogenous opioid system and putative risks for alcoholism in humans. Much of this work has been targeted towards the MOR gene. One polymorphism of interest involves an A118G nucleotide exchange and it is plausible that this polymorphism alters processes under opioidergic regulation (Kreek et al., 2004; LaForge et al., 2000). The MOR A118G variant has been associated with alcohol dependence mostly in males (Town et al., 1999). Further studies are however required to understand how genetic effects contribute in the development of vulnerability to drug dependence.

Various studies have also been performed to determine the role of mesocorticolimbic dopamine in ethanol consumption. Lesioning of dopamine neurons was shown not to affect maintenance of ethanol self-administration (Fahlke et al., 1994a; Ikemoto et al., 1997; Koistinen et al., 2001; Rassnick et al., 1993; Shoemaker et al., 2002). Acquisition of ethanol intake was substantially reduced by this manipulation suggesting that different neural mechanisms mediate acquisition and maintenance of ethanol intake (Ikemoto et al., 1997). However, in other studies, dopamine lesions were found not to affect acquisition of ethanol intake (Koistinen et al., 2001; Shoemaker et al., 2002). Furthermore, using genetically modified animals it was shown that mice deficient in dopamine D1- or D2-receptors consumed less ethanol than wild-type controls, indicating a role for dopamine D1- and D2-receptors in the motivation for ethanol consumption (El-Ghundi et al., 1998; Risinger et al., 2000).

Behavioral studies in experimental animals

One goal of behavioral neuroscience is to understand the biological mechanisms underlying brain function and behavior. An ethoexperimental approach in the study of behavior combines ethology and experimental psychology. The approach aims at the use of biologically relevant laboratory test environments and to include descriptions of the animal's behavior as a part of the analysis in the study of meaningful behaviors (Blanchard and Blanchard, 1988; Brain et al., 1991).

Risk assessment

In the wild, animals are exposed to a wide range of threats and dangers. Predation carries a much higher risk than that of temporarily losing food, water or a mate. An animal's normally observed behavior is a compromise between predator avoidance (cost) and the benefit of an alternative activity (foraging, mating) and takes into account differences in the costs and benefits from alternative options. Gain and risk are traded off so that the largest gain comes at the expense of the lowest risk (Lima and Dill, 1990).

Risk assessment is perhaps one of the most common behavioral patterns for any higher animal and occurs in situations involving any considerable degree of unfamiliarity or unpredictability. Risk assessment in rodents has been well summarized by Blanchard and Blanchard (Blanchard and Blanchard, 1988). There are two major variants of risk assessment in rodents. When there is a place to hide, animals tend to flee or seek shelter when exposed to a potential threat. If escape or shelter seeking is not possible, animals become immobile in an attempt to try to avoid danger. With no further signs of danger, the animal may reenter the threatening area to explore it using brief forward movements followed by rapid retreats. While in the threatening area the animal moves slowly, stays close to walls (thigmotaxis) and avoids unnecessary activities. Objects within the area may be explored using scanning and a stretched attended posture (SAP), a stretched motion of leaning far forward to sniff and then rapidly withdraw. When an animal assesses that there is no further indication of danger it slowly goes back to more normal activities, usually beginning with compensatory grooming. It has been suggested that reactions to poorly discriminate threat stimuli might be related to behaviors of emotional reactivity. Defensive motivational states are likely to involve partly separable systems, one for risk assessment and avoidance (with its primary goal of identifying a threatening stimulus or event) and one for defense elicited by specific threat (Blanchard and Blanchard, 1988). Defensive behaviors seen in wild rats, particularly in response to human approach and handling, have been reduced during the domestication of the laboratory rat (Blanchard et al., 1986).

Emotional reactivity

The term emotional reactivity is used here as a description of behaviors related to the emotional state induced by exposure to potentially dangerous or threatening objects, individuals or situations, which are often referred to as fear or anxiety. However, human fear and anxiety involve highly subjective components, which can be difficult to assess in experimental animals. Emotional reactivity is an appraisal of a particular situation that can only be measured in indirect terms in animal experiments. Emotionality may have evolved as an adaptation to modify an animal's behavior and thereby reduce the risk for being negatively affected by a potential dangerous situation and is an important response for the animal. Lack of this ability can be a serious survival disadvantage while overexpressed emotionality, on the other hand, can also be damaging to the animal. Risk assessment is related to emotional reactivity by being the information gathering process. Emotional reactivity comprises an array of behaviors and is not just mirrored by the adrenocortical hormones measurable in the blood (Boissy, 1995; Brain et al., 1991). Emotionality may not always be easy to measure in laboratory animals and perhaps it is more appropriate to focus on behavioral descriptions in different situations without putting functional relevance to humans into the interpretation, at least at an early stage. Even though caution is needed when translating results from animal experiments to humans, findings from human studies were found to show parallels to measures of emotional

reactivity in animal experiments (Blanchard et al., 2001). A great number of brain areas, circuits and transmitter systems comprise in the processes of risk assessment, learning and emotional reactivity and along with gene-environment interactions it forms the behavioral output (e.g. Blanchard and Blanchard, 1988; Boissy, 1995; Meyerson, 1979).

Animal models of emotional reactivity

Several animal models have been developed for research on the behavioral, pharmacological and neurochemical effects of emotional reactivity. Two common models include the open field (OF) test and the elevated plus-maze (EPM) test. The OF test is a commonly used test for measures of general exploration or ambulation in a novel and unfamiliar environment. The test consists of a large open arena. In this paradigm, animals are placed in the arena and allowed to explore it for a certain period of time. Emotional reactivity is assessed based on an emotional component in exploration of the inner field of the arena versus staying close to the walls. The outcome from the test may depend upon factors such as size and shape of the testing arena, level of illumination and duration of testing (e.g. Lister, 1990). The EPM test is another commonly used test to investigate and measure the behavioral, physiological and pharmacological aspects of emotional reactivity. The EPM apparatus consists of an elevated plus-shaped maze with two open arms and two closed arms. The conventional concept is that there is a conflict between exploration of the open arms and staying in the closed arms and that the height of the arena along with the open arm versus the closed arm response has an emotional component related to the open arm choice (e.g. Hogg, 1996; Lister, 1990; Pellow et al., 1985).

The literature on the effects of short or prolonged periods of MS on emotional reactivity is extensive. Reports do not always conform most likely due to dissimilarities in experimental protocols including environmental conditions, treatment of control groups and differences that can be attributed to strain characteristics. Short periods of MS have been shown to decrease emotional reactivity and enhance learning performance and attention abilities while more contradictory results have been obtained after prolonged periods of MS, but indications for an increased emotional reactivity have been presented (e.g. Ladd et al., 2000; Lehmann and Feldon, 2000; Pryce and Feldon, 2003). One previous study has used an ethoexperimental approach in order to study the effects of early life experiences on risk assessment behavior. In that study, rats individually separated for 15 min and control rats were compared in three different behavioral tests and a decreased emotional reactivity was found in the separated rats (Roy and Chapillon, 2004).

Choice of statistics in experimental animal models

In the choice of statistical tests one should consider aspects such as the manner in which the data was obtained, the nature of the samples and the particular hypothesis to be tested. In brief, parametric statistics are based on the assumption that the data was drawn from a normally distributed population and from populations having the same variance. In group comparisons, parametric statistics focus on the difference between the means of the groups. Nonparametric statistics also referred to as distribution-free, focus on the ranking of the scores and not their numerical values. Depending on the test, non-parametric statistics can be based on the ranking of the scores or the difference between the medians. Nonparametric tests are recommended when the sample size is very small and the nature of the sample distribution is not exactly known. With few animals and skewed data, the use of parametric tests is not appropriate since it can increase the risk for type I errors (Siegel and Castellan Jr., 1988). However, with sufficiently large samples the central limit theorem reveals that regardless of the shape of the samples, the means will be nearly normally distributed. For most sampled populations used in the behavioral sciences, a sample size of 50-100 is sufficient to produce a nearly normal sampling distribution (Kirk, 1994). This sample size is often not reached in the kind of experiments presented herein. Statistical programs enable testing of data distribution for normality and thereby determining which of the appropriate statistical tests, parametric or nonparametric, should be employed.

In studies of short and prolonged periods of MS the experimental design sometimes complicates the statistical analyses, as pointed out by Lehmann and Feldon (Lehmann and Feldon, 2000). In order to minimize the number of litters needed, studies sometimes use complete litters, i.e. littermates within the same experimental group. Related individuals then do not represent independent data points and this should be corrected for in the statistical analyses. Not all laboratories have the possibility to house the number of litters enabling the use of unrelated pups in the groups. In an attempt to minimize the number of litters, cross-fostering is sometimes used in order to spread the genetic influences. The littermates are then, as far as possible, spread among the dams so that the new litters contain a mix of pups from several dams. This procedure results in the same maternal influences impacting on pups of different genetic background.

Aim of this thesis

As discussed above, environmental and genetic factors contribute to the individual vulnerability to develop psychopathology and/or increased intake of drugs of abuse, which may lead to drug dependence. However, the underlying mechanisms are not fully elucidated. In this thesis are described studies of both short and prolonged periods of MS to investigate long-term effects on neurochemistry, voluntary ethanol intake and behavior. The work is divided into three parts with the following specific aims.

- Neurochemistry
  To investigate long-term effects of short and prolonged periods of MS on 1) opioid peptide levels in the pituitary gland and various areas of the brain, 2) opioid and dopamine receptor density in various areas of the brain as well as ethanol-induced effects on receptor density. For this purpose, male Wistar rats were used.

- Voluntary ethanol intake
  To examine long-term effects of short and prolonged periods of MS on acquisition of voluntary ethanol intake and subsequent ethanol intake. For this purpose, male and female Wistar rats as well as male ethanol-preferring AA and ethanol-avoiding ANA rats, with an inherent high and low voluntary ethanol intake, respectively, were used.

- Exploration and risk assessment behavior
  To explore long-term effects of short and prolonged periods of MS on the adult animal's behavior with specific consideration to information gathering and risk assessment. For this purpose, male Wistar rats were used.

Materials and methods

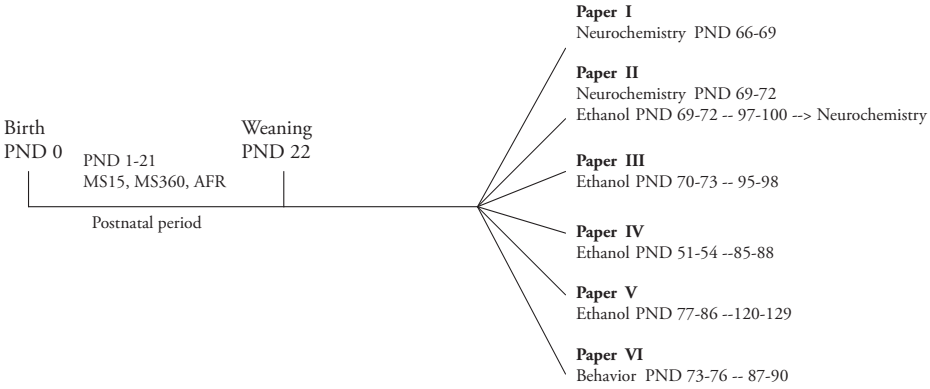


Figure 3. A schematic overview illustrating the design of the experiments presented herein.

Animals

In Papers I through III and VI, time-mated Wistar rats (Scanbur BK AB, Sollentuna, Sweden) were used. Pregnant ethanol-preferring AA rats, Paper IV, as well as pregnant AA rats and ethanol-avoiding ANA rats, Paper V, were kindly provided from the National Public Health Institute, Helsinki, Finland. All pregnant females were singly housed in standard macrolon cages (59x38x20 cm) containing wood chip bedding material and nesting material. After birth, the rat pups were used according to the overview in Figure 3. The animals were housed in a temperature (22 ± 1°C) and humidity (50 ± 10%) controlled environment on a 12:12 light/dark cycle with lights on at 6:00 a.m., except in Paper VI. Standard laboratory chow (R36 Labfor; Lactamin, Vadstena, Sweden) and tap water were available *ad libitum*. Gloves were used in all physical contact with the animals. All animal experiments were performed under a protocol approved by the Uppsala animal ethical committee and in accordance with the Swedish Animal Protection Legislation.

Short and prolonged periods of maternal separation

The litters were sexed, cross-fostered and culled on the day of birth (PND 0) and randomly assigned to one of three different rearing conditions: 15 min (MS15) or

360 min (MS360) of MS and normal AFR. The separations occurred once daily from PND 1-21. In the process of MS, first the dam and then the whole litter were removed from the home cage. The litters were at all times kept close together. Each litter was placed in macrolon cages (26x20x14 cm) containing wood-chip bedding material and moved to an adjacent room, with a higher temperature (24-27°C), for 15 min or 360 min, respectively. The cages in which the litters were placed during the separation were changed every day. The dams of the MS360 litters were returned to their home cages during the separation procedure but taken out prior to the return of the litters. The dams of the MS15 litters were transferred to another cage during the separation and the litters were returned to the home cages before the dams. The separation sessions were performed in the same room and at the same time every day, between 9.00 a.m. and 9.15 a.m. for the MS15 groups and between 9.00 a.m. and 3.00 p.m. or between 9.30 a.m. and 3.30 p.m. for the MS360 groups. The AFR litters were disturbed as little as possible and were only handled when cages were changed. During cage changes, a small part of the old bedding material was mixed with the clean bedding material and the old nesting material was transferred to the new cage in order to limit potential stressful experiences. This cage changing procedure occurred three times during the period from the arrival of the dams until PND 22 for AFR litters as well as for MS15 and MS360 litters. A limited number of persons had permission to enter the animal room during the period from the arrival of the pregnant dams until PND 22 and these persons performed the separations as well as the cage changes. The MS15 and MS360 litters were inspected for furring (Paper V) and eye opening (Papers IV-V) as a control for normal development. Measurements of litter weights were taken during the postnatal period whereas individual body weights were taken later on during the respective experiment. After weaning, on PND 22, the animals were group-housed in the same sex and treatment groups and left undisturbed except for normal animal facility care, Figure 3. Short and prolonged periods of MS were used as an experimental method to study the long-term effects of early life experiences on adult neurochemistry in male Wistar rats (Papers I-II), voluntary ethanol intake in male and female Wistar rats (Papers II-III) as well as male ethanol-preferring AA rats (Papers IV-V) and ethanol-avoiding ANA rats (Paper V) and exploration and risk assessment behavior in male Wistar rats (Paper VI).

Figure 4. The USV apparatus.

USV test

In Paper I, an USV test was performed in the morning, before the separation. The number of USVs emitted was measured during two consecutive days, PND 5 and PND 6. A randomly selected number of pups from the MS15, MS360 and AFR litters were transferred to an adjacent room with the same conditions as in the animal room. The rat pups were placed singly in a circular recording chamber made of aluminium with a diameter of 17 cm (Figure 4). The USV apparatus was kept at room temperature. After 1 min of adaptation, the number of USVs emitted during 1 min was recorded. A bat detector (Petterson Elektronik AB, Uppsala, Sweden) linked to an electronic counting device (developed by the Department of Medical Pharmacology, Uppsala University, Uppsala, Sweden) was used. The recording chamber was wiped clean with water after each test.

Neurochemical analyses

Drugs and chemicals

DYNB and Met-enkephalin-Arg^{6}Phe^{7} (MEAP) used for radioimmunoassay (RIA) in Paper I were purchased from Bachem AG (Bubendorf, Switzerland). In the autoradiographic analyses (Paper II), the radioligands [^{3}H]CI-977 and [^{125}I]iodosulpride were obtained from Amersham (Little Chalfont, United Kingdom) while [^{125}I]SCH23982 and [^{3}H]Ile^{5,6}-deltorphin II were purchased from NEN Life Science Products (Boston, MA) and Izotop (Budapest, Hungary), respectively.

Tissue extraction, purification and RIA

Tissue samples from the pituitary gland and dissected brain areas were placed in 1 M acetic acid (Paper I). The samples were heated at 95°C for 5 min and, after cooling on ice, homogenized using sonication. The heating procedure was repeated, the samples were again cooled on ice and then centrifuged. The tissue extracts were then subjected to a purification step using an ion exchange procedure (Christensson-Nylander et al., 1985) before RIA. The endogenous opioid peptides were analyzed using specific RIAs for DYNB and MEAP, used as markers for the prodynorphin and proenkephalin systems, respectively. RIA is highly specific and sensitive. It is based on the competition between an unlabeled antigen and the corresponding labeled antigen for a limited number of binding sites. When equilibrium is reached some antigen will remain free and some will be bound in an antigen-antibody complex. After separation of free antigen and antigen-antibody complex, the radioactivity in the bound fraction can be measured using a gamma counter. The percentage of labeled antigen that is bound decreases as the amount of unlabeled antigen increases. A standard curve is used for determination of the amount of unlabeled antigen. The DYNB and MEAP tracer peptides were labeled using chloramine-

T and purified by high-performance liquid chromatography. Samples subjected to MEAP assay were oxidized prior to the RIA procedure. The samples were dissolved and incubated with antiserum and ^{125}I-labeled peptide. The MEAP (90:3D II) and DYNB (113+) antisera, generated in rabbits (Christensson-Nylander et al., 1985), were diluted in gelatin buffer. The DYNB antiserum did not show cross-reactivity with either DYNA (1-17) or DYNA (1-8). Cross-reactivity with DYNB 29 was 1% and with big dynorphin (DYN 32) 100%. Other opioid peptides did not cross-react with the DYNB antiserum. For MEAP, cross-reactivity with the MEAP antiserum and Met-enkephalin, Met-enkephalin-Arg^{6}, Met-enkephalin-Arg^{6}Gly^{7}Leu^{8}, Leu-enkephalin or DYNA (1-6) was less than 0.1%. Following the incubation period, the DYNB samples were incubated with a sheep-antirabbit antiserum (Pharmacia Decanting Suspension; Pharmacia Diagnostics, Uppsala, Sweden) or a charcoal suspension (MEAP assay) in order to separate free antigen and antigen-antibody complex. Values were expressed as fmol peptide/mg tissue (wet weight).

Receptor autoradiography

Receptor autoradiography is a technique in which radioligands are bound to tissue slices to localize receptors. Radioligand binding sites are detected in tissue sections by exposure to a photographic emulsion. After decapitation, intact brains were removed and frozen immediately (Paper II). Coronal brain sections from intact brains were used to determine opioid (DOR and KOR) and dopamine (D1- and D2-like) receptor densities. The autoradiography procedure was modified from a protocol described by Kitchen et al. (Kitchen et al., 1997). Slides were preincubated in a buffer chosen for each receptor investigated, before binding was carried out. Different concentrations of radioligand, [^{3}H]Ile^{5,6}-deltorphin II (DOR), [^{3}H]CI-977 (KOR), [^{125}I]SCH23982 (dopamine D1-like receptors) and [^{125}I]iodosulpride (dopamine D2-like receptors), respectively, were used for labeling. Non-specific binding was determined using naloxone (DOR and KOR), SCH23390 in addition with ketanserin to inhibit ligand binding to 5-HT1A and 5-HT2A receptors (dopamine D1-like receptors) and apomorphine (dopamine D2-like receptors), respectively. The slides were then washed and dried followed by exposure to [^{3}H]-Hyperfilm for various time periods depending on the receptor of interest along with [^{3}H]- and [^{125}I]-microscales. The films were manually developed (Kodak D19, Unifix) and the optical densities were converted to fmol/mg wet weight based on the co-exposed standards using NIH Image software (NIH Image 1.62; NIMH, Bethesda, MD). Brain areas were identified using a rat brain atlas (Paxinos and Watson, 1997).

Voluntary ethanol intake

The acquisition of voluntary ethanol intake and subsequent daily ethanol intake was investigated in adult rats subjected to short and prolonged periods of MS during

the postnatal period (Papers II-V). To this end, the two-bottle free choice paradigm was used. The animals were housed in individual cages (42x26x18 cm) in order to measure individual ethanol intake. The ethanol solutions (v/v) were made from 95% ethanol and tap water. Bottle (plastic 150 ml-bottles with ballvalve nipples; Scanbur BK AB, Sollentuna, Sweden) positions were randomly changed in order to avoid position preferences. Starting at 2% ethanol, the concentrations were increased during a period of about two weeks and the rats were thereafter maintained on 8% ethanol (Papers II-III), 10% ethanol (Paper IV) and in Paper V, 10% ethanol (AA rats) or 6 % ethanol (ANA rats). The animals thereafter had continuous access to the final concentration for 11-27 days. In AA rats (Paper V), the ethanol intake was further investigated using the limited access paradigm (modified from (Sinclair et al., 1992)). The period with limited access to 10% ethanol started immediately at the beginning of the active period, when the light turned off at 6.00 p.m. The period with limited access was gradually decreased, starting with 4 h/day (4 days), then 2 h/day (3 days) and finally 1 h/day for 6 days before the end of the experiment. Measurements of water and ethanol intake were made daily while measures of food intake and body weight were taken every third day.

Restraint stress

Restraint stress was used in order to study the effects of a stressful situation in adulthood on voluntary ethanol intake and to investigate whether this procedure would alter the ethanol intake. Restraint stress can be viewed as a forced inability to explore the novel environment and escape potential threats. The animals were placed in a transparent plastic tube with a size appropriate to induce immobilization for 1 h during four consecutive days (Papers II-III), 30 min at a single occasion (Paper IV) or for 30 min at two unpredictable occasions (Paper V). The restraint stress sessions were performed during the afternoon.

Behavioral tests

In Paper VI, the rats were transferred to an animal room (temperature 22 ± 1°C and humidity 50 ± 10%) with reversed 12:12 light/dark cycle (lights off at 9:00 a.m.) and allowed to adapt for two weeks before the start of the testing. Three behavioral tests were used: the Concentric Square Field (CSF) test, the OF test and the EPM test. The tests were designed to explore effects of short and prolonged periods of MS on adult general activity, exploration, approach and avoidance performance and the animals' staying in open versus sheltered areas. In addition some behaviors such as rearing (standing on hind legs) and grooming (face, body and genital washing) were recorded. The purpose of the multivariate design was to gather information that taken together should illustrate if MS alters risk assessment and risk taking strategies in the adult animal.

Each animal was run in the three behavioral tests during three consecutive days during a period of two weeks starting with the CSF, then the OF and finally the EPM. The rats in each experimental group were tested using a running schedule in order to avoid time and order bias. All testing took place in an adjacent room with similar temperature- and humidity-conditions as in the animal room. Observations were carried out during the afternoon, during the dark (active) period of the light/dark cycle. The rats were weighed at the end of the first day of testing.

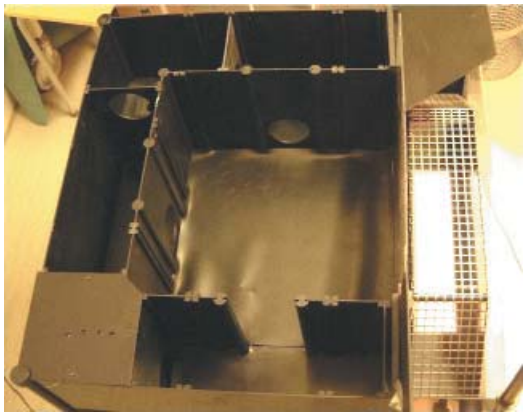


Figure 5. The CSF arena.

The CSF test

The CSF test was originally designed to measure risk and benefit assessment in rats after experimental brain lesions (Clausen et al., 2001; Roos et al., 2003). The technique has also been used to characterize differences in exploration and risk assessment in wild house mice and domesticated mice (Augustsson, 2004; Augustsson and Meyerson, 2004). The method provides multiple areas for the animal to attend (Figure 5). The physical construct of the various areas of the field differs. The idea behind the construction is that the various areas should provide different elements of risk versus shelter and explorative incentives. The apparatus consists of a square field (70x70 cm) with a smaller square field (41x41 cm) located in the centre of the larger one. The outer walls are 26 cm high and the inner walls 25 cm. Openings in three of the walls of the centre field lead to the outer field and as a consequence a corridor is formed around the central field. The following items are placed in this corridor: an obstacle consisting of a wall with a round ($\subseteq$=8 cm) opening placed 10 cm above the floor with a hole board with two holes ($\subseteq$=2.5 cm) inside (HURDLE), a stainless steel wire mesh construction (10 mm between the bars) that bridges over an illuminated opening in the floor (BRIDGE), a dark room covered by a piece of board, to which the animal has access via one entrance (Dark Corner Room; DCR). Walls are built in black poly vinyl chloride plastics (4 mm thick) except the outer wall of the BRIDGE, which is transparent, and a

black rubber mat covers the floor. The entire arena was divided into different zones (Figure 18, in the Results and Discussion section), which constituted the basis for the description and the variables of the animal's performance in this test. The animal was transferred to the CSF apparatus from the home cage and released in the centre square field facing the wall without openings (Figure 5). The test session lasted for 20 min. Dimmed light was used during the testing, except for the BRIDGE area (see above). The behavior was monitored by means of a TV-video set up (Panasonic Super Dynamic WV-BP 550/B camera, Panasonic AG-TL 300E VHS recorder) from which the recordings were taken. After each test, the floor was wiped with a cloth containing 70% ethanol. Sufficient time was allowed for the floor to dry before the next animal was placed in the arena.

Zone definitions and behavioral scoring

The following zones were defined: *CENTRAL SQUARE (CENTRE)*, the centre field of the arena; *CENTRAL CIRCLE (CTRCI)*, a circular zone in the middle of the centre field used for measures of activity in an open area; *HURDLE*, a high passage to a hole board introduced to test the motor ability of the animal and the exploratory drive; *BRIDGE (BRG)*, the elevated and illuminated bridge construction, considered as an open and elevated area; *SLOPE*, the slope leading up to the BRIDGE, considered as an area where the animal has to assess the risk of visiting the BRIDGE; *BRIDGE ENTRANCE*, the area just in front of the entrance to the SLOPE, considered as an area where the animal has to asses the risk of entering the SLOPE; *DARK CORNER ROOM (DCR)*, a shaded room where the animal could seek shelter. The test was analyzed by direct manual scoring and by use of Ethovision (Noldus Information Technology, Wageningen, The Netherlands). The numbers of rearing and grooming as well as the latency to leave the centre field were measures taken by direct manual scoring. The latency (LAT) to first visit a zone, the frequencies (FREQs) of visits as well as the duration (DUR) of staying in a zone were all measures taken either by direct manual scoring or by using the Ethovision computer program. General locomotion was measured by the sum of entries to the corridors *(FREQ TOTCORR)* that leads from the centre field to the BRIDGE, DCR and HURDLE as well as measurement of the distance traveled in the total arena *(DIST TOTARENA)*. Number of rearing, latency to leave the centre field, latency to first visit the HURDLE, BRIDGE, DCR, SLOPE and CTRCI as well as frequency of visits to HURDLE were used as measures of exploration. The occurrence, frequency and duration of visits to CTRCI, BRIDGE and DCR were used as measures of occupying open versus sheltered areas. The behavioral scoring was performed during the entire 20 min trial as well as during the first 5 min in order to investigate potential differences between the three experimental groups during the period immediately after start.

Figure 6. The OF arena with the start box attached to the wall.

The OF test

The OF test used in the present experiment is shown in Figure 6. The OF arena consisted of a black circular stainless steel arena with a diameter of 90 cm surrounded by 35 cm high walls and a black stainless steel wire mesh floor (10 mm between the bars). Two cages were attached to the wall opposite to each other. One cage was empty and wire bars blocked the entrance so the animal did not have access to this cage. The other cage was used as a start box (25x25x25 cm with an opening $\subseteq$=8 cm). The rat was transferred from its home cage and placed in the start box. Thereafter the test session started and the animal was free to explore the arena with the possibility to return to the start box. The location of the experimental animal was recorded by an infrared-sensitive video camera-computer linkup (Höglund et al., 1983). The arena was divided into pre-programmed zones for the animal to explore. The test sessions were run in darkness and lasted for 30 min. After each rat, the OF arena was wiped clean with water and dried.

Behavioral scoring

The following parameters were recorded: latency to leave the start box and duration of time spent in the start box as well as entering and leaving of the defined zones within the arena (Figure 20, in the Results and Discussion section), scored as latency to first visit, frequency of visits and duration of visits into the zones. The total number of visits to the defined zones was taken as a measure of locomotor activity. Visits and staying in the inner open area of the arena (CENTRE) versus more sheltered areas close to the walls (OUTER CIRCLE) as well as measures of exploratory drive by staying in the zone in front of the closed cage in the wall (GOAL ZONE) were also analyzed. The possibility to return to the start box enabled assessment of shelter seeking. The test was analyzed using a video camera-computer linkup (Höglund et al., 1983).

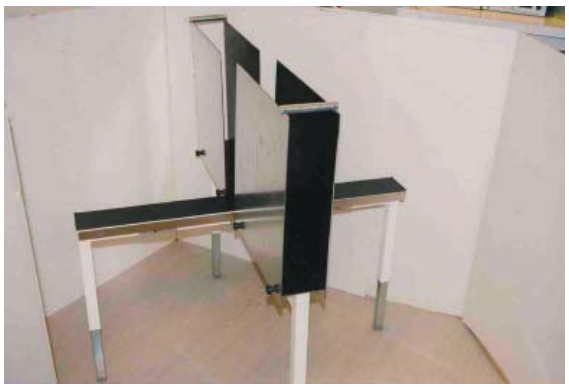


Figure 7. The EPM test.

The EPM test

The EPM is shown in Figure 7. The maze consisted of four arms, each 40 cm long and 10 cm wide, arranged in the shape of a plus sign and elevated 51 cm off the floor. Two opposite arms were open, whereas the other two were closed with 40 cm high walls. The area inside the centre of the EPM (10x10 cm) was not considered being either an open or closed arm. The entire EPM was made of black metal with a black rubber mat covering the floor of the arms. The test began with each rat being placed in the centre facing an open arm, after which the experimenter left the room. Each rat was tested for 5 min and dimmed light was used during the testing. The behavior was recorded via an Ikegami CTC 4600 video camera mounted over the maze connected to a SABA M3705M monitor and a Panasonic AG-TL300E VHS recorder in a room next to the testing room. After each rat, the EPM was wiped clean with 70% ethanol and sufficient time was allowed for the apparatus to dry before the next animal was placed in the maze.

Behavioral scoring

The following parameters were measured: latency to first enter an open or closed arm as well as the frequency and duration of visits into the open and closed arms and the centre portion. Furthermore, the total frequency of stretched attended postures (TOT SAP) and head dipping (TOT DIP) as well as numbers of rearing and grooming were scored. The total crossings were used as a measure of locomotor activity. The test was analyzed manually using our software Score v 1.0 (developed by Pär Nyström, Department of Psychology, Uppsala University, Uppsala, Sweden).

Statistical analyses

In Papers I-V, the normal distribution of the data was estimated based on mean and median values and by the data distribution. In Paper VI, the data distribution was tested for normality using the Shapiro-Wilk's W test. The parametric one-way Analyses of Variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) post-hoc tests were used for data showing a normal distribution. Data not showing a normal distribution was analyzed using the non-parametric Kruskal-Wallis test for between-group comparisons. Whenever a statistical significance was found, the Mann-Whitney *U* test was used for further analyses. Furthermore, in Papers V-VI, where significant effects were found with Mann-Whitney *U* test, significances were further corrected using the Bonferroni-Holm (Holm, 1979) correction for type I error.

For comparisons of body weight during the postnatal period (Papers I-VI), during the period of access to ethanol (Papers II-V) as well as at the time for the behavioral testing (Paper VI), ir peptide levels (Paper I), receptor density (Paper II), day for eye opening (Paper IV) and food intake (Paper IV), ANOVA followed by Fisher's PLSD post-hoc tests were used. Body weight gain during the MS period (Papers I-IV) was analyzed using repeated measures ANOVA. Correlations between receptor density and ethanol intake (Paper II) were evaluated with the paired correlation analysis followed by Fisher's *r* to *z* test.

The number of USVs (Paper I) and fluid intake data (Papers II-IV) were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney *U* test. In Paper V, significant differences in fluid intake and food intake data as well as the behavioral data (Paper VI) after the Mann-Whitney *U* test were further corrected using the Bonferroni-Holm correction. The Wilcoxon signed rank test was used to analyze the effects of restraint stress on voluntary ethanol intake (Papers II-V) in animals subjected to the three different rearing conditions.

The Chi-Square test was used to evaluate significant effects within and between the groups in the change of USVs (Paper I). In Paper VI, animals that did not enter a zone in the CSF test were considered as missing values in the statistical analyses and therefore the Chi-Square test, followed by the Bonferroni-Holm correction, was used for analyses of occurrence.

StatView 4.5 (Papers I-II) and 5.0.1 (SAS Institute Inc., Cary, NC) (Papers III-VI) and Statistica 6.0 (StatSoft Inc., Tulsa, OK) (Paper VI) software's were used for the statistical analyses. Differences were considered statistically significant at $p<0.05$ (Papers I-V) and $p\leq0.05$ (Paper VI).

In Paper VI, the traditional statistical analyses were further supplemented by use of a multivariate data analysis (Soft Independent Modeling of Class Analogy Principal Component Analysis [PCA]). The PCA is a multivariate pattern based approach designed to extract and display the systemic variation in a data set. This was done to determine whether the behavioral data registered in the CSF test permitted a class prediction corresponding to experimental groups (i.e. if there were behavioral profiles corresponding to experimental rearing conditions) and to identify parameters important for this class prediction. The PCA creates a score plot, showing a summary of the relationship among the individuals, and a loading plot, identifying variables important for creating these relationships. The SIMCA-P+10.02 (Umetrics, Umeå, Sweden) software was used for the PCA analysis.

Results and discussion

Comparisons of results from MS experiments are complicated because of a large variation in experimental protocols used, especially for prolonged periods of MS. The outcome of such experiments is dependent upon several critical factors, such as rat strain, as well as experimental protocols, e.g. time and duration of separation as well as litter or individual separation but also the choice of control group i.e. non-handled animals or AFR animals (Lehmann and Feldon, 2000; Pryce and Feldon, 2003). In the present thesis, an experimental protocol consisting of 15 min or 360 min of daily MS in litters during PND 1-21 was used and AFR animals were used as controls. A confounding factor is that the pregnant dams were transported during gestation. Furthermore, the rats were housed individually during the period of access to ethanol in order to measure individual ethanol intake. The rats had visual, auditory and olfactory contact but not the normal social interaction with other rats. These factors may affect the later outcome of the postnatal separations while at the same time these factors are similar in all experiments presented herein.

Pup weights and USV during the postnatal period

The litters were kept close together at all times. The litters were never cold to the touch when they were put back after the prolonged MS and the pups always had milk left in their stomach as could be seen during the first postnatal week.

The litters were weighed during the postnatal period and at the time of weaning in all experiments. In Papers I-IV and VI statistically significant effects on pup weight or pup weight gain were not found between the three experimental groups. In Paper V, significant differences were not observed in body weight of ANA litters in either rearing condition, Figure 8. However, a significant difference in mean pup weight (g) in the AA MS15, MS360 and AFR litters was found on PND 7 while no difference was found on PND 16. On PND 7 the mean pup weight was significantly lower in AA MS360 litters compared to AFR litters. The AA MS15 and MS360 litters were weighed on additional days during PND 1-21 and no differences between these two groups were found. No differences were found in mean pup weight between experimental groups, either in AA litters or ANA litters, on PND 22.

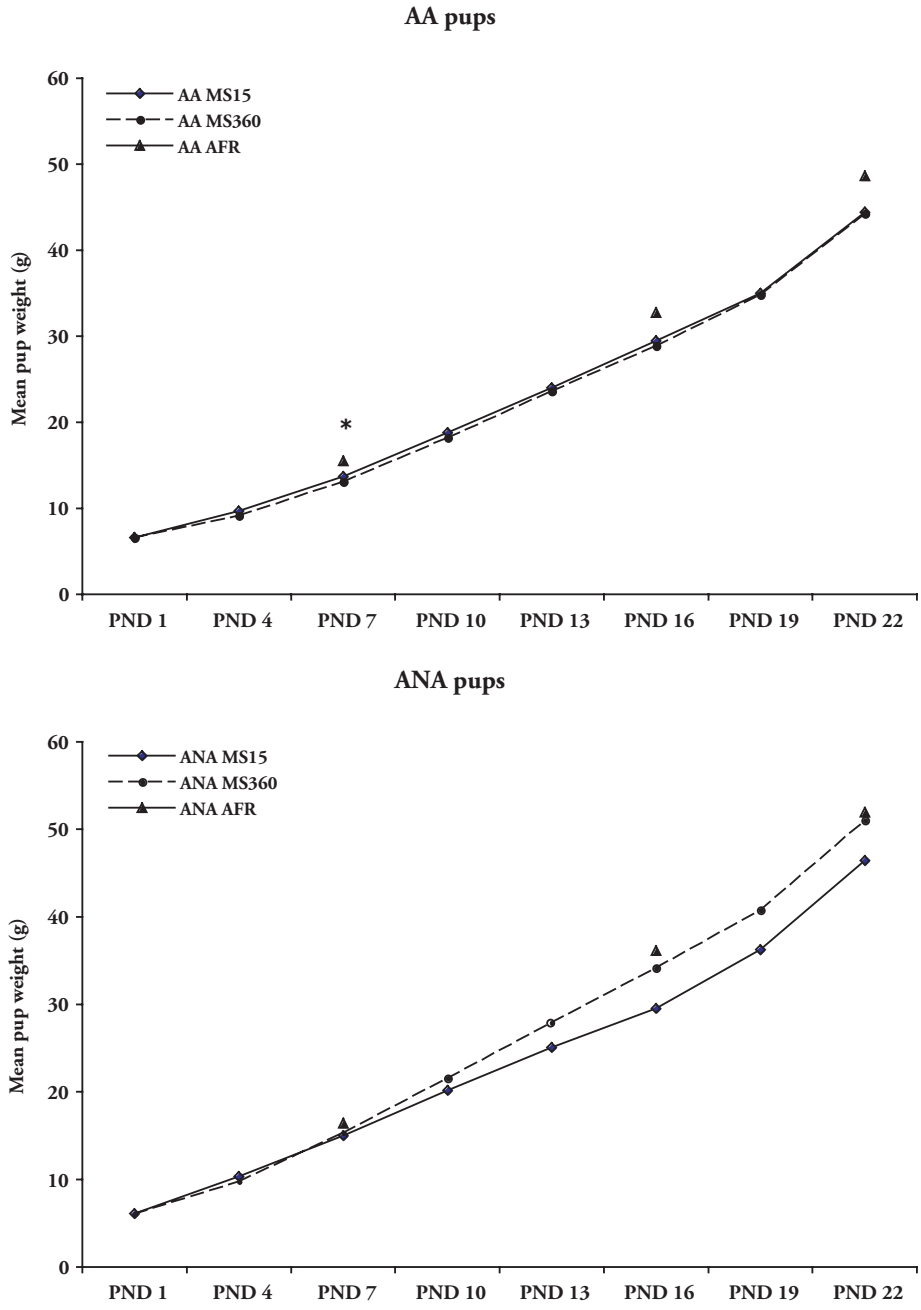


Figure 8. Mean pup weight (g) in AA and ANA MS15 (AA n=5, ANA n=2), MS360 (AA n=5, ANA n=3) and AFR (AA n=4, ANA n=3) litters during the postnatal period and at weaning on PND 22 (Paper V). * indicate differences between the three experimental AA groups.

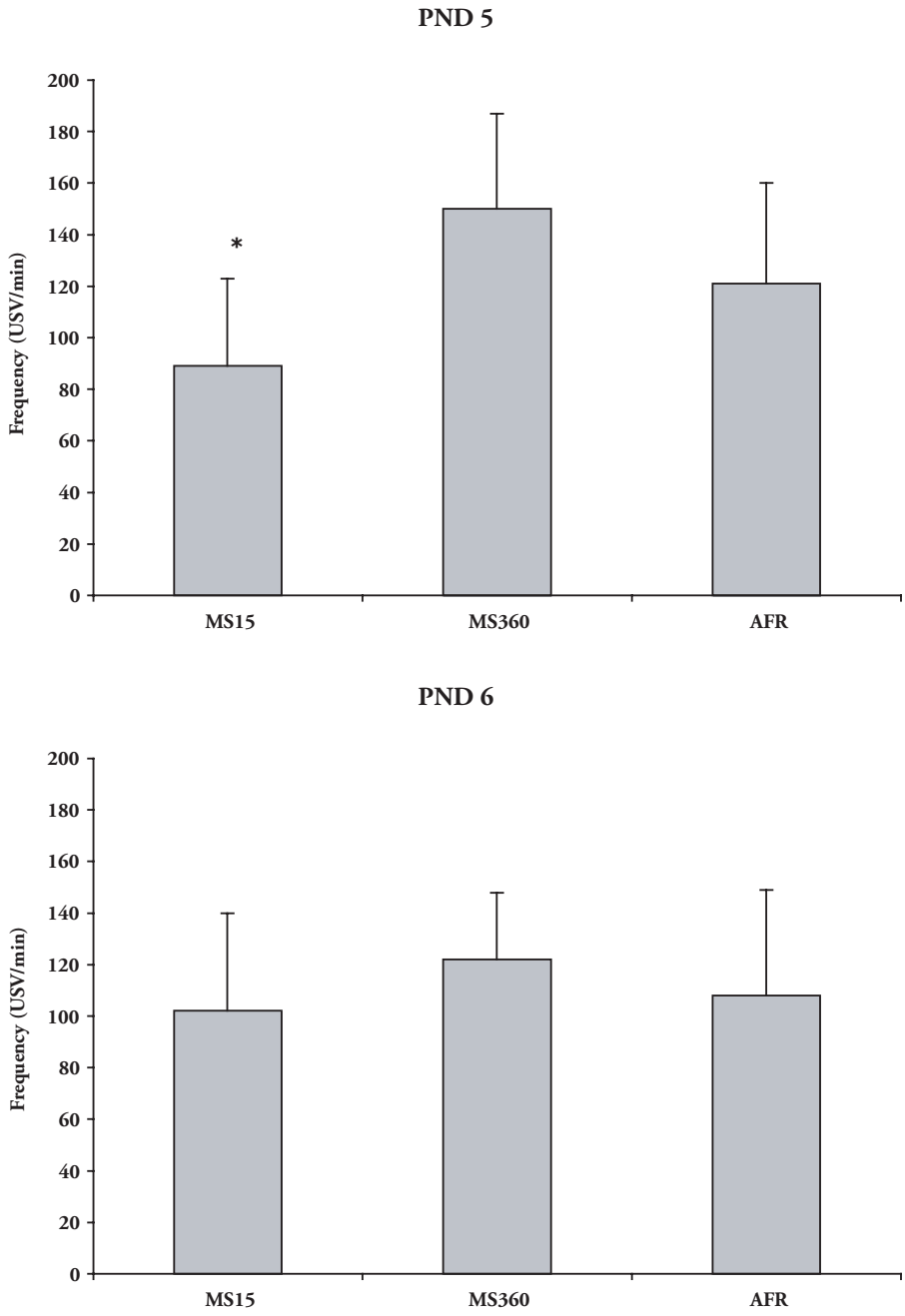


Figure 9. The median ± MAD number of USVs during 1 min on PND 5 and PND 6 in Wistar MS15, MS360 and AFR pups (n=12 pups/group, Paper I). *p<0.05 compared to MS360 pups (Kruskal-Wallis, Mann-Whitney *U* test).

In Paper I, the number of USVs emitted during 1 min was recorded before the separation on PND 5 and 6 and the results are shown in Figure 9. On PND 5, MS360 pups had a significantly higher number of USVs compared to MS15 pups, whereas there were no significant differences in response between MS360 and AFR pups or MS15 and AFR pups. No significant effects between the three experimental groups were seen on PND 6. A detailed analysis of the change of USVs from PND 5 to PND 6 revealed no significant difference within groups over days but an obvious difference between groups as to number of pups with higher frequency of calls. Seventy-five percent of MS360 pups, 25 % of MS15 pups and 50 % of AFR pups had higher frequency of USVs on PND 5 than on PND 6 and this was found to be statistically significant when comparing MS360 and MS15 pups. The frequency of USVs was expected to be the same on PND 5 and PND 6, during the mentioned plateau level (Johansson-Wallsten, 1993). This was true for the AFR pups. However, the MS360 group had more pups with higher frequency of vocalizations on PND 5 than 6 compared to the MS15 pups indicating a difference in the time course of the development of the USV response.

In Papers IV and VI the litters were inspected for furring and eye opening as a control for normal development and no differences were found between the experimental groups in any of the two studies. However, previous results using the present experimental protocol have shown differences between the three groups in the time point for eye opening. MS360 pups were found to open their eyes significantly later than the other two groups, while no difference was seen between MS15 and AFR pups (Ploj et al., 2002).

Taken together, it can not be excluded that the present experimental protocol induce developmental changes between the MS15, MS360 and AFR groups, even though indices have not been found in all the experiments.

Neurochemistry (Papers I-II)

The DYNB and MEAP ir peptide levels in male MS15, MS360 and AFR Wistar rats (n=7-10 rats/group) were measured at 10 weeks of age (Paper I). MS15 and MS360 rats had significantly higher DYNB ir levels in the neurointermediate pituitary lobe and MS15 rats had significantly higher DYNB ir levels in the hypothalamus compared to those of the AFR rats. In the hypothalamus, significantly higher MEAP ir levels were found in the MS15 rats compared to both MS360 and AFR rats. In the substantia nigra, MS15 rats had significantly higher DYNB ir levels compared to those of the MS360 and AFR rats. Furthermore, significantly lower DYNB ir levels were detected in the amygdala in the MS15 rats compared to MS360 rats. MS360 rats had significantly higher DYNB ir levels in the periaqueductal gray compared to those of the MS15 and AFR rats.

In Paper II, the effects of MS15, MS360 and AFR on opioid and dopamine receptor density were investigated in male Wistar rats at 10 weeks of age (n=7-8 rats/group). Analysis of DOR density indicated a significant effect in the pontine

nuclei, where both MS15 and MS360 rats had higher density of binding sites compared to AFR rats. Quantitative analysis of KOR binding demonstrated that there were no significant differences in binding sites between the three experimental groups. A significant effect on dopamine D1-like receptor binding was found in the hippocampus, where MS15 rats had an increased number of binding sites compared to AFR rats. Finally, quantitative analysis of dopamine D2-like receptor binding indicated a significant effect in the VTA and the periaqueductal gray. MS15 rats had increased dopamine D2-like receptor binding in the VTA compared to MS360 and AFR rats and in the periaqueductal gray compared to MS360 rats.

A different protocol for short periods of MS consisting of individual separation for 15 min and normal AFR, has been used to investigate long-term effects on the endogenous opioid system in Sprague-Dawley rats (Ploj and Nylander, 2003; Ploj et al., 1999; Ploj et al., 2001). In male rats, ir tissue levels of DYNA and DYNB were altered in certain brain areas that were dissected out using a different technique than in Paper I. Higher DYNB ir levels were detected in the pituitary gland and areas of the brain including the hypothalamus, hippocampus, striatum, medulla oblongata and the midbrain in rats individually separated for 15 min (Ploj et al., 1999). In contrast, less pronounced effects were found in female rats, where DYNB ir levels were higher in the periaqueductal gray and lower in the frontal cortex and the amygdala in rats subjected to 15 min of individual MS (Ploj et al., 2001). Furthermore, 15 min of MS during the postnatal period was shown to increase DOR density in the amygdala whereas KOR density was unaffected (Ploj and Nylander, 2003). In another study, comparing 240 min and 5 min MS in male and female Wistar rats, male rats separated for 240 min had higher MEAP ir levels in the hippocampus. No differences were detected in MEAP ir levels in female rats and neither male nor female rats showed significant differences in DYNB ir peptide levels (Marmendal et al., 2004). Together these data further indicate that the outcome of MS is dependent on experimental protocol, sex and rat strain.

The results in receptor density in Paper II indicated minor changes in opioid receptor density after MS. This may be relevant since experiences from opioid gene knockout studies have shown that a small change in receptor binding may well have significant consequences for function (Kitchen et al., 1997). Furthermore, changes in receptor coupling mechanisms, or other intracellular processes that affect the functional response, are not detected in autoradiography and therefore cannot be excluded. The absence of effect on receptor density in areas where altered peptide levels were found after MS may also depend on differences in sensitivity in the methods used to quantify peptides and receptors, respectively. The present study also presents information about MS-induced effects on dopamine D1- and D2-like receptors. Of special interest is the finding of MS-induced effects on dopamine D2-like receptors in the VTA where the mesocorticolimbic dopamine neurons originate. The present results on dopamine D1- and D2-like binding are however not in concordance with results reported recently (Brake et al., 2004; Meaney et al., 2002) using a different experimental protocol for MS.

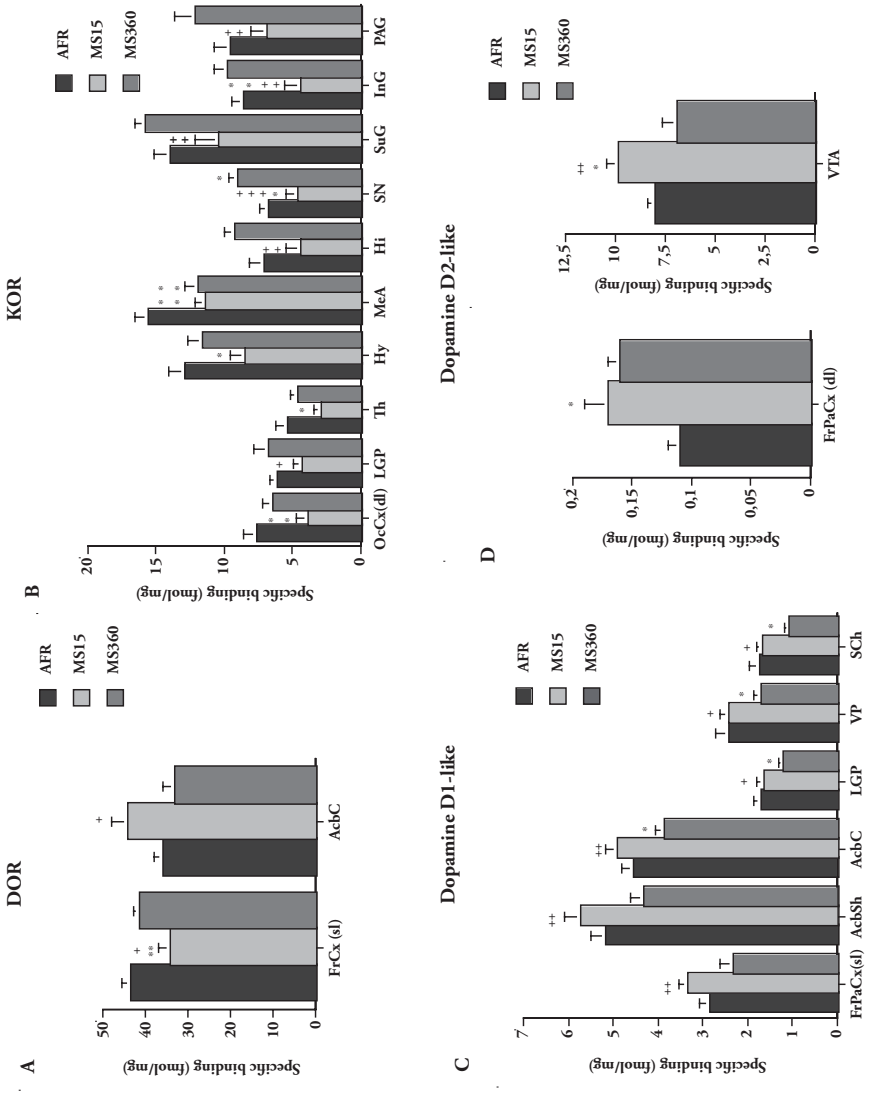


Figure 10. Quantitative autoradiography of receptor binding in MS15, MS360 and AFR rats 26 days after first access to ethanol. Values represent mean ± SEM specific binding of **A.** [^{3}H]Ile^{5,6}-deltorphin II (DOR), **B.** [^{3}H]CI-977 (KOR), **C.** [^{125}I]SCH23982 (dopamine D1-like receptors) and **D.** [^{125}I]iodosulpride (dopamine D2-like receptors) expressed as fmol/mg brain tissue (n=7-8 rats/group). *p<0.05, **p<0.01 compared to AFR rats; +p<0.05, ++p<0.01, +++p<0.001 compared to MS360 rats (ANOVA, Fisher's PLSD post-hoc test). Abbreviations: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; FrCx (sl), frontal cortex superficial layers; FrPaCx (dl), frontal-parietal cortex deep layers; FrPaCx (sl), frontal-parietal cortex superficial layers; Hi, hippocampus; Hy, hypothalamus; InG, intermediate gray layer of the superior colliculus; LGP, lateral globus pallidus; MeA, medial amygdala; OcCx (dl), occipital cortex deep layers; PAG, periaqueductal gray; SCh, suprachiasmatic nucleus; SN, substantia nigra; SuG, superficial gray layer of the superior colliculus; Th, thalamus; VP, ventral pallidum; VTA, ventral tegmental area.

The opioid and dopamine receptors were also measured after 26 days of voluntary ethanol intake in the rats selected for the highest ethanol intake in each group, see below. Brain receptor density was analyzed in the same areas as after MS. The differences detected are shown in Figure 10. In this study, the KOR density was altered in MS360 and/or AFR rats after ethanol consumption in brain areas involved in drug intake behavior and/or emotional responses. After ethanol drinking, a lower KOR density was found in MS15 rats, the group with a low ethanol intake (see below). Thus, ethanol drinking may have induced an upregulation of KORs in distinct brain areas in MS360 and AFR rats. Furthermore, in accordance with human studies, where alcoholics and cocaine abusers show a significant reduction in brain dopamine D2 receptors and cigarette smokers have indications for a reduction in dopamine D1 receptors (Volkow et al., 2004; Volkow et al., 1996), this study showed that ethanol consumption resulted in a downregulation of brain dopamine D1- and D2-like receptors in MS360 and/or AFR rats. Interestingly, the higher density of dopamine D2-like binding sites in the VTA in MS15 rats detected after MS was still higher than in the other two groups after access to ethanol. This finding may relate to the lower tendency to initiate drinking and the lower ethanol intake in MS15 rats compared to the other rats.

Voluntary ethanol intake behavior (Papers II-V)

In the light of previous findings in humans, rats and non-human primates showing that early life experiences could affect intake of drugs of abuse, e.g. ethanol, it was hypothesized that rats subjected to MS15 and MS360 would differ in voluntary ethanol intake.

Wistar rats (Papers II-III)

When analyzing ethanol intake (g/kg/day) in all male Wistar rats (n=26-28 rats/group, Paper II), MS15 rats consumed less ethanol than MS360 and AFR rats throughout the period of 26 days of voluntary ethanol consumption. However, a high variation in intake was found within the groups and statistical analyses of ethanol intake at the different ethanol concentrations revealed no significant differences in ethanol intake or preference (% of total fluid intake). At 8% ethanol, 23% of MS360 rats had an ethanol intake over 1 g/kg/day whereas only 4% of the MS15 and AFR rats had an intake over 1 g/kg/day. The ethanol intake after MS in those male rats that initiated drinking and had the highest ethanol intake in each group (n=8 rats/group) is shown in Figure 11. MS15 rats consumed significantly less ethanol than MS360 and AFR rats at 2%, 4% and 6% ethanol and significantly less than MS360 at 8% ethanol. At 8% ethanol, the AFR rats reduced their intake whereas the MS360 rats consumed significantly more 8% ethanol than both MS15 and AFR rats. Analyses of the ethanol preference in the selected male rats showed a similar pattern as the ethanol intake. At 8% ethanol, the preference in the male

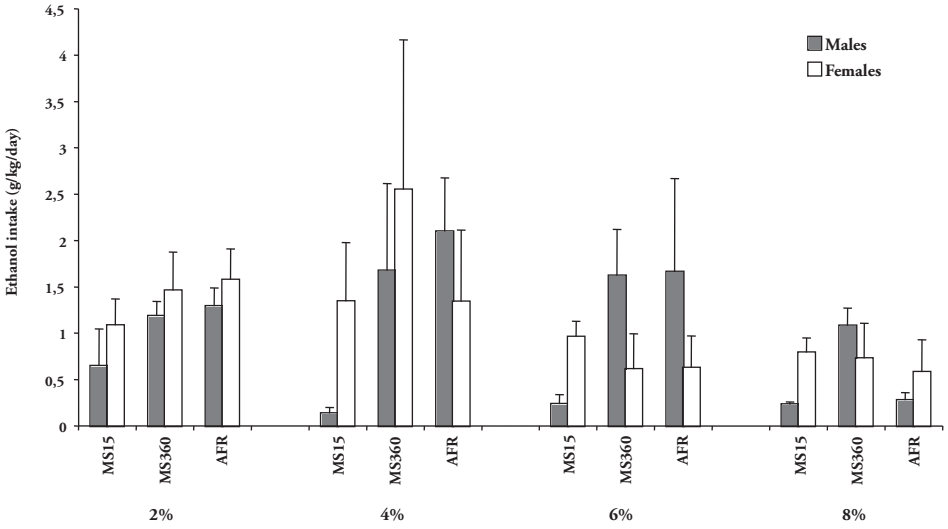


Figure 11. Ethanol intake (g/kg/day) in male responders (dark bars) and female (light bars) Wistar MS15, MS360 and AFR rats during the different concentrations of ethanol (n=8 rats/group). Data are expressed as median ± MAD.

MS360 rats (19%) was significantly higher compared to both MS15 rats (5%) and AFR rats (11%).

Ethanol intake in female Wistar rats (n=8 rats/group, Paper III) is shown in Figure 11. The respective ethanol intake pattern during the period of access to ethanol was similar for the three experimental groups. Thus, no statistically significant differences between the female MS15, MS360 and AFR groups were observed in ethanol intake or ethanol preference at any ethanol concentration.

The findings in female Wistar rats add further support to recent data describing sex differences various parameters after different MS procedures (e.g. Kalinichev et al., 2002; Lehmann et al., 1999; McIntosh et al., 1999; Papaioannou et al., 2002; Park et al., 2003). The sex difference in ethanol intake found herein might to some extent be explained by developmental differences between males and females (Andersen, 2003). The difference in ethanol consumption between females and males was most pronounced in the MS15 group of animals, Figure 11, where male MS15 rats had a very low intake in contrast to female MS15 rats. In the males more rats in the MS360 group initiated drinking, and those rats also had a higher ethanol intake, than in the MS15 and AFR groups, indicating the presence of responders and non-responders in the MS360 group. Due to a small sample size in each group of female Wistar rats, it was not possible to characterize animals as responders or non-responders. Further studies are required to identify possible subgroups in the outcome of short and prolonged periods of MS in female rats and to fully evaluate sex differences in the long-term effects of MS on voluntary ethanol intake.

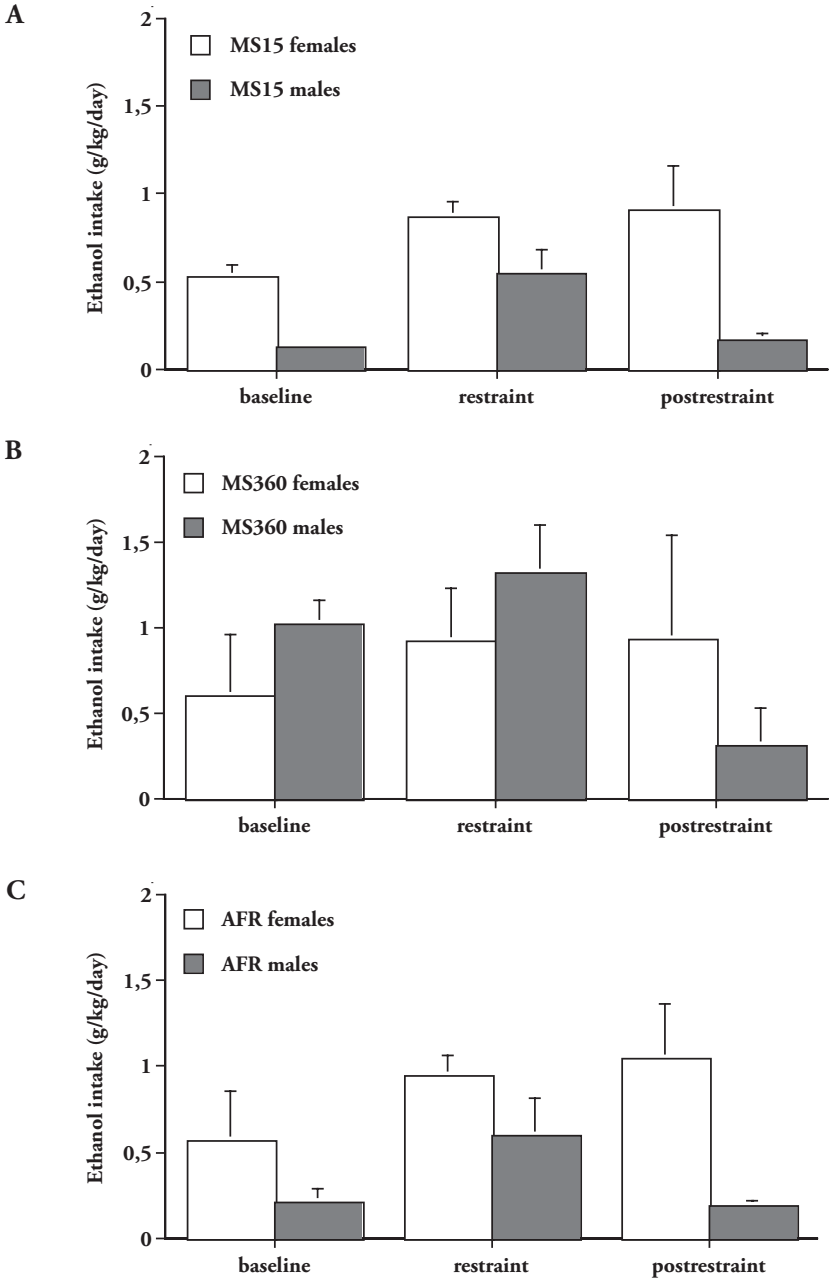


Figure 12. Ethanol intake (g/kg/day) at 8% ethanol before, during and after four daily restraint stress (1 h) sessions in MS15, MS360 and AFR female rats (light bars) and male responders (dark bars). Baseline represents three days before restraint stress used to make restraint-induced comparisons. The restraint period represents the four consecutive days of 1 h restraint stress and the postrestraint period represents the two days after restraint stress. Data are expressed as group median ± MAD (n=8 rats/group).

Restraint stress was used to investigate the consequences of a stressful experience in adulthood on voluntary ethanol intake in the three experimental groups. Different restraint procedures have been shown to alter voluntary ethanol intake in rats (Chester et al., 2004; Lynch et al., 1999; Rockman et al., 1986). In Wistar rats the effects of restraint stress for 1 h for four consecutive days, during the period of access to 8% ethanol, are shown in Figure 12. In male Wistar rats, comparison of ethanol intake in each experimental group before and during restraint stress showed that all three groups increased their intake during the restraint period. The increase was statistically significant in the male MS15 and MS360 groups but not in the AFR rats.

In females, a tendency towards a higher ethanol intake was seen in all three female groups during the restraint period but this increase did not reach statistically significance in the MS15 and MS360 rats. The AFR rats had a statistically significant increase in ethanol intake during the restraint period and the higher levels persisted during the postrestraint period compared to baseline levels. Both MS15 and MS360 rats had a statistically significant higher ethanol intake during the postrestraint period compared to their respective baseline levels. The present study examined only short-term effects of restraint stress and it cannot be excluded that the effect on ethanol intake persists even longer. Results have shown that effects of repeated stress can induce a considerably delayed effect on ethanol intake (Sillaber et al., 2002). The present data thus indicate that interesting sex differences exist in the later effects of restraint stress in adulthood on voluntary ethanol intake.

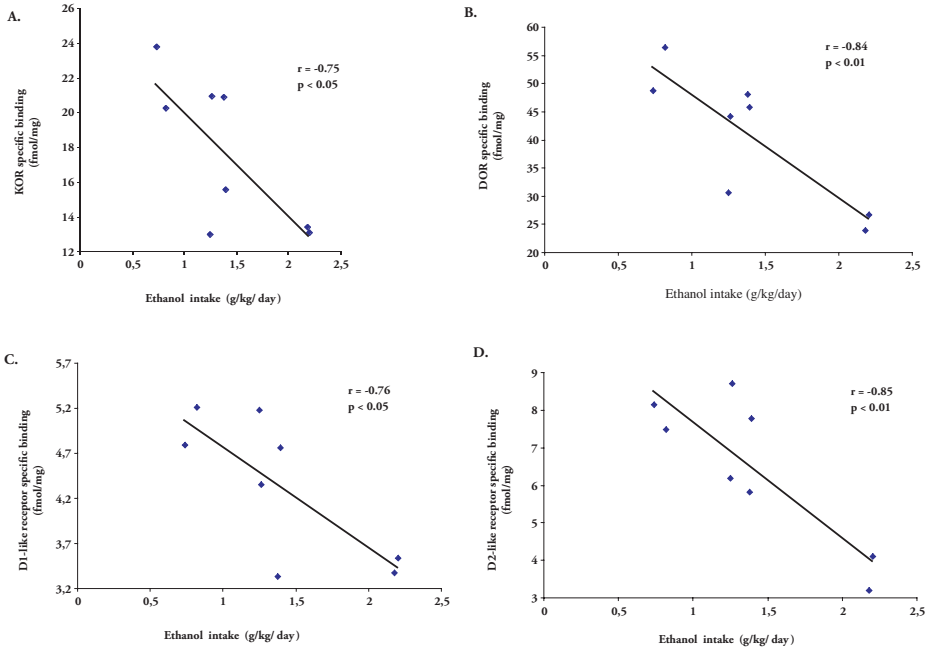


Figure 13. Correlation between mean **A.** KOR binding, **B.** DOR binding, **C.** dopamine D1-like receptor binding and **D.** dopamine D2-like receptor binding (fmol/mg) in the nucleus accumbens shell and mean ethanol intake (g/kg/day) during the restraint stress period in the male Wistar MS360 responder rats.

In Paper II, a correlation between ethanol intake and receptor density was examined in the male responders (Figure 13). In MS360 rats a significant negative correlation was found between KOR, DOR, dopamine D1-like and dopamine D2-like receptor binding in the nucleus accumbens shell and ethanol consumption during the restraint stress period. A significant negative correlation was also found between dopamine D2-like receptor binding in the nucleus accumbens core region of the MS360 rats and ethanol consumption. Thus, the higher ethanol intake, the lower opioid and dopamine receptor densities in the nucleus accumbens shell and/or core. No such correlation was found in the MS15 and AFR group. A significant correlation in other areas, where a significant change in receptor binding between the groups was found after ethanol consumption, could not be detected. These findings give further evidence for the relationship between high levels of dopamine D2 receptors and low ethanol intake. Furthermore, in other studies an overexpression of dopamine D2 receptors in the nucleus accumbens was associated with a reduction in ethanol intake and preference in rats, including the ethanol-preferring P rats (Thanos et al., 2004; Thanos et al., 2001). In the current study, a low D2-like dopamine receptor density in the nucleus accumbens was accompanied by a high ethanol intake.

Ethanol-preferring AA and ethanol-avoiding ANA rats (Papers IV-V)

In these two papers it was possible to investigate the impact of early environmental factors in male rats selectively bred for a high and low ethanol intake and preference, respectively. Paper IV was performed as a first study in the ethanol-preferring AA rats (n=6-9 rats/group). The number of animals in this study was limited and the period of access to ethanol was relatively short. The respective ethanol intake pattern indicated that the MS15 rats had a delayed acquisition phase and thus, it took longer time to reach a high ethanol intake in MS15 rats compared to both MS360 and AFR rats. AFR rats reached a high, stabilized ethanol intake in a pattern similar to what is generally seen in AA rats, at 8% ethanol whereas MS360 rats reached a stabilized intake already at 6% and MS15 rats not until 10% ethanol.

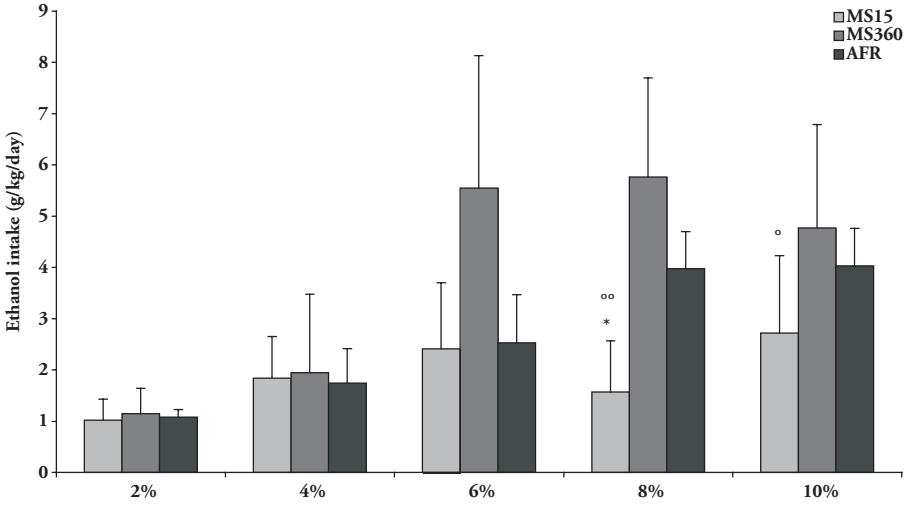


Figure 14. Ethanol intake (g/kg/day) during the different ethanol concentrations in AA MS15 (n=9), MS360 (n=7) and AFR (n=6) rats (Paper IV). Data are expressed as median ± MAD. *p<0.05 compared to AFR rats; °p<0.05, °°p<0.01 compared to MS360 rats (Kruskal-Wallis, Mann-Whitney *U* test).

Figure 14 shows the median ethanol intake at the different ethanol concentrations. The MS15 rats had a significantly lower ethanol intake at 8% ethanol in comparison with both MS360 and AFR rats and at 10% ethanol compared to MS360 rats. The ethanol preference was similar to the ethanol intake. At 6%, 8% and 10% ethanol MS15 rats had a lower preference compared to AFR rats, while MS360 rats had the highest ethanol preference. High-preferring animals have been defined as covering more than 60% of their daily fluid consumption from the ethanol solution (Fahlke et al., 1994b). When this definition was applied at 8% ethanol, large differences between the groups were found. Only 22% of MS15 rats had an ethanol preference over 60% compared to 50% of AFR rats and 71% of MS360 rats.

In Paper IV, restraint stress was again used to examine whether this procedure could differentially affect ethanol intake in AA MS15, MS360 and AFR rats, since alterations was found in Wistar rats (Paper II-III). A single 30 min restraint procedure during the period of access to 10% ethanol was used. The four days prior to restraint stress were used as baseline in the statistical comparisons. At baseline the MS15 rats had a statistically significant lower ethanol intake in comparison with MS360 rats, but not compared to AFR rats. A different alteration, than in Wistar rats, was detected in the AA rats. After restraint stress, a decrease in ethanol intake was seen in the MS15 and MS360 groups in comparison with baseline levels. When the individual reduction in ethanol intake was used for comparisons, the MS15 rats had 21% lower ethanol intake and MS360 rats 33% reduced intake the day after restraint compared to baseline levels. The ethanol intake in AFR rats was not affected by restraint stress. During the last eight days of the experiment, the ethanol intake in the MS15 and MS360 groups was more similar to that of the AFR group and the ethanol intake was thus merging in the three experimental groups. The

current findings are in accordance with findings in ethanol-preferring P rats and HAD rats. During a period of unpredictable immobilization stress, P rats showed a significant decrease in ethanol intake followed by an increase in intake immediately after the restraint period. In the HAD rats, immobilization stress resulted in a trend towards a moderate suppression in ethanol intake (Chester et al., 2004). The increasing ethanol intake in MS15 rats after restraint presented herein may indicate that MS15 slows the acquisition phase but in the long term the MS15 rats might reach the same high ethanol intake as other AA rats. However, it is also possible that exposure to restraint stress causes a temporary increase in ethanol intake that then slowly would return to the low levels prior to restraint stress, as shown for P rats (Chester et al., 2004).

The aim of Paper V was to more thoroughly investigate the impact of MS on acquisition of voluntary ethanol intake and the subsequent ethanol intake in male ethanol-preferring AA rats (n=15-20 rats/group). Long-term effects were examined using a relatively large number of animals, to identify possible subgroups as previously shown for male Wistar MS360 rats (Paper II). Ethanol-avoiding ANA rats (n=8-12 rats/group), the counterparts to AA rats, were also studied as a contrast group.

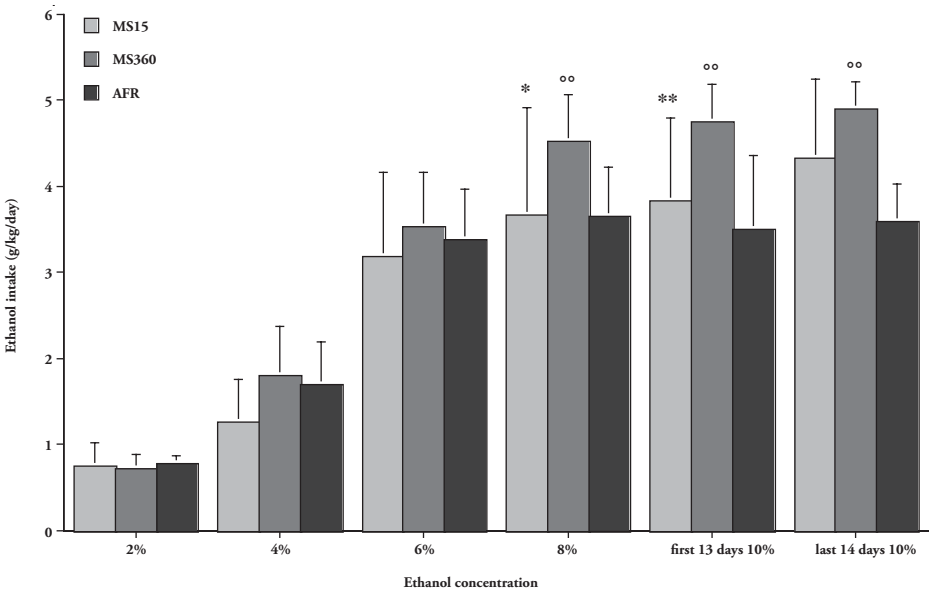


Figure 15. Ethanol intake (g/kg/day) during the different ethanol concentrations in AA MS15 (n=20), MS360 (n=20) and AFR (n=15) rats during continuous access to ethanol (Paper V). Data are expressed as median ± MAD. *p<0.05, **p<0.01 compared to MS360 rats; °°p<0.01 compared to AFR rats (Kruskal-Wallis, Mann-Whitney *U* test, Bonferroni-Holm correction).

The AA MS360 rats had an overall higher ethanol intake compared to both AA MS15 and AA AFR throughout the experiment. At a 10% ethanol concentration, the daily ethanol intake within each group increased to some extent during the first 13 days after which the curves showed a more stabile pattern during the last 14

days, with a tendency to merge. The period of access to 10% ethanol was therefore divided into two phases for the further statistical analyses. Figure 15 shows the ethanol intake in AA MS15, MS360 and AFR rats during the different ethanol concentrations used. Statistical analyses demonstrated that the AA MS15 rats had a significantly lower ethanol intake at 8% ethanol and the first 13 days in comparison with AA MS360 rats. In the MS360 rats, a significantly higher ethanol intake was found during 8% ethanol and during the entire period of 10% ethanol compared to AFR rats. No differences were found between AA MS15 and AFR rats at any concentration. The preference data was in concordance with the ethanol intake with the AA MS360 rats having the highest preference. However, no statistically significant differences in ethanol preference were found at any concentration.

In Paper IV, a low voluntary ethanol intake was found in the AA MS15 rats, whereas AA rats subjected to MS360 showed less pronounced effects with only a trend towards a higher ethanol intake compared to AFR rats, possibly due to a small sample size. There have however, been good reasons to believe that AA rats may be resistant to manipulations that further increase the innate high ethanol intake. Most attempts to increase the ethanol intake in AA rats have been studies of ethanol deprivation. While other lines of rats, including ethanol-preferring P rats, show a deprivation-induced increase in ethanol intake, no major effects have been found in the AA rats (Sinclair and Li, 1989; Vengeliene et al., 2003). It is therefore an interesting finding that MS360 indeed can increase voluntary ethanol intake also in AA rats with an inherent predisposition to high voluntary ethanol intake, as shown in Paper V.

The results in male AA rats and the results found in female AA rats (Gustafsson L, Roman E, Hyytiä P, Nylander I. Manuscript submitted for publication) from the same experiment as Paper V, add further evidence for sex differences in the outcome of MS on voluntary ethanol intake, as shown in Wistar rats, where MS induced distinct effects in males (Paper II) but no effects on ethanol intake in female rats (Paper III). Here, male AA rats subjected to MS360 were found to have a higher voluntary ethanol intake compared to AFR and MS15 rats. In contrast, both separation procedures were shown to lower ethanol intake in female AA rats (Gustafsson L, Roman E, Hyytiä P, Nylander I. Manuscript submitted for publication).

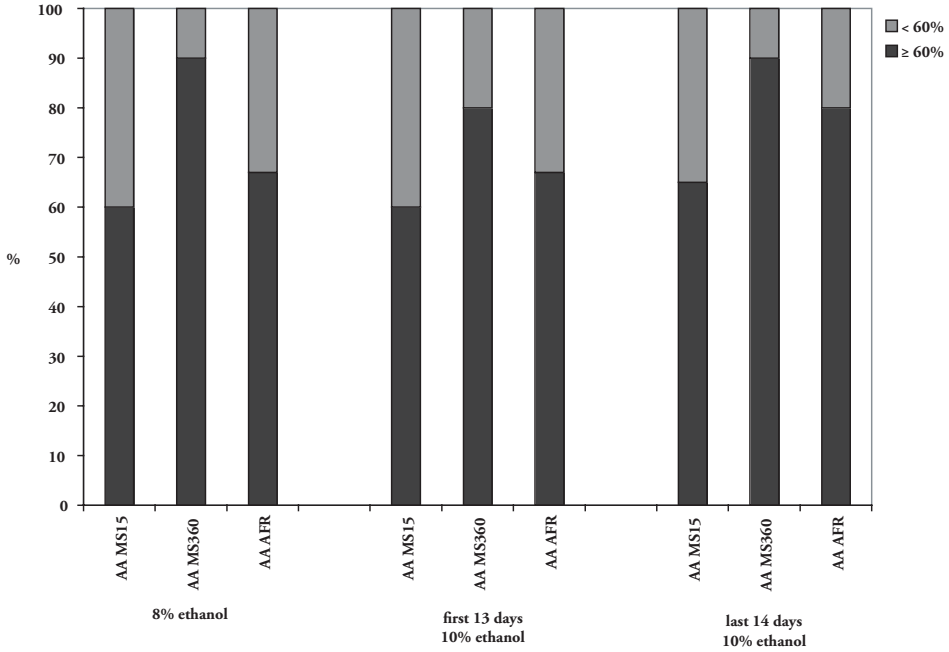


Figure 16. The percentage of animals with an ethanol preference (% of total fluid intake) above or below 60% during the period of continuous access to ethanol in AA MS15 (n=20), MS360 (n=20) and AFR (n=15) rats (Paper V).

As illustrated in Figure 16, at both 8% and 10% ethanol there were more high-preferring rats in the MS360 group (80-90%) while the MS15 group had the lowest number of animals (≈60%) with a high ethanol preference. The results from previous studies in male rats indicate that MS15 may serve as a protection against high voluntary ethanol intake later in life. Wistar rats (Paper II) that experienced MS15 during the postnatal period showed a low ethanol intake as adults and AA rats (Paper IV) showed a delayed acquisition of ethanol intake along with a low intake. Also in the present study, AA MS15 rats showed a lower ethanol intake and ethanol preference, as compared to MS360 rats. Furthermore, fewer high-preferring animals (ethanol preference >60%, (Fahlke et al., 1994b)) were present in the MS15 group than in the MS360 group. In contrast to the previous AA study, no differences were found when comparing ethanol intake in the MS15 and AFR rats. When comparing the ethanol preference in the present study, similar numbers of high-preferring animals were found in the MS15 and AFR groups, except towards the end of the experiment where the number of AFR rats with a high preference had increased. These results indicate a slower acquisition of ethanol intake in MS15 rats, although the difference between MS15 and AFR was not as pronounced as shown in Paper IV.

During the last part of the experiment in Paper V, voluntary ethanol intake during limited access to ethanol was investigated in male AA rats. The ethanol intake in the three experimental groups was in concordance with what has previously been

reported for AA rats during limited access for 1 h/day (Sinclair et al., 1992). However, no differences between the three experimental groups could be detected most probably because limited ethanol intake was investigated during a later part of the experiment, when the ethanol intake in the three experimental groups was merging.

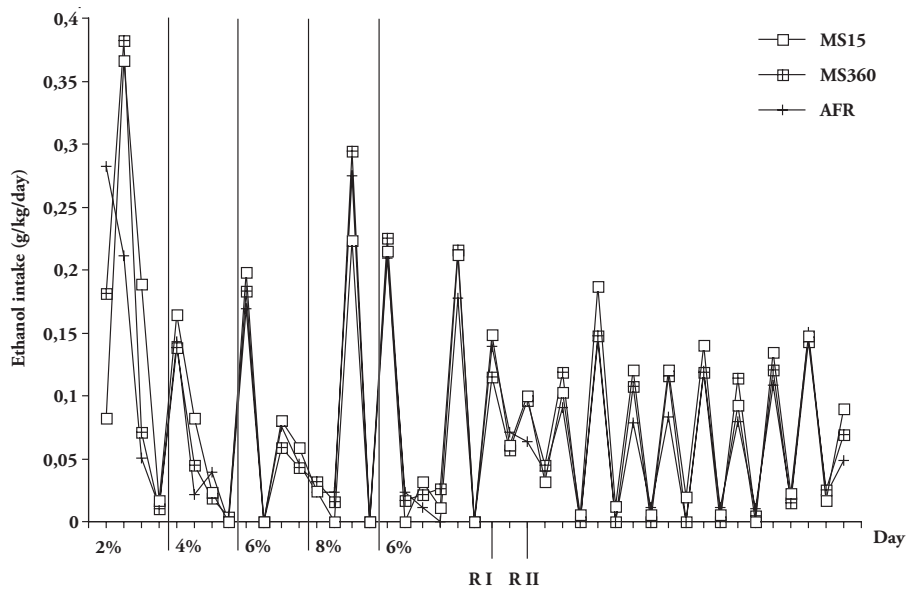


Figure 17. The median daily ethanol intake (g/kg/day) patterns in ANA MS15 (n=8), MS360 (n=12) and AFR (n=8) rats (Paper V). R I and R II, respectively, mark the two days when a 30 min restraint stress was performed.

The ethanol-avoiding ANA rats have a well-documented low voluntary ethanol intake (e.g. Nylander et al., 1994; Viglinskaya et al., 1995). In Paper V, the intake was only 0.1-0.2 g/kg/day. The daily ethanol intake drinking patterns in ANA MS15, MS360 and AFR rats are shown in Figure 17. The rats in the three experimental groups were found to have a similar ethanol intake during continuous access to ethanol without major separation-induced effects. The respective ethanol intake pattern in the three ANA groups was disturbed during the time period when two 30 min restraint stress procedures were performed, as compared to the daily intake before and after the restraint sessions. This disturbed pattern could be seen also for water intake and thus total fluid intake, in a similar fashion in ANA MS15, MS360 and AFR rats. Furthermore, in the ANA rats a digging and burying behavior was observed when cages were changed during the period of access to ethanol, as previously reported (Sandbak et al., 1998), and along with the restraint stress effects this provides further evidence for an increased susceptibility to stressful events in this line of rats. These findings are in line with the common notion that the ANA rats have a different behavioral profile than AA rats (e.g. Möller et al., 1997; Overstreet et al., 1997; Sandbak et al., 1998; Viglinskaya et al., 1995). This proposed difference in behavioral responses in the ANA rats might also affect the

outcome of the MS procedure since the ANA dams could be differently affected compared to the AA dams. This might result in a different response to MS in the offspring, as indicated here with no differences between the three experimental ANA groups while distinct effects on ethanol intake were demonstrated in the studies using male Wistar (Paper II) and AA rats (Paper IV-V). When comparing the male and female ANA rats (Gustafsson L, Roman E, Hyytiä P, Nylander I. Manuscript submitted for publication), from the same experiment, no separation-induced effects were found either in males or in females, in contrast to what was found in AA rats (Paper V; Gustafsson L, Roman E, Hyytiä P, Nylander I. Manuscript submitted for publication) and Wistar rats (Papers II-III).

Exploration and risk assessment behavior (Paper VI)

The object of this study was to use an ethoexperimental approach to investigate the impact of short and prolonged periods of MS on the adult animal's behavior with specific consideration to information gathering and a behavioral strategy that implies risk assessment in male Wistar rats (n=15 rats/group). To this end three methods were used, the CSF test, the OF test and the EPM test, which together constitute a multivariate experimental design. The behavioral analyses were mainly kept at a descriptive level rather than relating inter-group differences to certain central nervous processes. However, given statistical differences between the three experimental groups, the various behavioral variables were assigned to functional categories such as general locomotion, exploration and visits to open versus sheltered areas. This approach was adapted from recent studies (Augustsson, 2004; Augustsson and Meyerson, 2004) using the same methodology for scoring differences in wild and laboratory mice. The sequential order of the three tests was based on a pilot study in mice using the same battery of behavioral tests. By alternating the order of the different tests, it was found that parameters recorded in the CSF test, but not in the other two tests, were affected. It was therefore concluded that the CSF test was the most sensitive to previous experience and should therefore be performed as the first test in the battery of tests in order to reduce the likelihood of any carry over effect (Augustsson, 2004).

One previous study has used an ethoexperimental approach to study the effects of early life experiences on risk assessment behavior. In that study, rats individually separated for 15 min and AFR control rats were compared in the EPM test, the OF test and the free exploration paradigm. Rats that were individually separated and handled for 15 min, spent longer time on the open arms and had fewer closed arm returns along with a lower frequency of SAPs in the EPM test compared to AFR control rats. Differences were also detected in the OF test and the free exploration paradigm and the results together indicated a decreased emotional reactivity in the separated rats (Roy and Chapillon, 2004).

The CSF test

Compared to the EPM and OF tests, the CSF test includes areas that provide the animal with different explorative incentives, elements of risk and the possibility to seek shelter.

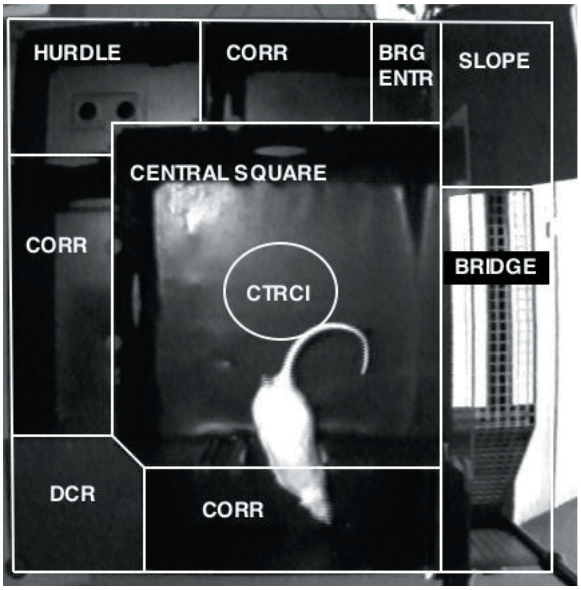


Figure 18. The CSF test with its defined zones.

The results from the 20 min trial in the CSF test are shown in Table 2. The MS360 rats differed from the AFR rats in several parameters, with the MS15 rats constituting an intermediate group. The total number of entries to the corridors was considered as a measure of general locomotion. No differences between groups were seen in this parameter or in the total distance moved in the arena, another measure of locomotor activity. In general, the CTRCI was initially avoided. Entrance into the CTRCI had a latency of about one and a half to two minutes. The MS360 group of animals had a shorter latency to the SLOPE of the BRIDGE than the AFR rats, however, this difference did not reach significance using the Bonferroni-Holm correction. The MS360 rats had significantly shorter latencies to HURDLE and BRIDGE as well as higher frequency of visits to the SLOPE of the BRIDGE and the HURDLE than the AFR group. The frequency of visits to the HURDLE was significantly higher also compared to the MS15 rats. The MS360 group of animals had a significantly higher velocity in the CTRCI than AFR rats. MS15 rats had a higher frequency of visits to the ENTRANCE of the BRIDGE than AFR rats and a similar trend was found also for the MS360 rats, but not statistically significant. No other differences in measures of exploration or behavior in open or sheltered areas were found between groups.

PARAMETERS	MS15	AFR	MS360
LAT LEAVE *	42.0 ± 10.8	67.7 ± 18.5	32.3 ± 8.0
LAT DCR *	368.9 ± 69.8	253.5 ± 47.8	247.3 ± 46.4
LAT SLOPE	161.4 ± 31.6	295.9 ± 65.5	138.0 ± 28.6
LAT BRIDGE *	227.3 ± 56.8	302.9 ± 46.5	182.1 ± 51.5 †
LAT HURDLE *	425.4 ± 70.3	534.3 ± 70.7	258.8 ± 76.7 ††
LAT CTRCI	101.9 ± 25.3	83.8 ± 25.4	132.0 ± 34.8
FREQ DCR *	7.9 ± 1.2	7.2 ± 0.8	7.5 ± 0.8
FREQ BRIDGE ENTRANCE	27.6 ± 1.5 †	21.7 ± 1.4	26.2 ± 2.4
FREQ SLOPE	10.5 ± 1.6	6.7 ± 0.9	10.7 ± 1.2 †
FREQ BRIDGE *	4.5 ± 0.4	3.8 ± 0.5	5.0 ± 0.5
FREQ HURDLE *	2.5 ± 0.3 #	2.1 ± 0.2	3.5 ± 0.3 †
FREQ CTRCI	20.8 ± 2.2	22.3 ± 3.6	17.1 ± 1.8
FREQ TOTCORR	58.3 ± 2.4	55.6 ± 2.6	66.3 ± 4.3
DUR DCR	141.4 ± 22.4	162.9 ± 28.1	147.6 ± 19.2
DUR BRIDGE *	205.3 ± 27.5	164.3 ± 22.4	221.1 ± 24.4
DUR HURDLE	47.9 ± 7.8	56.1 ± 8.4	71.4 ± 7.7
DUR CTRCI	24.5 ± 4.4	29.9 ± 5.6	17.0 ± 2.2
DUR TOTCORR	334.5 ± 26.6	360.0 ± 38.0	310.0 ± 22.1
DUR / FREQ DCR	8.7 ± 1.5	10.7 ± 1.7	9.6 ± 1.2
DUR / FREQ BRIDGE	48.1 ± 6.6	46.3 ± 4.2	45.6 ± 4.6
DUR / FREQ HURDLE	16.1 ± 2.4	17.6 ± 2.9	18.5 ± 3.1
DUR / FREQ CTRCI	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.1
% DUR DCR	11.8 ±1.9	13.6 ± 2.3	12.3 ± 1.6
% DUR BRIDGE	17.1 ± 2.3	13.7 ± 1.9	18.4 ±2.0
% DUR HURDLE	4.0 ± 0.6	4.7 ± 0.7	6.0 ± 0.6
% DUR CTRCI	2.0 ± 0.4	2.5 ± 0.5	1.4 ± 0.2
% DUR TOTCORR	27.9 ±2.2	30.0 ±3.2	25.8 ± 1.8
OCCURRENCE DCR *	14 / 15	13 / 15	15 / 15
OCCURRENCE BRIDGE *	15 / 15	14 / 15	14 / 15
OCCURRENCE HURDLE *	14 / 15	14 / 15	13 / 15
REARING *	28.1 ± 2.1	31.1 ± 2.2	31.9 ± 2.3
DIST TOTARENA	9056.8 ± 398.6	8708.0 ± 338.4	9328.9 ± 342.0
VELOCITY CTRCI	22.2 ± 2.2	17.3 ± 1.3	24.3 ± 2.2 †

Table 2. *Behavioral parameters during the 20 min trial time in the CSF test in male Wistar MS15, MS360 and AFR rats. Data are expressed as mean ± SEM. % DUR represents the duration expressed as percentage of total trial time. Occurrence is shown for the zones that were not visited by all animals in each group (n=15 rats/group). * indicates recordings taken by manual scoring; †p<0.05, ††p<0.01 compared to AFR rats; #p<0.05 compared to MS360 rats (Kruskal-Wallis, Mann-Whitney U test, Bonferroni-Holm).*

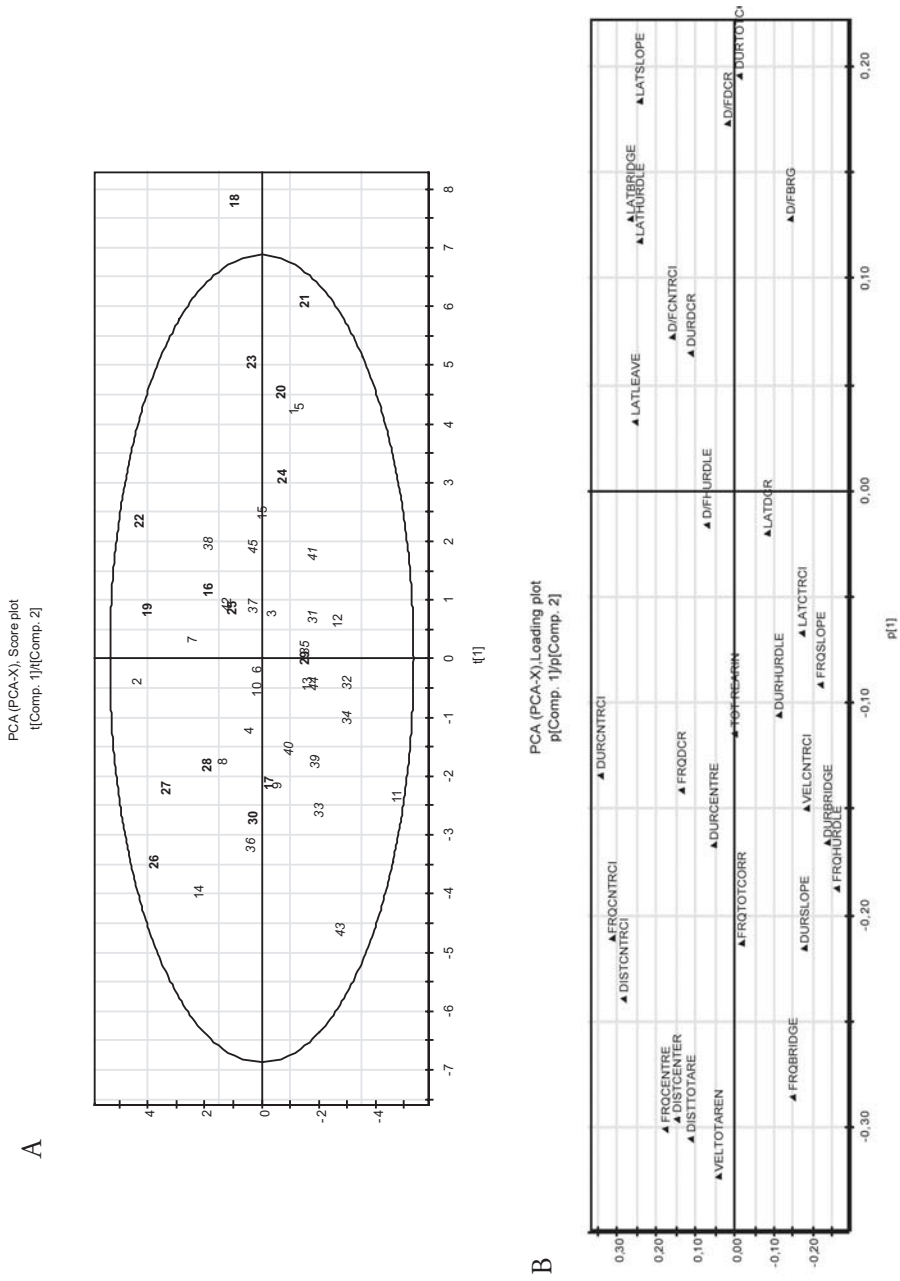


Figure 19. PCA charts showing the score plot (**A**) and the corresponding loading plot (**B**). The numbers 1-15 (regular) in the score plot corresponds to MS15 rats, 16-30 (bold) to AFR rats and 31-45 (italic) to MS360 rats.

In order to gain more information from the large amount of raw data from the CSF test, the PCA analysis was used. The score plot (Figure 19 A) is a summary of the relationship among the individuals and the loading plot (Figure 19 B) identifies

the variables that are important for these relationships. The direction of the score plot corresponds to the direction in the loading plot. The MS360 animals were more gathered in the lower left-hand quadrant compared to the other two groups, which were more dispersed in three quadrants. The corresponding loading plot illustrated that the more distinct grouping of the MS360 rats was mainly due to the following parameters: FREQ BRIDGE, SLOPE and HURDLE, DUR BRIDGE, velocity CTRCI and to a lesser degree DUR HURDLE and LAT CTRCI. The outcome of the PCA analysis therefore generally corresponds with some of the findings in the traditional statistical analyses.

PARAMETERS	**MS15**	**AFR**	**MS360**
FREQ DCR *	2.3 ± 0.8	2.1 ± 0.3	2.0 ± 0.2
FREQ SLOPE	6.5 ± 1.8	3.1 ± 0.6	5.4 ± 0.9
FREQ BRIDGE *	1.7 ± 0.3	1.1 ± 0.1	2.0 ± 0.1 ††
FREQ HURDLE *	1.0 ± 0.0	1.0 ± 0.0	1.4 ± 0.2
FREQ CTRCI	4.2 ± 0.6	6.1 ± 1.5	4.0 ± 0.7
FREQ TOTCORR	12.9 ± 1.3	12.4 ± 1.4	15.7 ± 1.3
DUR DCR	45.2 ± 15.6	49.7 ± 18.0	28.1 ± 5.7
DUR BRIDGE *	51.7 ± 14.2	33.6 ± 7.8	77.5 ± 15.1
DUR HURDLE	15.3 ± 3.4	15.9 ± 3.6	20.0 ± 3.4
DUR CTRCI	4.3 ± 1.1	8.7 ± 2.3	4.0 ± 0.8
DUR TOTCORR	91.9 ± 10.1	93.8 ± 13.0	74.5 ± 6.4
DUR / FREQ DCR	7.9 ± 1.6	6.1 ± 1.0	7.4 ± 1.4
DUR / FREQ BRIDGE	26.5 ± 4.8	30.5 ± 6.9	38.3 ± 7.5
DUR / FREQ HURDLE	15.3 ± 3.4	1 5.9 ± 3.6	12.4 ± 1.9
DUR / FREQ CTRCI	0.9 ± 0.2	1.5 ± 0.3	1.0 ± 0.2
% DUR DCR	15.1 ± 5.2	16.6 ± 6.0	9.4 ± 1.9
% DUR BRIDGE	17.2 ± 4.7	1 1.2 ± 2.6	25.8 ± 5.0
% DUR HURDLE	5.1 ± 1.1	5.3 ± 1.2	6.7 ± 1.1
% DUR CTRCI	1.4 ± 0.4	2.9 ± 0.8	1.3 ± 0.3
% DUR TOTCORR	30.6 ± 3.4	31.3 ± 4.3	24.8 ± 2.1
OCCURRENCE DCR *	6 / 15	7 / 15	9 / 15
OCCURRENCE BRIDGE *	12 / 15	11 / 15	12 / 15
OCCURRENCE HURDLE *	6 / 15	3 / 15	11 / 15 †
OCCURRENCE CTRCI	14 / 15	14 / 15	13 / 15
REARING *	9.5 ± 1.5	11.3 ± 1.0	9.5 ± 0.8
DIST TOTARENA	2396.8 ± 129.0	2263.6 ± 98.4	2498.4 ± 132.2

Table 3. *Behavioral parameters during the first 5 min in the CSF test in MS15, MS360 and AFR rats. Data are expressed as mean ± SEM. % DUR represents the duration expressed as percentage of the 5 min time period. Occurrence is shown for the zones that were not visited by all animals in each group (n=15 rats/group). * indicates recordings taken by manual scoring; ††p<0.01 compared to AFR rats (Kruskal-Wallis, Mann-Whitney U test, Bonferroni-Holm); †p<0.05 compared to AFR rats (Chi-Square test, Bonferroni-Holm).*

In order to identify possible differences between the experimental groups in immediate exploration and risk assessment behavior, the CSF data from the first 5 min of the 20 min trial was analyzed (Table 3). The distance traveled in the total arena did not differ between the three groups, indicating a similar level of locomotor activity. Significantly more animals in the MS360 groups visited the HURDLE and the MS360 rats had a significantly higher frequency of visits to the BRIDGE compared to AFR rats. No other differences in measures of behavior during the first 5 min were found between the three experimental groups.

Taken together, the most evident effects of the 360 min separation in the CSF test was found in measures of exploration and risk assessment. The latencies to enter the risk area (BRIDGE) and HURDLE were significantly different from the AFR rats while the frequency of visits to HURDLE was significantly different from both AFR and MS15 rats. The velocity of passing the CENTRAL CIRCLE of the centre field was significantly higher in the MS360 group. Furthermore, a longer latency of entering and lower frequency of visits to the CENTRAL CIRCLE of the open area was indicated in the MS360 rats. The outcome from the PCA analysis was found to be in accordance with the traditional statistical analyses, thus giving further evidence for an anomalous behavior in the MS360 group of animals as compared to the other two groups. When analyzing the behavior during the first 5 min, the frequency of visits to the risk area (BRIDGE) was significantly higher in the MS360 group of animals along with an indication for a longer duration on the BRIDGE. Furthermore, more MS360 rats visited the HURDLE compared to the AFR and MS15 rats.

The OF test

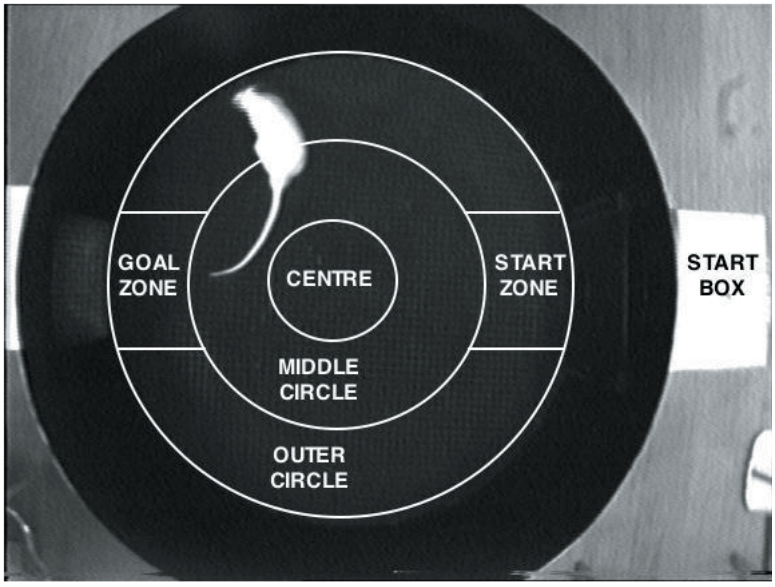


Figure 20. The OF test with its defined zones.

The three experimental groups had similar behavioral patterns in the OF test and no statistically significant differences between the three experimental groups were found for any of the parameters analyzed. The lack of effects in the OF test was most probably due to the fact that the OF test was run in darkness. Illumination has been observed as one of several significant factors in the outcome of behavior in the OF test (e.g. Lister, 1990). When run in darkness, the locomotor activity driven by the motive to explore are likely to be less governing by elements of emotional reactivity. The presence of the start box further enabled recordings of shelter seeking behavior as an additional measure to the regular locomotor activity. The start box gave the animals the opportunity to seek shelter and MS15 and MS360 rats spent approximately 20% of the total time in the start box, while this was found to be somewhat lower for the AFR group (14%).

The EPM test

PARAMETERS	**MS15**	**AFR**	**MS360**
CLOSED ARMS			
LAT	14.8 ± 5.6	21.8 ± 5.0	27.3 ± 5.0
FREQ	8.9 ± 0.6	8.7 ± 0.3	9.5 ± 0.5
DUR	224.3 ± 11.0	210.6 ± 11.1	227.0 ± 7.3
DUR / FREQ	26.3 ± 1.6	24.7 ± 1.7	25.3 ± 2.0
% DUR	74.7 ± 3.7	70.0 ± 3.6	75.6 ± 2.4
OPEN ARMS			
LAT	70.9 ± 28.0	20.7 ± 7.8	29.5 ± 18.0
FREQ	2.4 ± 0.4	2.6 ± 0.4	2.5 ± 0.4
DUR	34.0 ± 7.0	34.8 ± 6.9	34.2 ± 5.3
DUR / FREQ	14.5 ± 2.6	13.0 ± 1.5	16.1 ± 2.9
% DUR	11.3 ± 2.3	11.6 ± 2.3	11.4 ± 1.7
OCCURRENCE	12 / 15	14 / 15	13 / 15
CENTRE			
FREQ	10.8 ± 0.6	11.4 ± 0.7	11.7 ± 0.8
DUR	48.9 ± 5.6	57.6 ± 6.1	43.8 ± 4.4
DUR / FREQ	4.6 ± 0.5	5.2 ± 0.6	3.9 ± 0.4
% DUR	16.7 ± 1.9	19.2 ± 2.0	14.6 ± 1.4
Total crossings	21.7 ± 1.4	22.5 ± 1.3	23.3 ± 1.6
FREQ TOT SAP	7.4 ± 0.5	5.7 ± 0.5	5.9 ± 0.5
FREQ TOT DIP	6.1 ± 0.6	5.8 ± 0.8	5.8 ± 0.6
Rearing	5.5 ± 0.6	8.1 ± 0.9	7.7 ± 0.9

Table 4. *Behavioral parameters recorded during 5 min in the EPM test in MS15, MS360 and AFR rats. Data are expressed as mean ± SEM. % DUR represents the duration expressed as percentage of total trial time. Occurrence is shown for the open arms since this part of the maze was not visited by all animals in each group (n=15 rats/group).*

The results from the EPM test are shown in Table 4. An indication for a difference was found in the latency to first enter an open arm where the MS15 rats had a longer latency compared to the other two groups. Moreover, a somewhat higher TOT SAP frequency was noted in the MS15 group of animals compared to AFR and MS360 rats. However, no statistically significant differences were found between the three experimental groups. The interpretation of the open versus closed arm data in the

EPM is not entirely clear. The conventional idea is that there is a conflict between exploration of the open arms and staying in the closed arms and that the open arm versus the closed arm response has an emotional quality with a fear response related to the open arm choice. However, this has been disputed and in a recent study comparing wild and domestic mice in the same behavioral battery as in the present study, wild mice tended to seek more shelter than domestic mice in the CSF and OF tests but spent more time on the open arm in the EPM test (Augustsson and Meyerson, 2004). This might give an indication for escape behavior being measured in the EPM, as also demonstrated in another study in wild mice (Holmes et al., 2000). A MS-induced behavioral response has been shown in the EPM using the present MS protocol. Just after weaning, the MS360 rats were found to have the longest latency to enter the open arms along with fewer open arm entries than AFR rats. When tested again at two months of age, this was shifted and the MS360 rats spent longer time on the open arms compared to AFR rats, with intermediate duration in MS15 rats (Ploj et al., 2002). These results were not replicated in the current study. The finding that MS360 and AFR rats had a shorter latency to first enter an open arm compared to MS15 rats might indicate an increased vigilance towards the open arms in the MS15 group of animals. However, after entering an open arm, no differences between the three groups were detected in the duration of time spent on the open arm as percentage of total time in the maze. The frequency of SAPs was used as a measure of risk assessment in the EPM test and in this analysis the MS15 rats were found to have the highest frequency of SAPs. This result contrasts to findings where rats that were individually separated and handled for 15 min showed lower frequency of SAPs in the EPM test compared to AFR control rats (Roy and Chapillon, 2004). The low number of SAPs was interpreted as a reduced approach/avoidance conflict indicating a decreased emotional reactivity, a finding that is not reproduced in the present experiment. This contrasting finding might be due to different line of rats as well as differences in experimental protocols such as individual separation versus litter separation and differences between behavioral testing procedures.

Compared to the EPM and OF tests, the CSF test includes areas that provide the animal with different explorative incentives, elements of risk and the possibility for shelter seeking. Overall, the behavioral studies give no indication for increased emotionality in the MS360 rats, as suggested by e.g. Ladd et al. and Huot et al. (Huot et al., 2001; Ladd et al., 2000), using 180 min of MS. The current data show that the MS360 rats were more explorative and expressed an altered risk assessment and a higher risk-taking behavior but had a tendency to be more cautious about open areas in the CSF test but not in the EPM and OF tests. The higher exploratory drive found in the MS360 group of animals was not accompanied by higher locomotor activity, that is the rats were not hyperactive as demonstrated in a recent study (Brake et al., 2004). The present results further indicate more vigilance in the MS15 group of animals than MS360 rats especially in the EPM test, where the MS15 rats had the shortest latency to enter a closed arm and also the longest latency to enter an open arm. However, in the CSF test, that enabled measurement

of a broader behavioral repertoire, the MS15 rats were found to be the intermediate group. The present results showed minor effects of short and prolonged periods of MS on adult behavior in the traditional EPM and OF test. On the other hand, the CSF test indicated alterations in exploration, risk assessment as well as risk-taking behavior in the MS360 rats compared to AFR rats, while minor effects were seen in the MS15 group of animals. The present results show the advantage of a battery of tests instead of a single test. It is concluded that tests such as the CSF test, allowing the animal a choice of staying at places that from a risk assessment point of view have different qualitative meaning, provides a useful tool in the investigation of early environmental influences on the adult behavioral profile.

General discussion

The MS-induced effects on voluntary ethanol intake in male Wistar MS15 and MS360 rats (Paper II) are in accordance with previous findings in male rats (Hilakivi-Clarke et al., 1991; Huot et al., 2001; Weinberg, 1987) but contrasting to another study where no long-term effects on ethanol intake were detected in rats subjected to 5 min and 240 min of MS (Marmendal et al., 2004). Paper IV and V reveal effects of short and prolonged periods of MS on acquisition of voluntary ethanol intake and subsequent ethanol intake also in male ethanol-preferring AA rats with an innate predisposition for high voluntary ethanol intake. Thus, MS15 resulted in a lower ethanol intake whereas MS360 further increased ethanol intake in these rats.

Findings obtained from Paper III, showing a lack of effect of short and prolonged periods of MS on the acquisition of voluntary ethanol intake and subsequent ethanol intake in female Wistar rats are in accordance with a recent study (Marmendal et al., 2004).

A high risk-taking behavior and impulsiveness has been implicated in the development of drug dependence in humans (e.g. Dawe and Loxton, 2004). Evidence has been summarized by Bardo et al. (Bardo et al., 1996), indicating that high novelty seekers are more vulnerable to use of drugs of abuse compared to low novelty seekers in both humans and laboratory animals. Furthermore, genetic variances of the dopamine D2 receptor, resulting in reduced number of dopamine D2 receptors, have been shown to be implicated in alcoholism, sensation seeking and novelty seeking in humans (Noble, 2003). The results presented herein, demonstrate that male MS360 rats have reduced dopamine D2-like receptor density in the VTA (Paper II), higher voluntary ethanol intake (Papers II and V) along with a higher exploratory drive, altered risk assessment and somewhat higher risk-taking behavior (Paper VI). It is therefore tempting to speculate that the higher voluntary ethanol intake found after MS360 may be a consequence of a higher exploratory drive and novelty seeking. However, further studies are needed in order to fully evaluate the MS-induced effects on voluntary ethanol intake and risk assessment behavior.

Summary

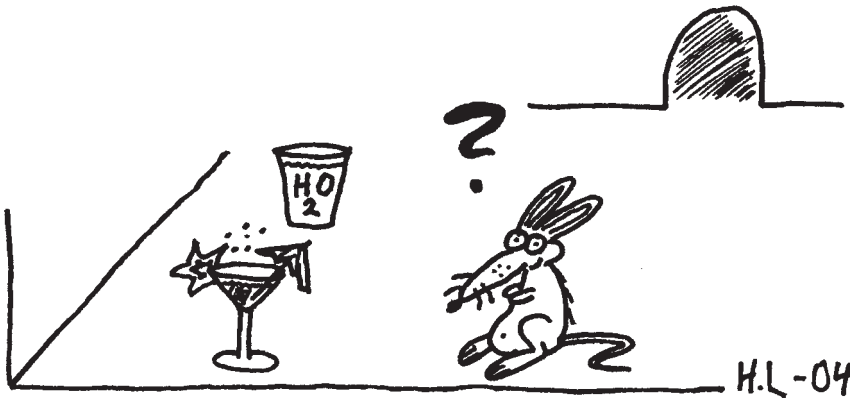
Daily short and prolonged periods of MS, MS15 and MS360 respectively, in litters during the postnatal period were used to investigate long-term effects on neurochemistry, voluntary ethanol intake behavior as well as exploration and risk assessment behavior in rats.

Neurochemistry

MS15 and MS360 induce long-term changes in ir levels of endogenous opioid peptides in the brain and the pituitary gland, especially DYNB whereas minor effects on MEAP levels were detected.

MS15 and MS360 induced only minor long-term effects on endogenous opioid and dopamine receptor density. However, of special interest was the finding of MS-induced effects on dopamine D2-like receptors in the VTA where the mesocorticolimbic dopamine neurons originate.

The major findings of voluntary ethanol intake in MS15, MS360 and AFR rats on brain opioid and dopamine receptor density were 1) a lower KOR density in MS15 rats, i.e. those rats showing a low ethanol intake. Thus, ethanol drinking may have induced an upregulation of KORs in distinct brain areas in MS360 and AFR rats and 2) the higher density of dopamine D2-like binding sites in the VTA in MS15 rats, after MS, was still higher than in the other two groups after access to ethanol. This finding may relate to the lower tendency to initiate drinking and the lower ethanol intake in MS15 rats compared to the MS360 and AFR groups.



Voluntary ethanol intake behavior

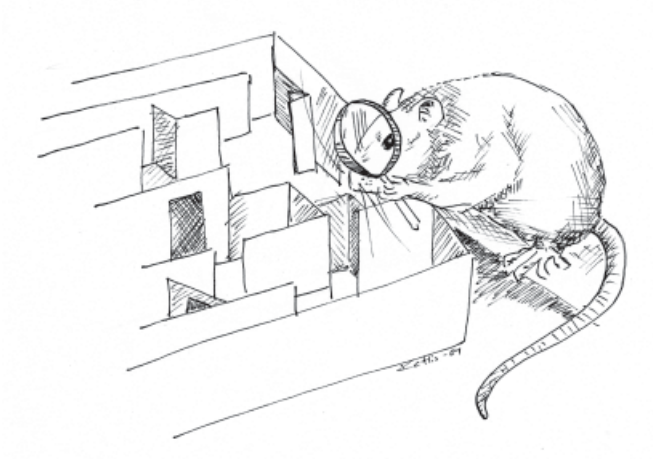
MS15 resulted in long-term effects on ethanol intake. Male Wistar and ethanol-preferring AA rats had a slower acquisition phase of voluntary ethanol intake along with a low subsequent intake. Also, fewer rats had a high ethanol preference in this group of male rats.

MS360 resulted in a high ethanol intake and more rats with a high ethanol intake and preference in adult male Wistar rats. In addition, MS360 could further increase voluntary ethanol intake in adult male ethanol-preferring AA rats with an already high voluntary ethanol intake.

The effects of MS15 and MS360 were sex dependent, since distinct long-term effects were found in male Wistar rats whereas female Wistar rats showed no MS-induced effects on voluntary ethanol intake. Further studies are needed to elucidate the mechanism(s) essential for the observed sex differences.

In male ANA rats with an inherent low voluntary ethanol intake, no specific separation-induced effects on voluntary ethanol intake were observed. An altered behavioral profile has been proposed in this line of rats and this may also affect the outcome of the MS procedure since the ANA dams could be differently affected compared to the AA dams, resulting in a different response to MS in the offspring, as indicated here with no differences between the three experimental groups.

In conclusion, results presented herein provide evidence to support the hypothesis that different environmental factors early in life can have profound effects on adult voluntary ethanol intake. Furthermore, evidence that environmental manipulations early in life can alter the inherited high ethanol intake in adult ethanol-preferring AA rats is presented. It is further suggested that MS15 might protect against a high voluntary ethanol intake while MS360 might serve as a risk factor in male rats.



Exploration and risk assessment behavior

The results showed minor MS-induced effects on adult behavior in the traditional EPM and OF tests. On the other hand, the CSF test indicated alterations in risk assessment as well as an increased exploratory drive and somewhat higher risk-taking behavior in the MS360 rats compared to AFR rats, while minor effects were seen in the MS15 group of animals.

The present results also show the advantage of a battery of tests. It is concluded that tests such as the CSF test, allowing more alternatives for visiting risk areas versus sheltered areas, provide a useful tool in the investigation of early environmental influences on the adult behavioral profile.

Svensk populärvetenskaplig sammanfattning

Arv och miljö spelar tillsammans en viktig roll för individens utveckling. Den tidiga perioden i livet är känslig då många funktioner anläggs och hjärnan utvecklas. Det mänskliga genomet är idag känt vilket ger nya möjligheter att studera den genetiska faktorns betydelse för utvecklandet av olika sjukdomstillstånd. När det gäller risken att utveckla ett alkoholberoende är ca 60% relaterat till genetiska faktorer, vilket har kartlagts bl.a. i tvillingstudier. När det gäller miljörelaterade faktorer visar resultat att uppväxtmiljön är av betydelse för individens utveckling. Olika stressfyllda händelser (trauman, incest, emotionell försummelse) under barndomen kan öka risken för att utveckla psykopatologi, t.ex. ångest och depression, och/eller drogberoende senare i livet. Longitudinella studier tar dock mycket lång tid att genomföra och studier som kontrollerar för miljöfaktorer är näst intill omöjliga att genomföra på människa främst ur ett etiskt perspektiv. Kontrollerade djurstudier fyller därför en viktig roll i den prekliniska forskningen. Studier på både gnagare och primater har visat att uppväxtmiljön kan ge neurokemiska förändringar som i sin tur kan påverka risken att initiera ett högt drogintag senare i livet. Det är därför av intresse att försöka klargöra orsakerna till att negativa upplevelser tidigt i livet kan ge långvariga effekter på neurobiologi och beteende och därmed påverka individens sårbarhet. Den här avhandlingen sammanfattar studier där uppväxtmiljöns betydelse för förändringar i hjärnan, frivilligt alkoholintag och utforskande och risktagande beteende senare i livet har studerats på råtta. Arbetshypotesen har varit att en trygg uppväxtmiljö kan skydda mot ett högt alkoholintag medan en otrygg uppväxtmiljö ökar risken för att etablera ett högt alkoholintag.

Maternal separation (MS) på råtta är en djurmodell för att påverka uppväxtmiljön. De tre första levnadsveckorna är en mycket känslig period för råttans utveckling då råttan delvis är helt beroende av mamman för att få mat, för fysiologiska funktioner, skydd och normal utveckling. Genom att separera ungarna från mamman olika tidsperioder under de tre första veckorna kan man simulera trygg och otrygg uppväxtmiljö. En kort daglig separation från mamman resulterar i att mamman pysslar om ungarna mer när de återförenas. Dessa djur blir bättre på att hantera stressfyllda situationer både fysiologiskt och i olika beteendemodeller som vuxna. Efter längre dagliga separationer från mamman störs mammans relation till ungarna, men här är resultaten mer skiftande främst beroende på olika experimentella protokoll som används. I de studier som ingår i den här avhandlingen har samma protokoll för MS använts. Ungarna har separerats kullvis från mamman 15 min (MS15) eller 360 min (MS360) dagligen under de tre första levnadsveckorna. Dessa djur har jämförts med ungar som fått växa upp tillsammans med mamman men

med minimal hantering och bara i samband med burbyten (normal animal facility rearing (AFR)). Djuren har sedan studerats i vuxen ålder.

Få MS-inducerade effekter på hjärnans endogena opioida system, som bl.a. är involverat i drogberoendemekanismer, noterades och betydelsen av dessa förändringar är delvis oklar. Frivilligt alkoholintag mäts genom att råttan har fri tillgång till en flaska med vatten och en flaska med en alkohollösning i buren. Här sågs en tydlig effekt på hanråttor (Figur 11). MS15 råttorna hade en långsammare initiering av alkoholintag, ett lågt intag och det var få råttor i den här gruppen som hade ett högt alkoholintag. Motsatsen noterades bland MS360 djuren som hade ett högt frivilligt alkoholintag. Hos honråttor sågs inga MS-inducerade effekter på frivilligt alkoholintag (Figur 11), vilket antyder att effekterna är könsrelaterade. Även alkoholprefererande hanråttor undersöktes. Dessa råttor har avlats selektivt för ett högt alkoholintag. Genom att man hela tiden har parat de råttor som har det högsta alkoholintaget har man fått fram råttor som har ett medfött högt alkoholintag. Även hos dessa råttor kunde MS15 sänka alkoholintaget medan MS360 resulterade i att de drack mer än de normalt gör (Figur 14-15). Resultaten visar därmed att uppväxtmiljön även kan påverka det genetiska arvet. Utforskande och risktagande beteende undersöktes i tre olika beteendemodeller hos vuxna MS15, MS360 och AFR hanråttor. Det mest intressanta testet, Concentric square field test, är ett test som baseras på råttans drift att utforska okända miljöer samtidigt som dessa kan vara potentiella riskområden. I testet registreras råttans utforskande beteende, hur mycket tid den spenderar i de områden som är mer riskfyllda samt hur mycket skydd råttan söker. Testet ger därför en indikation om råttans förmåga att bedöma risker. Dessa studier är relevanta då det bl.a. visats att ett risktagande, impulsivt beteende kan kopplas till ett ökat drogintag hos människor. Här visade resultaten att MS360 råttorna var mer utforskande och risktagande i vissa situationer (Tabell 2-3). Dessa resultat kan vara av betydelse för det högre alkoholintaget hos dessa råttor, men här krävs fler studier för att säkerställa resultaten.

Sammanfattningsvis visar resultaten i den här avhandlingen att en simulerad trygg och otrygg uppväxtmiljö påverkar frivilligt alkoholintag hos vuxna råttor, även de råttor som har en genetisk predisposition för ett högt frivilligt alkoholintag.

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