Early Environment and Adolescent Ethanol Consumption

Effects on Endogenous Opioids and Behaviour in Rats

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Abstract

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Excessive and compulsive ethanol drinking is one of the most serious public health issues. Therefore, it is vital to increase the knowledge about risks and protection for alcohol use disorders (AUD) to optimize prevention and treatment strategies. Ethanol consumption commonly initiates during adolescence when extensive neuronal maturation and development also occurs. Early exposure to ethanol is a risk factor for AUD, but the effects of adolescent drinking and the basis for the individual susceptibility to AUD are not fully understood. The interactions between genotype and environmental factors determine the individual risk for AUD and this thesis aimed to examine the environmental impact. The specific aims were to investigate 1) how early-life conditions affect adolescent voluntary ethanol drinking, behavioural profiles, endogenous opioids and response to treatment with an opioid antagonist (naltrexone), and 2) whether alterations detected in the offspring may be mediated by variations in maternal behaviour. A rodent maternal separation (MS) model was used to mimic a protective and risk-inducing early-life environment, respectively, with the use of 15 min (MS15) or 360 min (MS360) of daily MS. The main findings were 1) the MS360, but not the MS15 rats, responded to naltrexone following adolescent ethanol drinking; all adolescent rats had a high voluntary ethanol intake independent of early environmental conditions whereas in the adult groups the MS360, but not the MS15 rats, increased their ethanol intake and preference over time; adolescent ethanol exposure resulted in higher dynorphin levels in hippocampus and higher Met-enkephalin-Arg^{6}Phe^7 in the amygdala, independently of rearing conditions, 2) behavioural profiling using the multivariate concentric square field™ test showed: the young MS360 rats had increased risk assessment and risk taking behaviour compared to the young MS15 rats; the young MS15 rats increased, whereas the young MS360 rats decreased, their risk assessment and risk taking behaviour over time; differences in pup-retrieval strategies where the MS360 dams retrieved some pups into a safe area but as compared to MS15 rats they left more pups in a risk area; increased risk assessment behaviour in the MS360 dams immediately after weaning. Taken together, early-life environmental conditions alter adult but not adolescent drinking, the response to naltrexone, and behaviour in dams and offspring. Adolescent rats consumed more ethanol independent of rearing conditions and displayed increased opioid levels in brain areas related to cognition and addiction.

Keywords: Alcohol, intermittent ethanol access, maternal separation, multivariate concentric square field™ test, maternal behavior, ultrasonic vocalization, adolescent, neonatal handling

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To Elie and Sebastian

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals (I-V).

I   Daoura, L., Haaker, J., Nylander, I. (2011) Early environmental factors differentially affect voluntary ethanol consumption in adolescent and adult male rats. *Alcoholism: Clinical and Experimental Research*, 35(3):506-515.

II   Palm, S*., Daoura, L*., Roman, E., Nylander, I. Behavioural profiles but not alcohol-induced effects are dependent on rearing condition in rats with alcohol access during adolescence. *Manuscript.*

III   Daoura, L., Nylander, I. (2011) The response to naltrexone in ethanol-drinking rats depends on early environmental experiences. *Pharmacology Biochemistry and Behavior*, 99(4):626-633.

IV   Daoura, L., Hjalmarsson, M., Oreland, S., Nylander, I., Roman, E. (2010) Postpartum behavioral profiles in Wistar rats following maternal separation - altered exploration and risk-assessment behavior in MS15 dams. *Frontiers in Behavioral Neuroscience,* 117(9):1077-1081.

V   Daoura, L., Nylander, I., Roman, E. Qualitative analysis of pup-retrieval strategies in a maternal separation paradigm. *Submitted manuscript.*

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Contents

List of abbreviations

AA	Alko, Alcohol
ABN	Arched-back nursing
ACTH	Adrenocorticotrophic hormone
AFR	Animal facility reared
AL	Anterior lobe of the pituitary
Ala	Alanine
Amy	Amygdala
ANOVA	Analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
AUD	Alcohol use disorders
BC	Before Christ
BEND	Beta-endorphin
BSA	Bovine serum albumin
CNS	Central nervous system
Corr	Corridor
CORT	Corticosterone
Cpm	Counts per minute
CTRCl	Central circle
DA	Dopamine
DCR	Dark corner room
DOPR	Delta opioid peptide receptor
DYNB	Dynorphin B
ED	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
ENK	Enkephalin
EPM	Elevated plus maze
FCx	Frontal cortex
GD	Gestational day
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
HC	Hippocampus
HCl	Hydrochloride acid
HPA	Hypothalamic-pituitary-adrenal
HT	Hypothalamus
Ile	Isoleucine
Ir	Immunoreactive
KOPR	Kappa opioid peptide receptor
Leu	Leucine
Lys	Lysine
MCSF	Multivariate concentric square field™
MEAP	Met-Enkephalin-Arg^6 Phe^7
Met	Methionine

LG	Licking grooming
MOPR	Mu opioid peptide receptor
MPFCx	Medial prefrontal cortex
MS	Maternal separation
MS15	Maternal separation for 15 min
MS15E	Maternal separation for 15 min, ethanol group
MS15W	Maternal separation for 15 min, water group
MS360	Maternal separation for 360 min
MS360E	Maternal separation for 360 min, ethanol group
MS360W	Maternal separation for 360 min, water group
NAc	Nucleus accumbens
NaCl	Sodium chloride
NIL	Neurointermediate lobe of the pituitary
NOP	Nociceptin orphanin FQ peptide
NOPR	Nociceptin orphanin FQ receptor
NTX	Naltrexone
OCC	Occurrence
OF	Open field
ORL	Opiate receptor-like protein
P	Preferring
PAG	Periaqueductal grey
PCA	Principal component analysis
PDYN	Prodynorphin
Phe	Phenylalanine
Pit	Pituitary
PLSD	Protected least significant difference
PND	Postnatal day
PNS	Peripheral nervous system
POMC	Proopiomelanocortin
Pro	Proline
RIA	Radioimmunoassay
S.E.M.	Standard error of the mean
Ser	Serine
SHRP	Stress hyporesponsive period
SN	Substantia nigra
Str	Dorsal striatum
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
USV	Ultrasonic vocalization
Val	Valine
VTA	Ventral tegmental area

Introduction

Alcohol consumption

The word "alcohol" is derived from the Arabic word "alkuhl" which means "something subtle", and the use of alcoholic beverages has been documented since 10,000 BC. The Greeks, Romans, and inhabitants of Babylon used alcohol (ethanol) as early as 3000 BC and incorporated ethanol into religious festivals, but it was also used for pleasure, to facilitate socialization, and for nutrition and medical reasons. Today, alcoholic beverages as well as the dangers of heavy consumption have been identified and recognized in most cultures (Schuckit 2008).

In many parts of the world, ethanol drinking is common, and excessive ethanol intake is associated with many negative consequences such as violence, child neglect and abuse. Excessive ethanol drinking compromises both individual development and social behaviour and can be harmful, not only for the drinker, but also to those around her/him, such as friends, relatives and co-workers. Ethanol is the world's third, and Europe's second, largest risk factor for premature mortality and loss of health. Two and a half million people die every year due to ethanol-related causes, such as individual health problems (e.g. cardiovascular diseases and liver cirrhosis), or violence and traffic accidents caused by ethanol-intoxications (WHO 2011). About one million Swedish people between 16 and 80 years are classed as at risk[1] for alcohol use disorders (AUD) due to their ethanol intake (CAN 2012). In Sweden, the societal costs for consequences of excessive ethanol consumption were estimated to 45 billion SEK during 2011 (Allebeck et al. 2012). Thus, there are multiple health, social and economic problems following excessive ethanol intake, and it is therefore of vital importance to find prevention and treatment strategies.

Alcohol abuse and alcohol dependence are diagnosed using the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV), or by the International Statistical Classification of Diseases and Related Health Problems 10th edition (ICD-10). The diagnosis of alcohol abuse and addiction is based on several defined criteria that are essentially the same in ICD-

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[1] Risk consumption is a term used when 14 standard drinks for men, or 9 standard drinks for women, is consumed within a week. One standard drink is approximately 12 gram ethanol and is included in 33 cl of beer (5% ethanol), 15 cl of wine (12% ethanol), or 4 cl of spirits (40% ethanol).

10 and DSM-IV (NIAAA 2003). The DSM-V is expected to be released during 2013 and it is proposed that alcohol abuse and alcohol dependence will be combined to create one unified disorder, AUD (http://www.dsm5.org 2013).

Adolescent drinking

Binge drinking among adolescence is common and 62% of male and 52% of female students (15-16 years old) report that they have tried ethanol before the age of 13 (Hibell et al. 2011). Approximately 16-17% of 15-16 years old and 37-43% of 17-18 years old Swedish students have a heavy episodic drinking[2] (CAN 2013). The risk for developing AUD later in life increases with earlier onset of drinking (WHO 2011), and about 40% of those who start to drink heavily before the age of 15, develop AUD later in life (Grant and Dawson 1997). A number of neurophysiological processes emerge during adolescence, and these developmental processes are critical for brain maturation (Crews et al. 2007; Kelley et al. 2004; Spear 2000). During this time period, the brain is highly sensitive to environmental influences that can change the course of development and thereby cause long-term change in brain function (De Wit et al. 2000; York 1999). An early onset of heavy drinking can disrupt the normal course of neuronal development and is, for some individuals, a risk factor for later development of AUD (Grant and Dawson 1997; 1998; Hicks et al. 2010). Indeed, it is well known that adolescent ethanol drinking can cause detrimental brain injuries, or worse, early death (Hibell et al. 2011). For example, approximately 320 000 between 15 and 29 years old die globally from ethanol-related causes, every year (WHO 2010).

The impact of the environment on ethanol consumption

Environmental factors interact closely with genetic factors and determine the individual vulnerability or resilience to AUD (Enoch 2006). Twin studies show that 50% of the variation in development of AUD can be explained by genetic factors (Enoch 2006), showing that the environmental factors are equally important. The impact of environmental factors is particularly important for the young developing brain. The individual genetic set-up combined with early-life environmental factors, has a large impact on the risk for developing AUD later in life (De Bellis 2002; Fenton et al. 2013). Variation in ethanol metabolism (Neumark et al. 2004; Thomasson et al. 1991) or in sensitivity to ethanol's sedative effects (Goldman et al. 2005; Schuckit 1994) is for instance related to genetic factors. Environmental factors early in life

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[2] Heavy episodic drinking is a term used when a bottle (750 ml) of wine (or an equivalent amount of ethanol by other beverages) is consumed at the same occasion.

such as ethanol availability (Grant and Dawson 1998), parental/sibling support (Langeland et al. 2004), and traumatic events (Koss et al. 2003; Wilsnack et al. 1997) also have a large impact on later ethanol consumption patterns. The mechanisms and mediators underlying the impact of early-life environment on individual susceptibility to AUD are not fully understood. Environmental factors can induce DNA and histone modifications and thereby alter developmental plasticity (Champagne 2013). These epigenetic mechanisms, which can alter gene expression without altering the DNA coding sequence, are gaining a great interest in AUD. It has been shown that ethanol induces modifications through epigenetic processes such as selective acetylation, methylation, and phosphorylation in histones (Shukla et al. 2008). Epigenetic studies contribute to the understanding of the interaction between genetic- and environmental factors, and reinforce the importance of early environment to modulate genetic influence, and thus act as risk or protective factors for later psychopathology.

Early-life environment

Early in life, the primary caregiver has a vital role in nursing and protecting, and, in addition, for social contacts that are essential for attachment and normal development of the infants (Murray and Murray 2010). Neuronal networks undergo extensive development after birth and the developmental reorganization and maturation processes continue all through adolescence (Crews et al. 2007; Lenroot and Giedd 2008). This developmental process stems from an on-going dialogue between the infant's genetic setup and the environment. The brain is therefore highly sensitive to environmental input early in life and the ability to change and adapt to environmental stimuli is important in processes shaping the brain (Crews et al. 2007; Romeo and McEwen 2006). Early-life adaptive alterations may be favourable for the individual (Feder et al. 2009; Kim-Cohen 2007) but may also have negative consequences, for example impaired ability to adapt to new situations, increased sensitivity to adverse events or impaired coping mechanisms, and thereby contribute to the individual vulnerability for later disease (McCrory et al. 2011; Nemeroff 2004; Sinha 2008). Disruption of these interactions between caregiver and offspring can therefore have major impact on the infant.

It was early shown that children exposed to prolonged separations from their parents, e.g. hospital admission, often experienced depressive-like behaviours. This has been demonstrated by a video-clip *"A two year old goes to hospital"* displaying the distress felt by the two year old Laura when spending 14 days at the hospital without her parents (Bowlby and Robertson 1953). This incident, that changed the hospital policies during the mid-1950s and resulted in permission for parents to visit their children at hospitals, also

inspired the intensive studies of social behaviour and social contacts between infant and parent. Later it was shown that a warm and healthy relationship was important for a normal psychological development of infants and that adverse early-life experiences had contrasting outcomes (Bowlby 1954).

Today, it is considered that an adverse environmental experience, such as physical, emotional or sexual abuse, during childhood and adolescence are one of the major risk factors for development of psychopathology later in life (Heim et al. 2010), such as anxiety disorders, depression (Gibb et al. 2007; Springer et al. 2007) and AUD (De Bellis 2002). The mechanisms underlying these long-term effects on AUD are still poorly understood. A better understanding of the interactions between genetic and environmental factors, and how these factors interact at different points during early development, may help to identify how and when to intervene to prevent AUD.

For ethical reasons, it is difficult to study causal relations between early environmental factors and AUD in humans. It is therefore important to develop and use experimental models to study the consequences of early-life environmental factors under controlled conditions. Rodents, e.g. rats and mice, are frequently used in experimental research. In rats and mice, similar to humans, the offspring are dependent on maternal care during the first weeks of life, not only for nursing but also for thermoregulation and endocrine, peripheral and central neuronal development (Hofer 1994; Levine 2001).

Besides tactile contacts, the communication within the litter, and between pup and dam, is made by ultrasonic vocalizations (USVs) within the frequencies of 40-60 kHz. For instance, USVs are emitted upon loss of maternal contact or poor thermoregulation. When the pups are separated from the mother and kept together in the litter, or when a pup is kept warm during the separation, fewer USVs are emitted (Hofer et al. 1993). Ultrasoic vocalizations are also involved in the dams' retrieval behaviour of the pups (Allin and Banks 1972) and usually cease within 10-15 min after separation (Kuhn and Schanberg 1998), most likely not to allow predators to hear. Pups placed in a cold temperature, such as room temperature (22 °C), will emit more USVs. The number of USVs peak around postnatal day (PND) 4-8, and then decrease with age (Allin and Banks 1971), most likely because the pups start to develop fur and can therefore thermoregulate on their own. Another possible reason for decreased USVs with increased age is the improved ability to orient and locate the nest as they grow older.

During the 2nd postnatal week, the pups open their eyes and ear canals, become more mobile and begin to eat on their own. However, they still nurse until the 3rd week, at which point weaning occurs (Lehmann 1999).

The stress hyporesponsive period

A key component of the stress response (i.e. the body's adaptations, which are designed to re-establish physiological or psychological equilibrium) involves activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in release of glucocorticoids, corticosterone in rodents. Upon stress, corticotropin-releasing hormone is released by the hypothalamus. Corticotropin-releasing hormone stimulates the production and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. Adrenocorticotrophic hormone acts upon specific receptors in the adrenal cortex to stimulate the synthesis and release of glucocorticoids from the adrenal cortex. The stress response is later inhibited by negative feedback of the glucocorticoids on the anterior pituitary and hypothalamus (Dallman et al. 2000).

During PND 1-2, the rat pups are capable of responding to stressful stimuli with a release of ACTH and corticosterone (Lehmann and Feldon 2000). Thereafter, the basal corticosterone levels decrease dramatically until PND 4 (Levine 1994). Between PND 4 and 14, the pups will display high resistance to environmental stressors and this period is defined as the stress hyporesponsive period (SHRP) (Levine 1994; Sapolsky and Meaney 1986). Elevated levels of corticosterone can be detrimental for the brain, and the SHRP may serve as a protection barrier (Sapolsky and Meaney 1986). Pups left undisturbed with their mother during the SHRP fail to release ACTH following a saline injection (Levine and Dent 2000). On the other hand, when pups are deprived of maternal care for a long period (24 h) during the SHRP they show a significant increase of corticosterone following high doses of exogenous ACTH (Levine et al. 1967a). These studies show that the maternal presence actively inhibit the release of ACTH during the SHRP and therefore serve to protect the brain of the pups. The first postnatal weeks are therefore critical and disruptions in the dam-pup interactions may result in negative consequences for the offspring.

Maternal behaviour

The behavioural repertoire of the pregnant and lactating dam includes building of the nest, pup retrieval, arched back nursing (ABN) and, licking and grooming (LG) of the pups (Stern and Johnson 1990). Nursing bouts vary among dams and seem to be mediated by a complex interplay of both pup cues and maternal motivation (Meaney 2001; Smotherman et al. 1974; Stern and Johnson 1990). Litter size and sex composition of the litter did not alter LG-ABN behaviour of the dam during the first week after parturition (Champagne et al. 2003). However, in another study differences in LG behaviour depending on the sex of the offspring were revealed when examining LG behaviour during the second week of life (Moore and Morelli 1979). These results suggest that the observed sex differences in behaviour towards

the pups observed are age-dependent. It has also been shown that different patterns of maternal behaviour have consequences for the physiological and behavioural development of the offspring (Denenberg et al. 1962; Ressler 1962). Furthermore, there is a natural variation in rodent maternal care such as LG-ABN behaviour expressed by the dam (Champagne et al. 2003) and the level of LG-ABN affects offspring neuroendocrinology and behaviour (Liu et al. 1997; Meaney 2001). For example, offspring of high LG-ABN mothers show a more explorative behaviour in the open field (OF) test and shorter latencies to eat in a novel environment in adulthood (Caldji et al. 1998). Furthermore, adult offspring of high LG-ABN mothers cope better with stress in adulthood (Meaney 2001). These long-term effects of maternal care are a result of epigenetic modifications of genes, and thereby gene expression, in the brain of the offspring, and these modifications can persist into adulthood (Graff et al. 2011). Epigenetic modifications can be reversed by environmental manipulations, such as cross fostering (Weaver et al. 2004), which further show the importance of maternal care during the first week of life.

Maternal separation paradigms

During the first weeks after birth, the dam leaves her offspring for short periods of time each day to search for food. Calhoun demonstrated this in a semi-naturalistic condition (Calhoun 1963) and it has also been confirmed in a dual-chambered apparatus (Grota and Ader 1969). It is therefore claimed that a brief separation of the dam from her litter mimics a natural environment. Early handling procedures in rodents originate from studies by Bernstein in the early 1950s (Bernstein 1952). Bernstein reported that rats that were handled from weaning (PND 21) into adulthood (PND 60) show more explorative behaviour and improved learning behaviour in adulthood compared to non-handled rats (Bernstein 1952). Later on Weininger reported that rats that were handled and gently stroked for 10 min daily during 21 days post weaning had less systemic and gastrointestinal damages and less affected glandular system following food and water deprivation. Moreover, handled rats had smaller adrenals than non-handled rats, which indicate a hypoactive stress axis in the handled rats. In addition, handled rats had increased locomotor activity in the OF test in adulthood (Weininger 1956).

During the late 1950s, Levine showed that brief daily separations of the pups from the mother during the first three postnatal weeks until weaning reduced the physiological responses to stress in adulthood compared to non-handled rats (Levine 1957). Furthermore, rats that were handled for 3 min daily, either 10 or 20 days after birth, had lower stress response following novel stimuli as compared to non-handled rats (Levine 1967). In addition, it

was shown that handling before weaning was more effective than handling after weaning (Levine and Otis 1958).

Along with these findings, the use of maternal separation (MS) paradigms emerged in studies examining the influence of early-life stress on endocrinology, neurobiology and behaviour. These MS paradigms are commonly based on a naturalistic environment and an adverse environment, respectively, and involve separations of pups from the dam during the SHRP. The naturalistic environment commonly consists of a brief (3-15 min) daily MS and is based on the duration of maternal absence in the wild (Grota and Ader 1969), but also on the endocrine (Levine 1967) and behavioural (Levine et al. 1967b) outcomes later in life compared to non-handled rats. The adverse environment generally involves repeated prolonged (180-360 min) periods of MS. The aim of longer periods of MS is to cause adverse consequences later in life by interfering with dam-pup interactions and thereby disrupt the normal neuronal and behavioural development (Lehmann and Feldon 2000).

Importantly, these MS paradigms should not be referred to as one MS model, as further discussed in Methodology. It is clear that the differences in physiological, social and environmental factors that can be noted in MS paradigms used worldwide influence the outcome of MS (Lehmann and Feldon 2000; Pryce and Feldon 2003; Roman and Nylander 2005). However, the compiled results from MS studies have increased our knowledge about how environmental factors early in life affect neurobiology and behaviour in rodents (Kuhn and Schanberg 1998; Lehmann and Feldon 2000; Macri and Wurbel 2006; Nylander and Roman 2012; Plotsky and Meaney 1993).

MS-induced effects on the offspring

Behaviour
In the majority of MS studies, behavioural assessments have been carried out in adult rats using various tests for emotional reactivity and learning and memory. Investigations of the effects of prolonged MS on behaviour in adolescent rats are however sparse. In many studies, the common elevated plus maze (EPM) or OF test have been used for interpretations of anxiety-like behaviour. In these tests, low activity on the open arms of the EPM or in the centre of the OF (i.e. increased thigmotaxis) are interpreted as increased anxiety-like behaviour. With regard to MS-induced effects on adolescent rats, it was revealed that MS360 rats exhibited higher velocity in the OF than MS15 and animal facility reared (AFR) rats (Colorado et al. 2006; Spivey et al. 2009), whereas another study showed a decreased spontaneous exploration in the OF following prolonged MS compared to AFR rats (Pautassi et al. 2012). Furthermore, adolescent MS360 rats immediately following weaning had lower activity on the open arms of the EPM compared to AFR rats (Ploj et al. 2002). Notably, these effects changed with time and in adulthood, the

MS360 rats displayed behaviours interpreted as less anxiety-like behaviour compared to AFR rats. In agreement, a lower anxiety-like behaviour has been observed following prolonged MS in the OF (Kaneko et al. 1994) but not in the EPM (Kaneko et al. 1994; McIntosh et al. 1999). In contrast, adult male rats exposed to prolonged (180-360 min) MS show higher anxiety-like behaviour compared to AFR (Huot et al. 2001), non-handled (Kalinichev et al. 2002; Wigger and Neumann 1999) and MS15 (Huot et al. 2001) rats in the EPM. Moreover, the inability to perceive or the lack of effort to seek something reinforcing by measuring sucrose or saccharin intake is commonly used for interpretation of anhedonia, one symptom of depression, where a low intake indicates depressive-like symptoms. A lower adult sucrose intake has been observed in rats exposed to prolonged (180 min) MS compared to both AFR and rats subjected to 15 min MS (Ladd et al. 2000).

When testing adult rats in a multivariate setting, i.e. the multivariate concentric square field™ (MCSF) test, it was shown that adult MS360 rats displayed more risk-assessment and risk-taking behaviour compared to AFR rats. Moreover, no differences between groups were evident using the OF and EPM tests (Roman et al. 2006). These findings are in agreement with other studies, where no effects of prolonged MS have been observed in the EPM (McIntosh et al. 1999). Thus, the compiled results reveal inconsistent results with regard to MS-induced effects on behaviour when assessed in adulthood.

Drug intake

As for studies on behaviour, the majority of studies on drug intake are performed in adult rats. Ethanol intake was measured during late adolescence in selectively bred ethanol-preferring Alko, Alcohol (AA) rats and revealed higher ethanol intake in MS360 rats compared to MS15 rats (Roman et al. 2003). However, there is still a gap in the knowledge about MS-induced effects on ethanol consumption in young rats. The MS15 condition is usually related to protection against high adult drug intake in the sense that self-administration of central stimulants and home-cage drinking of ethanol is lower as compared to prolonged (≥60 min) MS, which is associated with increased drug intake. This is shown in a number of studies where adult rats exposed to prolonged (60-180 min) MS, had an increased self-administration of cocaine (Kosten et al. 2000), and amphetamine and morphine (Vazquez et al. 2006) compared to either AFR or NH rats. Consistent effects are seen on ethanol intake; rats subjected to prolonged MS (180-360 min) have commonly increased ethanol intake compared to MS15 rats (Huot et al. 2001; Jaworski et al. 2005; Ploj et al. 2003a). Moreover, this was also the case when using alcohol-preferring AA rats (Roman et al. 2005), with an innate high voluntary ethanol intake. Furthermore, over time, MS360 rats increase their ethanol intake, change preference from low (5%) to higher (20%) concentration of ethanol, and increase their intake of the higher ethanol concen-

tration (Gustafsson and Nylander 2006). Notably, the protective effect on adult ethanol intake seen in the MS15 group requires litter-wise separations, since the protective effect is abolished with individual MS (Oreland et al. 2011).

MS-induced effects on maternal behaviour

It is proposed that the effects of MS on the offspring are, at least in part, mediated by differences in maternal behaviour in the various MS groups (Macri and Wurbel 2006). Several studies demonstrated that, after short periods of MS, the mother shows an increased LG behaviour of her pups upon reunion (Liu et al. 1997; Macri et al. 2004; Pryce et al. 2001). However, the experiments investigating the effect of MS on maternal behaviour are far from conclusive (Daskalakis et al. 2011; Liu et al. 1997; Pryce et al. 2001), and only few have studied the retrieval behaviour during MS (Huot et al. 2000; Marmendal et al. 2006; Marmendal et al. 2004). Moreover, the studies of behaviour in dams following MS revealed inconsistent results, perhaps due to the different rat strains used: Wistar (Eklund et al. 2009), Sprague-Dawley (Maniam and Morris 2010) and Long-Evans (Boccia et al. 2007) rats and the question about maternal mediation of MS-induced effects on the offspring remains controversial.

The endogenous opioids

History

Opium poppies (*Papaver somniferum*) have been cultivated over the centuries mainly for the capacity to produce a white, milky sap (latex) for both medical and non-medical purposes. The opium poppy is one of the most important medicinal plants in the history of pharmacology. The first reference to opium (*opos* in Greek) dates back to 300 BC. by Theophrastus. It is widely accepted that the ancient Greek and Romans used opium for the euphoric, narcotic and pain relieving properties. Morphine, named after the Greek god of dreams, Morpheus, was isolated from raw opium in 1806 by the German pharmacologist, Sertürner. It was not only the first alkaloid to be extracted from opium, but also the first alkaloid ever extracted from a plant (van Ree et al. 1999). The term *opiate* is limited to the natural alkaloids derived directly from the opium poppy whereas the term *opioid* refers to all substances and synthesized chemicals that produce effects characteristic of naturally occurring opiates.

Stereospecific opioid binding indicative of receptors in the central nervous system were demonstrated in the early 1970s (Pert and Snyder 1973;

Simon et al. 1973; Terenius 1973). A year following the first discovery of opioid receptor binding, in 1974, the Neurosciences Research Program Workshop was arranged and the majority of the early contributors to the opioid field attended the meeting, Figure 1. Some years later, pharmacological studies led to the identification of three different receptors referred to as mu, delta and kappa opioid peptide receptors (MOPR, DOPR and KOPR, respectively) (Lord et al. 1977; Martin et al. 1976). In parallel studies, opioid-like activity was detected in brain extracts indicating the presence of endogenous opioid ligands (Terenius 2000). The first endogenous opioids to be discovered were the enkephalins (from the Greek "in the head") (Hughes et al. 1975). The endorphin (from the Greek "endogenous morphine") and dynorphin (from the Greek *dynamis* meaning power) were discovered later (Goldstein et al. 1979; Simantov and Snyder 1976). Later on nociceptin/orphanin FQ (N/OFQ, NOP) (Meunier et al. 1995; Reinscheid et al. 1995) and the endomorphins (Zadina et al. 1997) were identified.

Subsequent cloning of the opioid receptors confirmed the presence of the MOPR, DOPR and KOPR. During the course of this work another receptor protein was discovered, the opiate receptor-like protein (ORL), the NOPR (Mollereau et al. 1994), that has molecular properties similar to the opioid receptors and the same origin but another pharmacological profile (Dreborg et al. 2008; Stevens 2009).

Figure 1. Members of the Neuroscience Research Program Workshop in 1974, USA (left to right): seated, Gavril W. Pasternak, William Bunney, John Hughes, Hans Kosterlitz, Steven Matthysse, Francis O. Schmitt, Solomon H. Snyder, Avram Goldstein, E. Leong Way, Vincent P. Dole and Horace Loh; middle row, L. Everett Johnson, Frederic G. Worden, Robert D. Hall, Candace D. Pert, Yvonne M. Homsy, Parvati Dev, Huda Akil, Floyd E. Bloom, Agu Pert, Peter A. Mansky, William H. Sweet, Albert Herz, William R. Martin and Harriet Schwenk; top row, Ian Creese, David J. Mayer, Eric J. Simon, Leslie Iversen, Diana Schneider, Pedro Cuatrecasas, A.E. Takemori, Arnold J. Mandell, Arthur E. Jacobson, Jose M. Musacchio and Lars Terenius. Reprinted from (Snyder and Pasternak 2003) with permission from Elsevier.

Endogenous opioid receptors

Four distinct genes encode the opioid receptors (Dreborg et al. 2008; Le Merrer et al. 2009; Stevens 2009). The opioid receptors exhibit extensive sequence homology and they differ mainly in their extracellular loops. The endogenous opioid receptors are G_i-protein coupled receptors that regulate the cAMP system by inhibition of adenylyl cyclase that, in turn, mediate a number of pharmacological actions. They are activated by endogenous (e.g. beta-endorphin, enkephalins and dynorphins) or exogenous (e.g. morphine) opioids.

Endogenous opioid peptides

Endogenous opioid peptides are major intermediate peptide products of larger precursor molecules (Table 1). They are generated by cleavage from proopiomelanocortin (POMC), proenkephalin and prodynorphin (PDYN), respectively (Kakidani et al. 1982; Nakanishi et al. 1979; Noda et al. 1982). Proopiomelanocortin is biosynthesized in the anterior pituitary lobe, arcuate nucleus of the hypothalamus and the nucleus tractus solitarius and generates beta-endorphin and several non-opioid peptides such as ACTH, and alfa-, beta-, and gamma-melanocyte-stimulating hormone. Proenkephalin is synthesized widely in the central nervous system (CNS) and is the precursor to leucine (Leu)- and methionine (Met)-enkephalin, met-enkephalin-Arg6-Gly7Leu8 and Met-Enkephalin-Arg6 Phe7 (MEAP) (Akil et al. 1998; Chang et al. 1980; Eipper and Mains 1980). Prodynorphin is synthesized widely in the CNS and it generates dynorphin A and B, neoendorphin and Leu-enkephalin (Akil et al. 1998).

The endogenous endorphins, enkephalins and dynorphins have a common four-amino acid sequence at their amino terminal (Tyr-Gly-Gly-Phe) (Table 1), which is important for their binding to opioid receptors and bioactivity (Feng et al. 2012). The receptor selectivity differs between the endogenous opioid peptides. For example, as shown in Table 1, beta-endorphin is more selective for the MOPR and DOPR while enkephalins are more selective for the DOPR (Akil et al. 1998). Further, the dynorphins show selectivity for the KOPR and MEAP has been shown to bind to all opioid receptors (Akil et al. 1998; Vats et al. 2009).

Table 1. *Amino acid sequences for some endogenous opioid peptides and their receptor affinity.*

Precursor	Endogenous peptide	Amino acid sequence	Receptor affinity
Proopiomelanocortin	Beta-endorphin	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	MOP=DOP
Proenkephalin	Met-enkephalin	Tyr-Gly-Gly-Phe-Met	DOP>>MOP
	Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	DOP>>MOP
	Met-enkephalin-Arg[6]Phe[7]	Tyr-Gly-Gly-Phe-Met-Arg-Phe	KOP=DOP=MOP
Prodynorphin	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln	KOP >> MOP, DOP
	Dynorphin B	Tyr-Gly-Gly-Phe-Leu- Arg-Arg-Gln-Phe-Lys-Val-Val-Thr	KOP >> MOP, DOP

Ontogeny of the endogenous opioid peptides and receptors

The CNS of the rat matures by 3-4 weeks following birth, which is a short time-period compared to the human brain (Marsh et al. 1997). In rat, the endorphins and enkephalins appear in diencephalon, midline telencephalon and medulla-midbrain region well before birth, around embryonic day (ED) 16. However, the endorphin levels are higher than the enkephalins at this time (Bayon et al. 1979). Leu-enkephalin immunoreactivity (ir) can be detected at ED 15 to 18 and beta-endorphin ir can be found on ED 13 (Bayon et al. 1979). Opioid receptors have been detected in rat brain at ED 14 (Clendeninn et al. 1976). The opioid system undergoes a significant developmental reorganization during the first postnatal weeks and the opioid levels detected before birth are therefore not comparable to those present later in life (McDowell and Kitchen 1987; Morita 1992; Wang et al. 1992). Immediately after ED 15, KOPR sites increases rapidly until ED 20, and decreases from PND 1 until PND 3 before increasing again. The KOPR peaks around PND 7 and reaches adult levels around PND 15 (Attali et al. 1990).

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Autoradiography studies have demonstrated that the density of the MOPRs peak at PND 4, then decline gradually to reach adult levels by the third postnatal week (Marsh et al. 1997). The number of MOPR and KOPR dominate at PND 6 whereas in adulthood, the MOPR and DOPR dominate (Marsh et al. 1997). The ontogeny of the DOPR is different from the one of the MOPR and KOPR. The DOPRs are present in low densities, if at all, in the brain at birth and then increase in density from 2nd week following birth and peak during the 4th week followed by a decline (Spain et al. 1985). The receptor development in the rat brain occurs rapidly during the first postnatal weeks, and the brain opioids play an important role in this neuronal development (Nelson and Panksepp 1998). It is however still unknown why the opioid peptides exist earlier than their receptors and whether present receptors are functional or not. Even though the opioid peptide system has been extensively studied during the last 40 years, it is still far from understood.

Distribution and function of endogenous opioids

The distribution is somewhat different between the opioid peptides (Hokfelt et al. 2000; Le Merrer et al. 2009). The enkephalin peptides are widely distributed in both the CNS and peripheral nervous system (PNS) whereas beta-endorphin is less spread and restricted to a pathway with cell bodies in the hypothalamus, and widespread projections throughout the brain. Further, the dynorphin neurons overlap with both the enkephalin and beta-endorphin neurons but they display separate pathways (Hokfelt et al. 2000).

The wide distribution of the opioid peptides in the CNS and PNS mirrors their involvement of opioids in numerous physiological functions. They regulate, for example, social and maternal behaviour, pain, stress, learning and memory and fluid and food intake. The progress in opioid research can be followed in an excellent series of reports reviewing the opioid field (Bodnar 2012). Of particular interest in this thesis is the role of opioids in social interactions early in life and in reward, reinforcement and addiction processes.

Endogenous opioids and early-life social behaviour

Endogenous opioids have been shown to be involved in several functions during the perinatal and postnatal period and seems to be important both for the dam and the offspring (Machin and Dunbar 2011). Opioids are implicated in social behaviour and in social interactions early in life and have an important role in both parental behaviour and social bonding (Nelson and Panksepp 1998; Panksepp et al. 1980; Panksepp et al. 1994). For example, mice lacking the MOPR were unable to selectively approach their mothers (Moles et al. 2004).

There is also an association between USVs and opioids. Morphine, an opioid receptor agonist, decreases USVs in rats (Kehoe and Blass 1986b).

The effect of naltrexone (NTX), an opioid receptor antagonist, on USVs, is however inconclusive (Carden and Hofer 1991; Carden et al. 1996; Kehoe and Blass 1986a). Fewer USVs have been observed in MOPR knockout mice (Moles et al. 2004).

Endogenous opioids and reward

In the early 1950s, Olds and Milner discovered that rats readily learned to press a lever that delivered an electrical impulse into certain brain regions, "reinforcing structures", and showed that electrical stimulation could produce positive reinforcement (Olds and Milner 1954). These areas, "pleasure centres", are rich in dopamine (DA) and it was later shown that DA receptor antagonists attenuated rewarding effects and resulted in anhedonia (Wise 1973; 1974). Dopaminergic pathways are present in brain areas such as the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (PFCx), hypothalamus, and amygdala. The mesocorticolimbic dopaminergic pathway involves projections from the VTA to the NAc, dorsal striatum (Str), amygdala and PFCx. This pathway has a key role in brain reward, reinforcement and addiction processes. The majority of drugs of abuse, including ethanol (Imperato and Di Chiara 1986), share the ability to increase extracellular DA in the NAc (Di Chiara and Imperato 1988a). However, DA is not the sole transmitter in reward and reinforcement processes, it is important to note that the mesocorticolimbic neurons are regulated by a number of transmitters such as acetylcholine, gamma-aminobutyric acid (GABA), glutamate and endogenous opioids and they can contribute to these processes.

   The endogenous opioids are involved in the positive reinforcement triggered by natural rewarding stimuli (Le Merrer et al. 2009) and has been shown to play a key role in the actions of several drugs of abuse, not only opioids like morphine and heroin but also central stimulants, nicotine and ethanol (Drews and Zimmer 2010; Trigo et al. 2009; Van Ree et al. 2000).

   Most studies report that the VTA is rich in MOPRs and includes only a trivial amount of KOPRs while the NAc contains all opioid receptor types (Mansour et al. 1988). Opioids have previously been shown to interact with the nigrostriatal DA neurons (Christensson-Nylander et al. 1986; Herrera-Marschitz et al. 1986; You et al. 1994). They also regulate mesocorticolimbic neurons; MOPR activation in the VTA increases DA in the NAc whereas KOPR activation in the NAc reduces DA in the NAc (Di Chiara and Imperato 1988b; Spanagel et al. 1992). The opposite actions of MOP and KOP receptor activation are further supported by findings that MOP but also DOP receptor activation induces euphoria (Herz 1997) whereas KOPRs mediate dysphoria (Akil et al. 1998).

   These studies have led to the hypothesis that endorphins and/or enkephalins and exogenously administered opioids mediate reward by activation of

the MOPRs in the VTA that inhibit the release of the inhibitory neurotransmitter GABA and thereby result in disinhibition of DA neurons and increase of DA in the NAc (Akil et al. 1998). However, opioid reward also includes non-dopaminergic mechanisms through direct effects in the NAc and further research is needed to elucidate these mechanisms.

Endogenous opioids, in particular dynorphin, are also involved in the negative consequences of long-term drug consumption. One example is the increase in dynorphin seen after chronic use of drugs, including ethanol, that can contribute to the dysphoria seen in withdrawal states (Koob and Volkow 2010). The brain adapts to long-term drug-induced DA release by activating counteracting systems and one example is the up-regulation of dynorphin (Walker and Koob 2008).

The opioid – ethanol link

Ethanol has a complex, and yet not fully understood, mechanism of action. Unlike other drugs of abuse like nicotine, cannabinoids or central stimulants that bind to specific target proteins, ethanol instead affects a number of targets in the brain. The presence of ethanol in the CNS affects, for example, G-protein function and the function of ligand-gated and voltage-gated ion channels and thereby interferes with a number of transmitter systems, such as GABA, glutamate, serotonin and acetylcholine (Soderpalm and Ericson 2013; Vengeliene et al. 2008).

Numerous studies support a link between endogenous opioids and ethanol; endogenous opioids are involved in ethanol-induced reward and in the propensity to develop AUD (Gianoulakis 2004; Nylander and Silberring 1998; Oswald and Wand 2004) and endogenous opioids regulate ethanol consumption. For example, opioid antagonists reduce ethanol consumption in experimental models using rats (Samson and Doyle 1985), ethanol-preferring AA and P rats (Froehlich et al. 1987; Hyytia 1993) and non-human primates (Altshuler et al. 1980). Clinical studies also reported reduction in ethanol intake (O'Malley 1996; Volpicelli et al. 1995). These results led to the use of opioid antagonists in treatment of AUD (see separate section). In line with these studies, it was also shown that MOPR knockout mice had lower ethanol consumption than wild-type mice and an ethanol intake more similar to that of ethanol avoiding mice (Roberts et al. 2000). Studies on the other opioid receptors are less conclusive; DOPR knockout mice instead had higher ethanol preference (Roberts et al. 2001). This effect has been attributed to increased anxiety-like behaviour due to loss of the anxiolytic DOPR function. Further, a more complex picture is described for the KOPRs; a reduced ethanol conditioned place preference is seen in KOPR knockout mice (Le Merrer et al. 2009) whereas a KOPR agonist reduces voluntary ethanol drinking (Lindholm et al. 2001).

The opioid receptor agonist morphine has a bimodal effect with higher ethanol intake after low doses whereas higher doses of morphine reduce ethanol intake (Herz 1997). Recent experiments showed that rats increase their ethanol consumption when morphine is administered into the NAc (Barson et al. 2009) or hypothalamus (Barson et al. 2010) and that these effects mainly are mediated by DOPRs rather than MOPRs.

Moreover, several studies have shown that the inherent opioid activity is important for the propensity to develop excessive and compulsive ethanol consumption (Gianoulakis 2004). For example, the ethanol-preferring AA rats display differences in basal opioid peptides compared to their non-preferring counterparts, the ANA rats (Nylander et al. 1994). Furthermore, ethanol-induced reward and variations in the gene coding for the MOPR, OPRM1, seems to be associated in humans. For example, the single nucleotide polymorphism (SNP) A118G, is linked to the ethanol-induced effects; humans with the minor G-allele display an enhanced DA response after ethanol administration and the ethanol-induced increase in DA in the NAc was higher in mice carrying the human G-allele than in those with the A-allele (Ramchandani et al. 2010). The G-allele has also been associated with higher sensitivity to the reinforcing effects of ethanol in adolescents (Miranda et al. 2010). Several studies also support the association between the G-allele and AUD (Bart et al. 2005; Schinka et al. 2002) whereas others, including a recent meta-analysis, do not support such an association (Bergen et al. 1997; Ray et al. 2012).

Many studies have analysed ethanol-induced effects on opioid peptides and on opioid gene expression and support the idea that ethanol increase opioid activity, at least after acute administration (Drews and Zimmer 2010; Trigo et al. 2009; Van Ree et al. 2000). However, there is no consistent answer in these compiled studies to the question about how ethanol affects endogenous opioid networks. One explanation for differences between studies is the use of a variety of ethanol administration paradigms and another is the measurement of opioids at different time points after administration (Gianoulakis 2004; Koob et al. 1998). Most studies report effects of passively administered ethanol and the information about effects of voluntary ethanol drinking is sparse.

Results from studies on ethanol-induced effects on opioids and the modulatory effects of opioids on ethanol consumption have led to different theories in attempts to describe the ethanol-opioid interactions (Gianoulakis 2004; Oswald and Wand 2004; Sanchis-Segura et al. 2005; van Ree et al. 1999). One theory is referred to as the "opioid deficit/compensation" theory, and it hypothesizes that an inherent low basal opioid activity will reinforce ethanol consumption and lead to increased ethanol intake in order to compensate for the low opioid activity (Trachtenberg and Blum 1987). Another theory is the "opioid surfeit" theory suggesting that vulnerable individuals develop or inherit excess/surfeit opioid activity, which stimulates ethanol

intake. Ethanol intake further increases opioid activity and maintains high ethanol drinking (Oswald and Wand 2004; Reid et al. 1991). A third theory postulates that the propensity for high ethanol intake is influenced by the individual differences in the sensitivity of the opioid system to ethanol (Gianoulakis 2004; Oswald and Wand 2004).

Endogenous opioids in treatment of AUD

Genetic deletion or antagonism of the MOPR decreases ethanol-induced mesolimbic DA increase in rodents (Benjamin et al. 1993; Job et al. 2007). This effect is suggested to contribute to reduced ethanol-induced reward and is the basis for the use of opioid antagonists in treatment of AUD. The opioid receptor antagonist NTX is used for relapse prevention in the treatment of AUD. Naltrexone was originally synthesized in 1963 for treatment of narcotic overdosing but its potential to treat AUD was acknowledged later by Volpicelli in the 1980s (Volpicelli et al. 1986). Following clinical studies (O'Malley 1996; Volpicelli et al. 1995), DuPont marketed NTX as treatment for AUD in 1995. Today it is a FDA-approved medication for AUD treatment. Another opioid receptor antagonist gaining more interest is nalmefene. In addition to the high MOPR affinity, nalmefene differs from NTX by having a high affinity for the KOPR (Soyka and Rosner 2010). Nalmefene is used for treatment of AUD and has been shown to reduce total ethanol consumption and the number of heavy-drinking days in patients (Mann et al. 2012).

One matter of concern regarding therapy with NTX is the large individual variation in efficacy (Kiefer et al. 2008; Spanagel and Kiefer 2008). A number of clinical studies have reported treatment outcome of NTX therapy and it is obvious that there are large differences between studies (Ray et al. 2012). The A118G polymorphism also relates to the efficacy of NTX (Oslin et al. 2003). Today several studies have investigated the impact of A118G polymorphism and although many support the notion that the G-allele is related to better NTX response (Anton et al. 2008; Oroszi et al. 2009), not all studies do (Ray and Oslin 2009). One explanation to the differences between studies is the populations studied (Ray et al. 2012).

The effect of MS on endogenous opioids

Previous studies using MS have shown that endogenous opioids are affected by early environmental factors as evidenced by immediate as well as long-lasting changes in opioid networks (Nylander and Roman 2012). The MS-induced effects on basal endogenous peptides have been studied using litterwise separations and the results provide evidence for distinct effects in young and adult Wistar rats (Gustafsson et al. 2008; Ploj et al. 2003a). MS-induced effects on opioids were altered in areas related to brain reward and

addiction processes at 10 weeks of age but these effects were not seen immediately after the MS period. These results suggest that MS influences opioid development and thereby cause long-lasting effects on opioids and consequences for reward and ethanol drinking behaviour. Furthermore, it was shown that the effects on opioid peptides were dependent on the rearing condition; in rats that were subjected to individual separations, that is, separated from both the dam and littermates, MS induced completely different effects (Gustafsson et al. 2008).

Moreover, neurobiological alterations caused by early environmental factors affect the sensitivity to drugs that act on the opioid system. For example, rats subjected to MS180 had an altered sensitivity to morphine compared to non-handled rats (Kalinichev et al. 2001). In addition, MS180 rats displayed enhanced effects of NTX on sucrose responses compared to non-handled Long-Evans rats (Michaels and Holtzman 2007). Interestingly, it was also reported that MS15 and MS360 rats differ in their response to ethanol, as evidenced by distinct ethanol-induced effects after voluntary drinking (Gustafsson et al. 2007; Roman and Nylander 2005). Long-term voluntary ethanol consumption elicits different effects on opioid peptides and receptors, which is dependent on previous experiences, with enhanced ethanol-induced responses in rats exposed to prolonged MS (Gustafsson et al. 2007; Ploj et al. 2003a).

MS-induced changes in the opioid system could be one of the underlying factors in the differences seen in the MS-induced adult ethanol-drinking rats. So far, little is known about the impact of environmental factors on the endogenous opioids in adolescent ethanol drinking behaviour.

Methodology

Maternal separation paradigms

There are several important parameters affecting the outcome of MS on behaviour, neuroendocrinology and neurobiology. A number of reviews address these issues and describe the impact of various experimental factors on the outcome of MS (Becker et al. 2011; Holmes et al. 2005; Lehmann and Feldon 2000; Moffett et al. 2007; Nylander and Roman 2012; Pryce and Feldon 2003; Roman and Nylander 2005). Some of these factors are:

- Control groups
  The experimental parameter that seems to be most important for MS-induced outcome is the choice of control group. Common control groups used as control for prolonged MS are NH (non-handled), AFR (animal facility reared) or MS0/15 (briefly handled). NH rats are generally left completely undisturbed with the dam and AFR are handled only when cages are changed. Both NH and AFR are suggested to be stressful environments for the rat and rats within both these groups are handled differently than the MS groups. These controls are therefore considered less suitable as control for the prolonged MS condition. MS0 or MS15 rats are handled as the rats that are subjected to prolonged MS and thereby a better choice to avoid confounding effects of experimenter handling.

- Age of the pup at onset of MS
  The timing of the MS is important and the compiled information from the literature generally shows that an earlier onset of MS, i.e. immediately after birth, results in more pronounced effects. Commonly, MS is performed during the first 2 or 3 weeks and it is generally acknowledged that separation during the first 1-2 weeks is most important to elicit effects in the offspring .

- Frequency of MS
  The number of separations varies from occasional MS to daily MS. It is a matter of debate whether daily MS or occasional MS at random days is the most optimal MS paradigm but there are, to my knowledge, no systematic studies on the impact of frequency.

- Duration of separations
  The duration of prolonged MS is commonly between 180 and 360 min, but can be as short as 60 min. The prolonged MS are used to investigate the consequences of disturbed dam-pup interactions. Shorter MS, commonly 3-15 min, can be used as control for prolonged MS, as mentioned above, but can also be used to examine effects of a shorter period of MS as compared to, for example, AFR.

- Ambient temperature
  To avoid cold stress in the pups, it is important to control the temperature during the separation as hypothermia in itself may elicit other effects than those aimed for using MS.

- Rearing conditions
  Separation of intact litters or individual placement of the pups during MS results in different outcomes. Individual MS might introduce confounding effects due to loss of tactile contact.

- Sex
  The general finding in the literature is that MS less affects female rats.

However, it is important to note that the propensity for high ethanol intake seen after prolonged MS is a robust finding, for example seen in studies using MS for 60, 180 or 360 min, in different rat strains and across several laboratories. In the present thesis, results are from MS studies using litterwise MS investigating outcomes in dam and male offspring using the experimental groups MS360, MS15, MS0 and AFR.

Ethanol drinking paradigms

There are a number of ethanol paradigms used today in animal experimental research. It is important to mention that although rats do drink ethanol voluntarily they generally do not freely drink intoxicating amounts of ethanol. However, there are individual differences in their ethanol consumption and in a batch of outbred rats some increase their ethanol intake and preference over time whereas others essentially do not drink. Many studies use selectively bred ethanol-preferring rat lines with an inherent propensity for high ethanol intake (Bell et al. 2012; Lumeng et al. 1995). Voluntary ethanol intake can be assessed in home-cage drinking or operant models. Forced administration paradigms can be used in studies where addictive behaviours are in focus. Ethanol can be administered in different routes such as oral, intravenous, intracranial and intragastric or vaporised for inhalation (Gilpin

et al. 2008; Green and Grahame 2008; June and Gilpin 2010; Koob et al. 2003; Meisch and Lemaire 1993; Spanagel 2003).

Voluntary ethanol intake

Home-cage drinking

A good model of choice, for studying the individual propensity to initiate a high ethanol intake, is the free-choice home-cage drinking model where the animal voluntarily chooses between two or more bottles, including one water bottle. The two bottle free-choice paradigm has been used since the beginning of the 1940s (Richter and Campbell 1940) and is today a conventional model for assessment of voluntary consumption. Three- or four-bottle choice paradigms can, for example, be used when preference for different ethanol concentrations is of interest. Three commonly used home-cage drinking models are the continuous, limited and intermittent ethanol access paradigms. The choice of model is dependent on the experimental aim stated. In the continuous access paradigm, ethanol is available at all times and this paradigm can be used for examination of acquisition patterns and is also common when studying ethanol intake over long periods of time (Spanagel and Holter 1999), e.g. for up to a year. Moreover, this model has been used for the establishment of the selectively bred ethanol-preferring rat lines (Lumeng et al. 1995). In adult Wistar rats the intake can vary from 1 to 4 g/kg/24h using continuous access models (Gustafsson and Nylander 2006; Hargreaves et al. 2009; Ortiz et al. 2004; Roman and Nylander 2005; Wolffgramm 1990). In limited access paradigms ethanol is available in shorter sessions, for example 2-4 h, either during the light or the dark phase. This paradigm is for example suitable to avoid confounding effects of differences between individuals in time since the last drinking bouts or when the blood ethanol concentration is measured. In the intermittent access model ethanol is available intermittently, for example in 24 h sessions every other day. Intermittent access to ethanol results in higher voluntary ethanol consumption in rats compared to continuous access (Sinclair and Senter 1967; Wise 1973). Ethanol consumption up to 6 g/kg/day has been described (Hargreaves et al. 2009; Simms et al. 2008).

The choice of ethanol concentration is important. In free-choice paradigms the ethanol concentration can either be fixed or gradually increased over time. An ethanol concentration between 2 and 6% will be consumed by all rats (Meisch and Lemaire 1993) most likely due to the experienced mild sweet taste of ethanol (Sanchis-Segura and Spanagel 2006) but concentrations above 6% are generally not liked by non-preferring rats (Hansen et al. 1994; Richter and Campbell 1940). Therefore, the preference for ethanol should be studied by using higher ethanol concentrations than 6%. Another option for making rats drink higher ethanol concentrations is to use sweetener in the ethanol solution, but the disadvantage is that the sweetener may

mask the motivational effects of ethanol (Koob et al. 2003; Meisch and Lemaire 1993).

Operant self-administration

Operant conditioning methods, whereby a lever press is reinforced by drug delivery, are usually referred to as self-administration (SA), and are commonly used for drugs such as central stimulants (Der-Avakian and Markou 2010; Matthews and Robbins 2003) but also ethanol (Roberts et al. 2000; Thorsell et al. 2005; Walker and Koob 2008). The operant technique can be used for intracranial, intravenous or oral delivery of the drug. Oral SA is less commonly used than intravenous SA. One reason is the aversive taste of ethanol at higher ethanol concentrations (Meisch 2001).

Forced ethanol intake or administration

The forced ethanol intake paradigm involves access to one ethanol bottle, without any free choice for water drinking. Furthermore, there are different methods used to force ethanol administrations, such as intravenous injection, gavage administration and inhalation of vaporized ethanol. These methods can be used in order to ensure that the animal has administrated a specific amount of ethanol and to induce an addictive-like state (Criado and Ehlers 2013; Schier et al. 2013; Walker and Ehlers 2009).

Unlike voluntary ethanol intake, forced intake will not provide the possibility to determine the individual propensity to initiate and acquire excessive and/or compulsive intake of ethanol. In the present thesis, home cage drinking was used with either two-bottle or three-bottle choice of ethanol and water.

The multivariate concentric square field™ (MCSF) test

The majority of the most commonly used behavioural tests are focused on specific predetermined mental states, e.g. anxiety-like behaviour using the EPM or the OF tests. The design of the MCSF arena instead provide the animal with a free choice of different environmental settings and items that provide the opportunity to detect essential features of the animal's mentality. In one single test situation the animal can choose between different environments designed to include opportunity for exploration, risk-assessment, risk-taking, shelter seeking, and approach and avoidance. In this way a behavioural profile is generated e.g. (Meyerson et al. 2006; Palm et al. 2011; Roman and Colombo 2009; Roman et al. 2012). The MCSF has been validated with regard to areas associated with risk and safety, respectively. Lactating dams retrieve their pups from the hypothesized risk area, i.e., the bridge, to a sheltered area, the DCR, but do not move the pups out of the sheltered area. Similarly, food pellets are hoarded from the risk area and consumed in the sheltered area (Meyerson et al. 2006). In a battery combin-

ing the MCSF, OF and EPM tests, the MCSF test was found to be the most sensitive to previous experience and should be performed as the first test in order to eliminate the risk of carry over effects (Augustsson 2004). Furthermore, the multivariate design of the MCSF generates more information than the OF and EPM tests, alone or in combination (Augustsson 2004; Roman and Colombo 2009; Roman et al. 2007), which suggests that the MCSF can be used as the sole test. In a multivariate test situation, several measures can be taken, which is advantageous when a profile is desired rather than a particular behaviour. Thus, the MCSF is launched as a complementary methodological possibility to understand mechanisms underlying various mental states.

When working with large data sets the use of multivariate data analysis techniques enables extraction of information from the data, that usually is not possible to obtain using traditional statistical approaches, and which also applies to data generated from the MCSF e.g. (Meyerson et al. 2006; Palm et al. 2011; Roman et al. 2012). Therefore, conventional statistics are usually complemented by multivariate data analysis procedures, such as principal component analysis (PCA), and the trend analysis.

The trend analysis is based on the fact that the individual may choose different behaviour strategies within the same or similar functional context, emanating from the same mental state. For each parameter, the animals are ranked against each other so that the animal with the lowest score is given the lowest rank and the animal with the highest score is given the highest rank value. Thus, the comparisons are based on the relative position of the animal within the population tested (Akerberg et al. 2012). The rank values for each parameter are then summed into a sum rank for each functional category (i.e. general activity, exploration, risk-assessment, risk-taking and shelter seeking) e.g. (Palm et al. 2011; Roman et al. 2012).

The PCA is a multivariate projection-based approach designed to extract and display the systemic variation in a data set. The most important use of PCA is to obtain an overview of the data, e.g., groups of observations, trends and outliers, and also to uncover the relationships between observations and variables, and among the variables themselves. The PCA creates a score plot showing a summary of the relationship between the individuals, and a loading plot identifying variables important for creating these relationships, i.e., parameters recorded in the MCSF. The direction of the score plot corresponds to the direction in the loading plot. The use of PCA on data generated in the MCSF test has been described in detail elsewhere (Meyerson et al. 2006; Roman and Colombo 2009; Roman et al. 2007).

The MCSF test is used in the present thesis to assess behavioural profiles in the offspring and also in the dams during or after MS.

Radioimmunoassay

Radioimmunoassay (RIA) is an extremely sensitive and specific method developed by Yalow and Bernson, during the 1950's, to measure insulin in the blood (Yalow and Berson 1960). The technique was one of the first immunoassay techniques developed and can be used to determine concentrations down to the picomolar range. The method is based on the competition between the unlabelled and the corresponding labelled antigen for a limited number of binding sites on an antibody. When equilibrium is reached some antigen will be free, and some will be bound and form an antigen-antibody complex. The free antigen and the antigen-antibody complex are then separated from each other by, for example, adding a charcoal suspension or a second antibody.

In this thesis, specific RIAs (Christensson-Nylander et al. 1985) were used to measure the ir levels of opioid peptides dynorphin B, MEAP and beta-endorphin in in the pituitary gland and the areas of the brain. MEAP, dynorphin B and beta-endorphin, respectively, were used as markers for the proenkephalin, the prodynorphin and the proopiomelancortin (POMC) systems.

Aims

Early-life adversity and adolescent ethanol consumption can cause brain damage, altered neuronal development and altered behaviour. However, the mechanisms are still far from understood. Previous studies have suggested a link between the early-life environment, adult ethanol intake and endogenous opioids but less is known about adolescent drinking in this link.

The overall aims of this thesis were:
1. To investigate how maternal separation affects adolescent voluntary ethanol drinking, behaviour profiles, endogenous opioids and response to treatment with an opioid antagonist.
2. To investigate if alterations detected in offspring may be mediated by variations in maternal behaviour.

Specific aims:
Paper I
To compare the establishment of voluntary ethanol consumption in adolescent and in adult male Wistar rats subjected to different early-life conditions.

Paper II
To investigate whether the early-life environment would affect behaviour in adolescent and in adult male Wistar rats.

To study interactions between early-life environment and voluntary adolescent drinking on behavioural development and on the opioid peptides.

Paper III
To examine the impact of early-life environment on individual response to naltrexone.
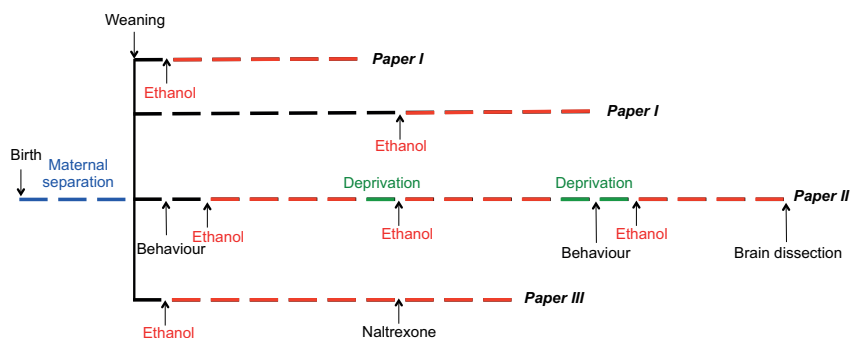
Paper IV
To profile post-weaning behaviour of dams previously exposed to pup-separations during the lactating period .

Paper V
To study the pup-retrieval strategies in dams exposed to pup-separations during the lactating period.

Materials and methods

A Offspring

Weaning

Ethanol

Paper I

Paper I

Ethanol

Birth

Maternal separation

Deprivation

Deprivation

Paper II

Ethanol

Behaviour

Ethanol

Ethanol

Behaviour

Brain dissection

Paper III

Ethanol

Naltrexone

B Dams

Parturition

Weaning

Maternal separation

GD

Papers IV-V

Behaviour

Behaviour

Pup-retrievals

USVs by pups

Approximately 1 week

Figure 2. An experimental outline for experiments performed on the A) offspring and B) dams, to illustrate the experimental set-up for the different studies presented herein.

Animals

Pregnant (Paper I-V) and virgin (Paper V) outbred Wistar (Sca:Wistar) rats were ordered from Scanbur BK AB, Sollentuna, Sweden. In Paper II, pregnant Wistar Han (RccHan:WI) rats were ordered from Harlan Laboratories B.V., Horst, the Netherlands. On arrival, the rats were singly housed in cages type IV (59 × 38 × 20 cm) containing wood chip bedding and nesting material and maintained on water and pellet food (Type R36; Labfor from Lactamin, Vadstena, Sweden or Lantmännen, Kimstad, Sweden) *ad libitum*. All animals were housed in temperature controlled (21 ± 1°C) and humidity controlled (50 ± 10 %) cabinets. All animal experiments were performed

under a protocol approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

Maternal separation

A schematic outline of the experimental designs is illustrated in Figure 2A (Papers I-III) and Figure 2B (Papers IV-V), respectively. Upon parturition (day 0), the pups were sexed and cross-fostered to avoid the use of only biological littermates in the same experimental group. Thereafter, the litters were randomly assigned to a rearing condition; housed under conventional animal facility rearing (AFR) condition, or exposed to 0 min (MS0), 15 min (MS15), or 360 min (MS360) of daily MS from PNDs 1 to 6 (Paper V) or 1 to 20-22 (Papers I-IV). Cages were changed once a week, and a small part of the old bedding material was always transferred to the bedding material in the new cage. The MS procedure was initiated by removing the dam from the maternity cage to a temporary cage (26 × 20 × 14 cm) containing wood chip bedding material, followed by moving the litter into a separation cage (26 × 20 × 14 cm) containing wood chip material. The MS15 and MS360 litters were moved to a heating cabinet (30 ± 2°C) in an adjacent room during the separation In the MS15 group, the dams were transferred to another cage during the separation and the litters were returned to the home cages before the dams. The dams in the MS360 group were returned to their maternity cages during the separation procedure, but taken out prior to the return of the litters. In the MS0 group, the litters were handled like the MS15 and MS360 litters but then immediately returned to the maternity cage and thus not separated from their mother more than approximately 45 seconds. The AFR groups were left undisturbed and only handled when weighed. The separation sessions were always performed in the same animal room, and only one person performed all separations and care giving of the rats.

In Papers I and III, the animals were weaned and thereafter group housed until PND 26 (n = 45) or PND 68 (n = 44), with three males per cage (59 × 38 × 20 cm). In Paper II the male pups were weaned and housed 5-6 males per cage until PND 34. The cages were changed once a week.

Voluntary ethanol consumption   [Papers I-III]

The animals were individually housed in cages type III (42 × 26 × 18 cm) containing wood chip bedding material and a wooden house and had *ad libitum* access to pellet food, and water. On PND 26, forty-five male rats (MS0, n = 15; MS15, n = 15; MS360, n = 15) were individually housed for assess-

ment of adolescent voluntary ethanol for the following 5 or 9 weeks. Forty-four male rats (MS0, n = 15; MS15, n = 15; MS360, n = 14) were group housed for a period of 7 weeks (until PND 67) and were then individually housed for the assessment of adult voluntary ethanol consumption for 5 weeks from PND 68 and onwards (Paper I). The rats in Paper II were individually housed on PND 34 and twenty rats from each MS group were randomly selected for assessment of voluntary ethanol consumption and ten from each group had access to water only.

During the ethanol-drinking period, the rats were given free access to a two-bottle (Paper II) or a three-bottle (Papers I, III) free choice paradigm with 24 h intermittent access on Mondays, Wednesdays and Fridays. The choice was between 20% ethanol (v/v using 96% ethanol) and water (Paper II) or between 5% and 20% ethanol (v/v using 95% ethanol), and water (Papers I, III). In Paper II, the water-drinking rats had access to two bottles, with tap water only and the ethanol-drinking rats had access to ethanol and water from PND 34 and 20 weeks onwards, until 20 weeks of age (Figure 2A). In Paper II, two deprivation periods were introduced during sessions 13-15 (week 5) and sessions 27-32 (week 10-11). During these sessions only water was accessible to the animals. Plastic bottles (250-mL) with ball-valve nipples (Scanbur BK AB, Sollentuna, Sweden) were used. Water was available at all times. The positions of the bottles were changed each session, to avoid position preference. Ethanol was changed each session, and water was changed every day. Measurements of ethanol and water consumption were made after each session by weighing the bottles.

In total, the rats had ethanol access for 5 weeks (15 sessions, Paper I), 12 weeks (36 sessions, Paper II), or 9 weeks (27 sessions, Paper III).

Naltrexone treatment [Paper III]

The NTX/saline injections in paper III started during the 7th week of ethanol access. The injections were performed once a week, on Mondays, over a three-week period. Each rat received three injections, saline and two doses of NTX (0.3 mg/kg or 3.0 mg/kg), respectively, with a one-week washout period between injections, according to a randomised injection schedule. Naltrexone (Sigma-Aldrich, Schnelldorf, Germany) was dissolved in saline and administered subcutaneously. The injection volume was 1 ml/kg, and the doses were chosen based on personal experience and previous reports (Mhatre and Holloway 2003; Williams and Broadbridge 2009). The drug solutions were prepared immediately before the injections, which were given 30 min prior to the beginning of the dark cycle and ethanol access. The ethanol consumption was measured 30 min, 2 h and 24 h after ethanol access, i.e., 1 h, 2.5 h and 24.5 h after the saline/NTX injections. Ethanol intake was also measured on Wednesdays and Fridays to assure that the duration of the

washout period was sufficient, i.e., that the effects of NTX did not persist. The difference between ethanol consumption after NTX and saline injections was calculated in each rat before the statistical analysis was performed.

Brain dissection [Paper II]

The animals in Paper II were decapitated at 20 weeks of age (immediately after the final ethanol-session for the ethanol-drinking animals) for assessment of endogenous opioid peptide levels. Immediately after decapitation, the brain and the pituitary gland were taken out and the pituitary gland was divided into the neurointermediate and anterior lobes (NIL and AL, respectively). The hypothalamus was removed from the brain, which was then placed in a cooled matrix (ASI Instruments, Inc., Warren, MI). The following nine areas from coronal sections were dissected manually with a razor blade: frontal cortex (FCx), medial prefrontal cortex (MPFCx), NAc, Str, hippocampus, amygdala, substantia nigra (SN), VTA and periaqueductal grey (PAG). All tissues were immediately frozen on dry ice and stored at -80 ^{o}C.

Tissue homogenization, extraction and separation of peptides

The opioid peptides MEAP, dynorphin B and beta-endorphin were extracted from the different brain areas by adding heated 1 M acetic acid to the frozen tissues, which were then heated for 5 min at 95 ^{o}C. The tissues were then cooled on ice and homogenized using a Branson Sonifier, after which the homogenate was reheated for 5 min at 95 ^{o}C. The samples were again cooled and centrifuged for 15 min at 15400 × g in a cooled Eppendorf 5417R centrifuge. For the purification of the supernatant, an ion exchange procedure was used according to Christensson-Nylander and co-workers (Christensson-Nylander et al. 1985). The samples were poured onto columns filled with Sephadex C-25 gel (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the opioid peptides were stepwise eluted in separate fractions using pyridine and formic acid buffers with increasing ion strength. The fractions were dried in a SpeedVac SC210A vacuum centrifuge and stored at -20 ^{o}C.

Radioimmunoassay

The MEAP and dynorphin B peptides were ^{125}I-labeled using chloramin-T and purified with high-performance liquid chromatography. For the MEAP RIAs, the samples were oxidized prior to the assays. They were dissolved in 100 µl 1 M acetic acid and 10 µl 30% H_2O_2 and incubated for 30 min at 37 ^{o}C, after which they were dried in the vacuum centrifuge. The dynorphin B antiserum and labelled peptide were diluted in a buffer containing 0.15 M

sodium chloride, 0.02% sodium azide, 0.1% gelatine, 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) in a 0.05 M sodium phosphate buffer. The MEAP antiserum and labelled peptide were diluted in a buffer containing 0.15 M sodium chloride, 0.025 M EDTA, 0.1% gelatine and 0.1% BSA in a 0.05 M sodium phosphate buffer. The beta-endorphin antiserum and labelled peptide were dissolved in a buffer containing 0.1 M sodium phosphate, 0.05 M NaCl, 0.01% NaN_3, 0.1% BSA, and 0.1% Triton X-100. All samples and standards were prepared in duplicates and dissolved in methanol: hydrochloric acid 0.1 M (1:1) and 25 µl aliquots were incubated with 100 µl antiserum for respective peptide and 100 µl ^{125}I-labeled peptide (100 µl, 4500 cpm) overnight at 4 $^{\circ}$C.

Antibody bound dynorphin B and beta-endorphin were separated from free peptides by adding 50 µl goat-anti-rabbit-IgG and 50 µl normal rabbit serum (Peninsula Laboratories LLC; San Carlos, CA) after which the samples were incubated for 90 min at 4 $^{\circ}$C. The samples were then centrifuged for 15 min at 12000 × g in a cooled Beckman GS-15R centrifuge. The pellet was counted in a Wallac 1470 Wizard gamma counter. For the MEAP assay, separation was performed by adding 200 µl of a charcoal suspension, containing 250 mg charcoal and 25 mg dextran T-70 in 100 ml 0.05 M sodium phosphate buffer, and the samples were incubated 10 min on ice before centrifugation for 1 min at 12000 × g. 300 µl of the supernatant was then counted in the gamma counter.

The dynorphin B antiserum did not show cross-reactivity with either dynorphin A (1-17) or dynorphin A (1-8). Cross-reactivity with dynorphin B 29 was 1% and with big dynorphin (dynorphin 32) 100%. Other opioid peptides did not cross-react with the dynorphin B antiserum. Cross-reactivity with the MEAP antiserum was less than 0.1% for met-enkephalin, Met-enkephalin-Arg[6], met-enkephalin-Arg[6]Gly[7]Leu[8], leu-enkephalin or dynorphin A (1-6). Cross-reactivity with the beta-endorphin antiserum was 0% for alpha-melanocyte stimulating hormone, met-enkephalin, and 60% for gamma-endorphin and 100% for alpha-endorphin.

The multivariate concentric square field™ (MCSF) test

The MCSF test arena (100 × 100 cm) includes exploratory incentives, open, sheltered and elevated areas with different illumination and is divided into zones (Figure 3), which forms the basis for the description and the variables of the animals' performance in this test.

All MCSF tests were performed in a room separate from the housing room, with a masking background noise. Each animal was transferred in a bucket from the home cage to the MCSF apparatus and released in the centre (Figure 3) facing the wall without openings, between the centre and bridge. After each animal/group of dams and litter to be tested, the arena was wiped

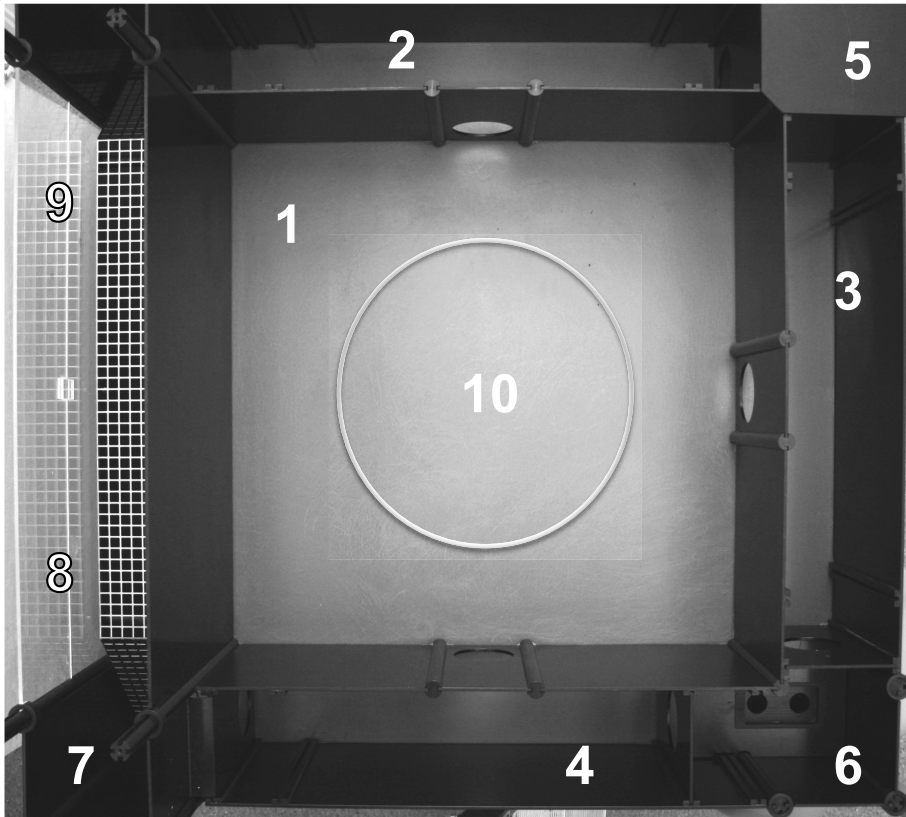with 10% ethanol solution and sufficient time was allowed for the arena to dry.

Figure 3. **1. Centre,** the centre field (70 x 70 cm), an open area; **2-4. Corridors,** the corridors surrounding the centre field with openings ($\varnothing$ = 8 cm), transit areas; **5. Dark corner room (DCR),** a shaded/covered room with access only through one entrance, for shelter seeking; **6. Hurdle,** a high passage to a hole board placed 10 cm above the floor with a photocell device measuring the animal's nose pokes into two existing holes ($\varnothing$ = 2.5 cm), exploratory incentive; **7. Slope,** the slope leading up to the bridge, a risk-assessment area; **8. Bridge entrance,** the very first part of the bridge, after leaving the slope, where the illumination is lower and the animal can assess the risk of visiting the bridge, a risk-assessment area; **9. Bridge**, the elevated stainless steel wire-mesh construction (10 mm between bars) and illuminated bridge construction, a risk area; **10. Central circle**, the circular zone (22 cm) in the middle of the centre, a risk area.

Dimmed light was used during the testing, except for the bridge area. The approximate light conditions (lx) in the MCSF arena were as follows: DCR: < 1; centre, corridors and hurdle: 10 – 20; bridge: 600 – 650.

The animals were monitored with a TV-video set-up (Panasonic Super Dynamic WV-BP 550/B camera connected to a SABA M3705M monitor and a Panasonic AG-TL 300E VHS) while the observer watched remotely

from an adjacent room. The numbers of rearing actions, grooming actions, faecal boli, and urinations were recorded by direct observation. The number of stretched attend postures (SAPs), a behaviour characterized by the animal stretching the body forward while retaining the position of the hind legs, from the corridors into centre, were also recorded by direct observation. Manual scoring of the behaviour in the MCSF test was performed using Score 3.3 (Soldis, Uppsala, Sweden). The latency (LAT, s) of first visiting a zone, frequency (FRQ) of visits, and duration (DUR, s) of time spent in each certain zone were all registered. Visits to the defined zones were only scored as such if both hind legs had crossed over into that section. The following parameters were calculated based on the descriptive parameters: the mean duration per visit to a zone (DUR/FRQ, s), the percentage duration spent in each zone, the sum of frequencies to corridors 2–4 (FRQ TOTCORR) and to all zones (total activity, TOTACT), and the total time spent in corridors 2–4 (DUR TOTCORR).

Behaviour before and after adolescent ethanol [Paper II]

At two occasions, PND 27-31 and PND 104-108, the animals (AFR, n = 10; MS15, n = 30; MS360, n = 30) were tested in a 20 min MCSF trial. The second test was performed during the ethanol deprivation period to avoid ethanol on board in the ethanol-drinking rats. The AFR rats were included as a control for the repeated testing (Paper II).

MS-induced behaviours in dams after weaning [Paper IV]

On postpartum days (PPDs) 24-25 (Paper IV), the dams in the MS15, MS360, and AFR groups (n = 6/group) were tested in a single 20-min trial in the MCSF test.

Pup-retrieval strategies during MS [Paper V]

On PNDs 3 and 5, the pup-retrieval tests (Paper V) were performed in a 10-min trial. The litter to be tested (MS0 brought from the home cage; MS360 brought from the heating cabinet) was placed on cellulose cotton at the end of the bridge before releasing the dam in the centre. Pup-retrieval related behaviours, i.e. the latencies in first visiting the bridge, in first checking of the pups, in retrieval of each pup from the bridge and the number of pups placed in the DCR (complete retrieval), or somewhere else in the arena or left on the bridge, were recorded. A latency of 600 s was given for any parameter in the pup-retrieval test that was not initiated or completed within the 10-min observation period (Paper V).

On PNDs 4 and 6, the individual USV calls emitted by MS0 and MS360 pups were measured at the end of the MS0 and MS360 procedures, respec-

tively. Each pup was individually placed in an insulated and circular aluminium chamber ($\varnothing$ 17 cm) connected to a microphone bat detector device (Petterson Elektronik AB, Uppsala, Sweden), interfaced with a USV counter device (developed by Department of Medical Pharmacology, Uppsala University, Uppsala, Sweden) and the number of USVs in the 40-60 kHz range were recorded.

Statistical analyses

Conventional analyses

Differences were considered statistically significant at $p \leq 0.05$. Statistical analyses were performed using Statistica 8.0, 9.1 or 10 (StatSoft Inc., Tulsa, OK).

Parametric statistics

A one-way analysis of variance (ANOVA) was used to analyse possible differences in body weights between groups (Paper V). The impact of rearing condition on behaviour in adolescent rats and the ir peptide levels in adult water-drinking rats were also tested with one-way ANOVA (Paper II).

For analysis of descriptive behavioural parameters in Paper II, the data was logarithmically transformed (log (x+1)) to achieve a normal distribution and analysed using parametric statistics.

A two-way ANOVA was used to analyse the effect of rearing environment, the effect of age, and the possible interaction between rearing and age (Paper I). The same analysis was performed to assess possible differences in body weight gain during the ethanol-drinking period in the adolescent and the adult rats (Paper I). Further, a factorial ANOVA with MS group and intake group as factors was used to analyse ethanol-induced effects on opioids in rats subjected to different MS conditions (Paper II).

A repeated measures ANOVA was used to analyse the impact of rearing condition in water-drinking rats on behavioural change over time (Paper II). Interactive effects of rearing conditions and adolescent voluntary drinking were also tested with repeated measures ANOVA by using MS group and intake group as factors to analyse behaviour after different MS conditions (Paper II).

Whenever a significant main effect was observed, the analysis was followed by Fisher's protected least significant difference (PLSD) post hoc test.

Nonparametric statistics

The Shapiro-Wilk's W test showed that the fluid intake, fluid preference, NTX-induced effects (Papers I, III) and the behavioural data (Papers IV-V) were not part of a normal distribution. Therefore nonparametric tests were used.

The Kruskal-Wallis analysis followed by the Mann-Whitney U-test or the Mann-Whitney U-test alone was used to analyse possible differences between the different groups (Papers I, III-V) and also between time-dependent changes in the groups (Paper I). Differences in ethanol intake and preference when compared with those at adulthood within each experimental group were also analysed using the Mann-Whitney U-test (Paper I).

The Friedman test followed by the Wilcoxon signed-rank test was used for comparison of fluid intake (Papers I-II) and ethanol preference (Paper I) over time within each experimental group and when comparing fluid intake parameters after NTX or saline administration (Paper III). The Wilcoxon signed-rank test was also used when comparing behavioural parameters between the first and last 5-min periods of the 10-min trial in the MCSF and between the first and second pup-retrieval and USV tests (Paper V).

The Spearman rank correlations test was used for calculating correlations (Paper V).

Trend analysis

Besides analysing each MCSF parameter, a trend analysis was used (see Methodology). In the trend analysis, behavioural parameters for each individual were ranked and summed into functional categories; general activity, exploratory activity, risk-assessment, risk-taking and shelter seeking (Papers II, IV-V) (Palm et al. 2011; Roman et al. 2012).

Multivariate data analysis

In addition to the conventional statistical analyses, a multivariate data analysis was performed using a PCA (Eriksson et al. 2006; Jackson 2003) in order to illustrate the relationship between the different MS dams used in Paper IV and for investigation of correlations between the behavioural parameters of the pregnant dams (GD20) and their later pup-retrieval strategies (Paper V). The impact of rearing condition on behaviour in the adolescent rats (Paper II) was, in addition to the one-way ANOVA test, also analysed using multivariate data procedures.

For the multivariate data analysis, SIMCA-P+ 12.0 (Umetrics AB, Umeå, Sweden) was used.

Results and discussion

Effects of MS on voluntary ethanol consumption and opioid peptides using an intermittent ethanol paradigm

Previous studies have shown that repeated long periods of MS result in higher ethanol intake in adulthood. However, little is known about adolescent consumption after MS. Moreover, previous studies assessing ethanol intake in rats subjected to MS have commonly used continuous ethanol drinking paradigms. Therefore, in Paper I, the adolescent and adult voluntary ethanol intake was compared in rats subjected to different early environmental settings in one and the same MS study. The MS-induced effects on adolescent ethanol intake in Paper I and Paper II, were assessed using an intermittent free choice between ethanol and water.

Results Paper I

Wistar rat pups were separated from their mother 0 min (MS0), 15 min (MS15), or 360 min (MS360) daily during first three weeks after birth. After weaning, the male rats were divided into two groups; rats were given free access to water, 5 and 20% ethanol at either PND 26 or 68. An intermittent schedule was used with ethanol available for 24h three days a week for 5 weeks.

Effects of MS on adolescent voluntary ethanol consumption

A decrease in total ethanol intake was observed with time, in all adolescent rats within the MS15, and MS360 groups (Table 2). Minor differences were observed in the 5% ethanol intake over time (Figure 4). A time-dependent increase in ethanol preference was also observed in all adolescent rats towards the end of the 5-week drinking period (Figure 5). There were no differences observed in ethanol intake or ethanol preference between the groups.
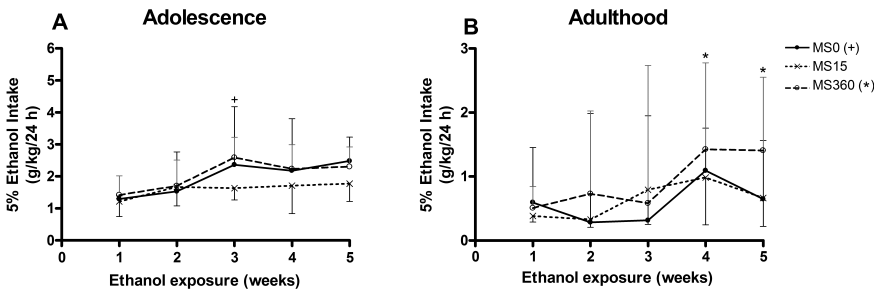
Table 2. The voluntary consumption of ethanol, i.e. the combined intake of 5 and 20% ethanol (g absolute ethanol/kg body weight/24 h), in rats exposed to 0 min (MS0), 15 min (MS15) or 360 min (MS360) of maternal separation. Ethanol access was provided either during adolescence (A) or adulthood (B).

Rearing environment	Week 1	Week 2	Week 3	Week 4	Week 5
(A) Adolescence					
MS0	4.69±2.90[+++]	5.85±2.92[+++]	6.24±3.23*[+++]	5.76±2.57[+++]	5.02±2.55[+++]
MS15	4.06±4.23[+++]	4.92±2.21[+++]	4.84±2.89[+++]	3.73±2.20*[+++]	3.82±1.22[+++]
MS360	5.02±2.93[+++]	5.64±2.75[+++]	6.30±3.58[+++]	4.97±1.86*[+++]	4.27±1.24*[++]
(B) Adulthood					
MS0	1.97±1.14	1.71±2.27	2.08±1.83	2.58±2.35	1.74±1.79
MS15	1.64±1.11	1.75±2.42	1.73±1.59	1.84±1.85	1.83±1.49
MS360	1.69±0.58	1.90±1.63	1.62±2.36	2.40±2.14*	2.72±1.67*

*The values show the weekly ethanol intake expressed as median ± interquartile range (IQR). *$p \leq 0.05$ as compared to the first week of ethanol consumption. [++]$p < 0.01$, [+++]$p < 0.001$ as compared to the ethanol consumption in adult rats the corresponding week.*

Figure 4. The weekly consumption of 5% ethanol (g absolute ethanol/kg body weight/24h) in rats exposed to 0 min (MS0), 15 min (MS15) or 360 min (MS360) of maternal separation. Ethanol access was provided either during adolescence (A) or adulthood (B). Data is expressed as median ± interquartile range (IQR). MS0: + p < 0.05 as compared to week 1. MS360: * p < 0.05 as compared to week one.

Effects of MS on adult voluntary ethanol consumption

The adult MS0 and MS15 rats maintained their total ethanol intake at a similar level over time (Table 2). On the other hand, the MS360 rats consumed more ethanol over time. In comparison to week one, they had higher total intake during weeks four and five. Analysis of the consumption of 5% and 20% ethanol, respectively, also revealed that the MS360 rats had a more pronounced increase in consumption of the 5% ethanol (Figure 4), than the

other groups and they also enhanced their ethanol preference (Figure 5) during the five-week drinking period. The last week the median ethanol preference was 35% in MS0, 40% in MS15 and 70% in MS360 rats.
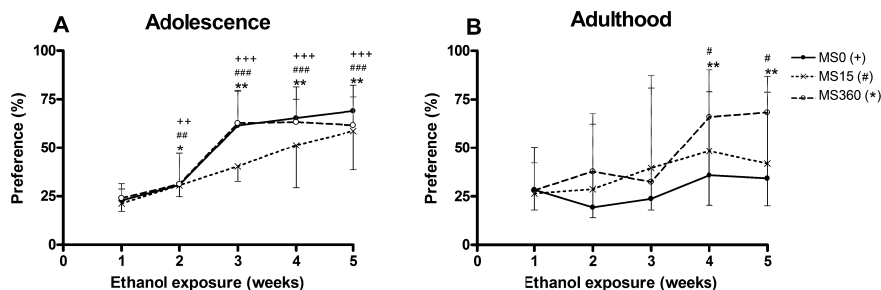

Figure 5. The weekly ethanol preference (%), calculated as the percentage of the combined intake of 5 and 20% ethanol in relation to the total fluid intake, in rats exposed to 0 min (MS0), 15 min (MS15) or 360 min (MS360) M of maternal separation. Ethanol intake was provided either during adolescence (A) or adulthood (B).

*Data is expressed as median ± interquartile range (IQR). MS0: ++ p < 0.01, +++ p < 0.001 as compared to week 1. MS15: # p ≤ 0.05, ## p < 0.01, ### p < 0.001. MS360: * p < 0.05, ** p < 0.01 as compared to week one.*

Comparisons of adolescent and adult voluntary ethanol consumption

The median intake in the adult rats was 1.5-2 g/kg/24h the first week but there was a large individual variation in ethanol intake within the groups with a consumption ranging from 1 to 4.5 g/kg/24h. The adolescent MS0, MS15, and MS360 rats initiated a high voluntary ethanol intake of 5-6 g/kg/24h and consumed more ethanol throughout the ethanol-drinking period as compared to rats given the same free choice in adulthood. Interestingly, initially the adolescent MS360 rats had higher 5% ethanol intake than adult MS360 rats, but during week 4 and 5, the adult MS360 rats increased their intake to the level initially seen in the adolescent rats, Figure 4. The same pattern was seen in consumption of 20% ethanol. Throughout the study, significantly higher intake of 20% ethanol was seen in adolescent MS0, MS15, and MS360 rats compared to the corresponding adult MS0, MS15, and MS360 rats, respectively. Moreover, significantly higher 5% ethanol intake was seen in adolescent MS0 and MS15 rats compared to the corresponding adult rats. However, the ethanol preference in the adolescent MS0, MS15, and MS360 rats was similar to adult rats in the corresponding groups throughout the study, Figure 5.

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Results Paper II

Wistar rat pups were separated from their mother 15 min (MS15), or 360 min (MS360) daily during first three weeks after birth. The ethanol access was a two-bottle free choice paradigm with 24 h intermittent access to 20% ethanol on Mondays, Wednesdays and Fridays. Sessions were initiated on PND 34 and lasted until the animals were 20 weeks old.

Effects of MS on voluntary adolescent ethanol consumption

The ethanol intake was similar in the MS15 and MS360 groups at all time points. In addition, no ethanol deprivation effects were found after any of the two deprivation periods. Both the MS15 and MS360 rats increased their preference significantly over time but there was no difference in ethanol preference between the MS groups.

Interaction effects of MS and adolescent ethanol intake on peptide levels

Ethanol-induced effects on peptides

An ethanol-induced increase in ir dynorphin B levels was found in the hippocampus and in the ir MEAP levels in the amygdala, Figure 6. In the hypothalamus there was a trend ($p=0.055$) to an ethanol-induced effect on ir beta-endorphin, with lower ir beta-endorphin levels in ethanol-drinking compared to water-drinking animals.
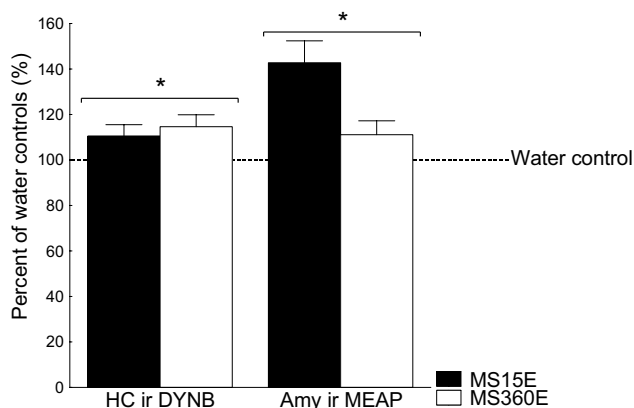
Figure 6. Ethanol-induced effects on peptide levels in the hippocampus (HC) and amygdala (Amy). The bars show the levels as percentage of the levels in the respective water-drinking control rats. Ir = immunoreactive, DYNB = dynorphin B, MEAP = Met-enkephalin-Arg^{6}Phe^7, MS15 = maternal separation 15 min, MS360 = maternal separation 360 min, E = ethanol. * $p < 0.05$ compared to water-drinking controls.

Effect of rearing condition on peptides

An overall effect of rearing condition was detected in ir beta-endorphin levels. The MS360 rats had lower levels of ir beta-endorphin than the MS15 rats, both in the pituitary gland and in the PAG, Figure 7.
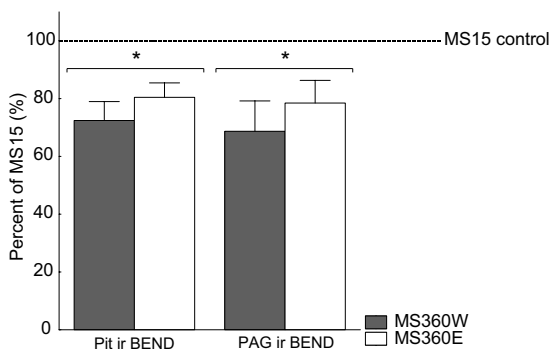
Figure 7. Rearing induced effects on beta-endorphin (BEND) levels. The bars show the levels in rats subjected to maternal separation for 360 min (MS360) as percentage of the levels in rats from the respective 15 min maternal separation (MS15) groups. ir = immunoreactive, E = ethanol, W = water, PAG = periaqueductal grey, Pit = pituitary. * $p < 0.05$ compared to MS15.

Analysis of basal differences in opioid peptides

The levels of opioids were also measured in water-drinking MS15 and MS360 rats and compared with water-drinking AFR rats to assess whether

the basal levels differed in adult rats after being first subjected to either MS15 or MS360 and then single-housed during adolescence.

Table 3. Mean ir dynorphin B (DYNB), Met-enkephalin-Arg[6]Phe[7](MEAP) and beta-endorphin (BEND) levels (fmol/mg tissue) ± SEM.

	AFR	MS15	MS360
ir DYNB			
AL	24.0 ± 5.2	36.0 ± 5.7	31.3 ± 3.7
NIL	392 ± 43	572 ± 66	442 ± 64
HT	28.8 ± 2.6	41.7 ± 3.5*	36.6 ± 3.5
FCx	2.41 ± 0.2	3.15 ± 0.3*	3.66 ± 0.2*
MPFCx	1.61 ± 0.1	2.65 ± 0.3*	2.58 ± 0.3*
NAc	26.0 ± 3.1	23.0 ± 3.3	28.1 ± 4.0
Str	11.5 ± 1.1	10.8 ± 0.8	11.5 ± 0.6
HC	11.9 ± 1.0	10.7 ± 1.0	11.1 ± 0.9
Amy	5.52 ± 0.6	8.12 ± 0.9*	9.10 ± 0.8*
SN	38.0 ± 4.1	48.6 ± 5.3	49.8 ± 5.9
VTA	3.60 ± 0.4	6.81 ± 0.7	8.62 ± 2.5
PAG	6.58 ± 0.9	6.82 ± 0.8	5.98 ± 0.7
ir MEAP			
AL	1.17 ± 0.1	1.62 ± 0.6	1.66 ± 0.8
NIL	2.62 ± 0.2	1.48 ± 0.2*	2.11 ± 0.4
HT	84.6 ± 8.6	73.4 ± 7.9	65.8 ± 6.9
FCx	4.86 ± 0.8	4.18 ± 0.4	5.24 ± 0.4
MPFCx	2.80 ± 0.4	5.79 ± 1.2*	5.05 ± 0.6*
NAc	70.4 ± 8.2	68.3 ± 7.6	64.1 ± 6.2
Str	74.2 ± 5.0	52.8 ± 5.4*	53.6 ± 7.8*
HC	7.07 ± 0.6	5.81 ± 0.8	5.69 ± 0.5
Amy	17.6 ± 2.0	24.5 ± 3.4	29.7 ± 3.3
SN	2.87 ± 0.4	6.55 ± 1.1*	6.52 ± 0.7*
VTA	11.6 ± 1.2	15.4 ± 2.0	13.7 ± 2.5
PAG	22.6 ± 3.3	23.4 ± 4.7	21.3 ± 2.9
ir BEND			
AL	8051 ± 666	8163 ± 727	8979 ± 693
NIL	20641 ± 3196	20824 ± 4734	9024 ± 1817
Pit	11839 ± 896	12332 ± 1445	8930 ± 585
HT	35.3 ± 2.5	42.5 ± 6.0	37.4 ± 2.7
NAc	13.0 ± 5.5	11.3 ± 4.4	13.3 ± 7.0
Amy	5.61 ± 1.3	5.67 ± 2.0	4.44 ± 0.5
PAG	16.1 ± 0.9	16.6 ± 1.9	11.4 ± 1.2*[§]

*AFR = animal facility reared rats, MS15 = maternal separation 15 min, MS360 = maternal separation 360 min, AL = anterior lobe of the pituitary, NIL = neurointermediate lobe of the pituitary, HT = hypothalamus, FCx = frontal cortex, MPFCx = medial prefrontal cortex, NAc = nucleus accumbens, Str = dorsal striatum, HC = hippocampus, Amy = amygdala, SN = substantia nigra, VTA = ventral tegmental area, PAG = periaqueductal gray area. * $p < 0.05$ compared to AFR, [§] $p < 0.05$ compared to MS15 (one-way ANOVA followed by Fisher's LSD test).*

The ir peptide levels are shown in Table 3. The MS15 and MS360 had higher levels or ir dynorphin B than the AFR rats in the amygdala, frontal cortex and MPFCx. In the hypothalamus the MS15 rats had higher levels than the AFR rats.

The MS15 and MS360 rats had higher levels of ir MEAP in the MPFCx and SN than the AFR rats. In the Str, the MS15 and MS360 rats had lower levels and in the NIL, the MS15 had lower levels than the AFR rats.
The MS360 rats had lower ir beta-endorphin levels in the PAG than the AFR and MS15 rats.

Discussion

One of the main findings in Paper I and II is that the consequences of early-life rearing conditions upon ethanol intake differ depending on when access to ethanol is given. The adolescent and adult rats subjected to MS0, MS15 or MS360 in Paper I were assessed in one and the same MS study giving the advantage of minimal influencing factors aside from MS. In Paper II the adolescent drinking in MS15 and MS360 rats was investigated further with a slightly different ethanol intake paradigm and the results from Paper I were confirmed.

A main and new finding was that in adolescent rats, the ethanol consumption and preference was not influenced by the early rearing conditions. Two studies using rats from different suppliers and two different intermittent access models describe the same outcome. Rats subjected to MS0, MS15 or MS360 and then given free choice between 5% ethanol, 20% ethanol and water during adolescence have no differences in ethanol intake or preference and, similarly, when adolescent MS15 and MS360 rats are given access to 20% ethanol and water they have the same consumption patterns.

The data on ethanol intake in adulthood in our study confirm previous reports of MS-induced effects on adult ethanol consumption using continuous paradigms. These studies have provided evidence for higher voluntary ethanol consumption after prolonged periods of MS, for example, 60 min (Hilakivi-Clarke et al. 1991), 180 min (Huot et al. 2001; Jaworski et al. 2005) and 360 min (Ploj et al. 2003a) as compared to short periods of MS.

However, one critique of continuous access is the generally low ethanol intake. Intermittent access paradigms have been shown to increase voluntary ethanol intake in laboratory rats (Sinclair and Senter 1967; Wise 1973). The present study confirms that adult rats establish higher voluntary intake and higher ethanol preference using the intermittent access paradigm when compared to Wistar rats from the same supplier given continuous ethanol access (Gustafsson and Nylander 2006; Oreland et al. 2011; Ploj et al. 2003a). Furthermore, the results show that the MS360 rats have a higher propensity for high ethanol intake and ethanol preference also with the use of intermittent access of ethanol. The compiled results thus strongly support the notion that

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MS360 constitutes a risk environment and contribute to adult high ethanol consumption.

The intermittent access ethanol paradigm used in the comparison of the drinking behaviour between adolescent and adult rats confirms data from previous studies using continuous (Doremus et al. 2005; Garcia-Burgos et al. 2009; Hargreaves et al. 2009; Vetter et al. 2007) and limited access (Walker et al. 2008) models showing that adolescent rats have higher ethanol intake than adult rats (Doremus et al. 2005; Garcia-Burgos et al. 2009; Hargreaves et al. 2009; Truxell et al. 2007; Vetter et al. 2007). There are several possible reasons for these age-dependent differences. The behavioural response differs; adolescence is for instance related to risk-taking, impulsivity, novelty seeking and increased social interactions (Chambers et al. 2003; Kelley et al. 2004; Spear 2000). Puberty comprises extensive endocrinological changes and, furthermore, adolescence is characterized by a number of neuronal maturation processes, including cortical development, with changes in plasticity, neurotransmission and neurogenesis (Crews et al. 2007; He and Crews 2007; Monti et al. 2005). Reward networks continue to develop (Spear 2000) and these neurobiological changes may result in altered sensitivity to ethanol (Matthews et al. 2008; Pian et al. 2008; Silveri and Spear 1998) that may affect ethanol consumption.

The main finding with regard to ethanol-induced effects on endogenous opioids was that ethanol drinking in adolescent rats affected levels in hippocampus and amygdala and that these effects were similar in rats exposed to different early-life environmental conditions. There is very little information in the literature about the effects of adolescent voluntary drinking, and ethanol-induced effects in adolescent rats that have been subjected to different early-life environmental settings have not previously been described. Therefore it is interesting to note differences in the hippocampus and the amygdala. These areas are related to learning and memory, emotional processing, addiction processes and regulation of activity in the HPA axis. Previous studies have shown that dynorphin injections into the hippocampus impair spatial learning in rats and because ethanol also impairs spatial learning it is possible that the two are connected through an ethanol-induced increase of dynorphin (Sandin et al. 1998). Altered dynorphin levels in the hippocampus has also been suggested to contribute to cognitive decline (Gallagher and Nicolle 1993) and involved in cognitive impairments seen in Alzheimers disease (Cai and Ratka 2012) and the present finding of ethanol-induced effects on the young brain is of interest to study further.

Moreover, ethanol drinking also induced higher ir MEAP levels in the amygdala. A previous study in adult rats following MS showed differences in ir MEAP levels depending on rearing conditions; the MS360 rats had lower basal ir MEAP levels in the amygdala than MS15 rats (Gustafsson et al. 2008). Furthermore, ethanol drinking in adulthood increased the ir MEAP levels in both MS15 and MS360 rats (Gustafsson et al. 2007). These results

are in line with our results showing that ir MEAP in amygdala is highly sensitive to adolescent ethanol exposure. Taken together, ethanol exposure increases ir MEAP levels in amygdala independent of early rearing condition and independent on the onset of drinking.

Levels of ir beta-endorphin in the PAG and the pituitary were found to differ between MS15 and MS360 groups, but MS had no effect in ir dynorphin B and MEAP levels in these regions. This is in contrast to previous studies using the MS model, where several differences in ir dynorphin B and MEAP levels have been found in adulthood when comparing the two MS groups (Gustafsson et al. 2008; Gustafsson et al. 2007; Ploj et al. 2003b). However, in this study the animals were individually housed and given access to ethanol at an early age and throughout adolescence, introducing two factors not previously studied. It is possible that the individual housing had a larger impact on opioid levels and therefore masked the effects of early rearing environment. This is in line with studies showing the importance of group housing and a social environment during adolescence (Trezza et al. 2010). Previously we have seen differences emerge in reward circuits in adulthood as a result of different early-life conditions whereas it was shown that MS360 rats differed from MS15 rats in areas related to stress regulation immediately after MS (Gustafsson et al. 2008). These results indicate that the development of opioid networks during adolescence in MS15 and MS360, respectively, also is an important factor for the differences in ethanol consumption to emerge. The results in the present study further indicate that single housing during adolescence may affect opioid development and attenuate the differences between MS15 and MS360 rats.

Efficacy of naltrexone in ethanol-drinking rats subjected to MS

The opioid receptor antagonist NTX is currently used in the treatment of AUD. However, substantial individual differences have been reported for the efficacy of NTX. Genetic factors are known to contribute to these differences, but little is known about the impact of early environmental influences. Since previous studies have provided evidence that endogenous opioids are targeted by early-life environmental conditions the question arose whether the opioid antagonist NTX would induce different effects in rats subjected to MS0, MS15 and MS360.

Results Paper III

Wistar rat pups were separated from their mother 0 min (MS0), 15 min (MS15), or 360 min (MS360) daily during first three weeks after birth. After

6 weeks of voluntary drinking, initiated at PND 26, the rats were treated with saline or NTX, 0.3 mg/kg or 3.0 mg/kg, respectively.. In the MS0 rats the ethanol consumption was reduced during the first 30 min of ethanol intake after treatment with both 0.3 and 3.0 mg/kg NTX compared to saline, Figure 8A. Additionally, 2 h after ethanol access, the ethanol consumption in the MS0 rats was reduced after both NTX dosages, Figure 8B.

In the MS360 rats, the low dose of NTX did not induce statistically significant changes in ethanol consumption during the first 30 min of ethanol intake compared to saline, but a decreased intake was seen after 3.0 mg/kg NTX, as shown in Figure 8A. The decrease was more pronounced after 3.0 mg/kg than 0.3 mg/kg. Two hours after access to ethanol, both NTX doses caused reduced ethanol consumption compared to saline (Figure 8B).

The analysis of the NTX-induced effects in the entire population of rats, that is MS0, MS15 and MS360, revealed decreases in ethanol intake after the NTX administration, which were significant at 30 min and at 2 h after ethanol access, i.e., 1 h and 2.5 h, respectively, after the injection, Figure 8. A further analysis was performed in the respective experimental groups. A decrease in total ethanol consumption was observed after NTX compared to saline injections in MS0 and MS360 rats, whereas NTX treatment had no effect in the MS15 group, Figure 8A. The only significant difference between the MS0 and MS360 groups was found at 30 min after ethanol exposure; the MS0, but not the MS360, group had reduced ethanol consumption following 0.3 mg/kg NTX.

The MS15 rats did not reduce their total ethanol intake following NTX treatment. On the other hand, they reduced their intake of 5% ethanol and increased their preference for 20% ethanol following NTX administration, Figure 9.
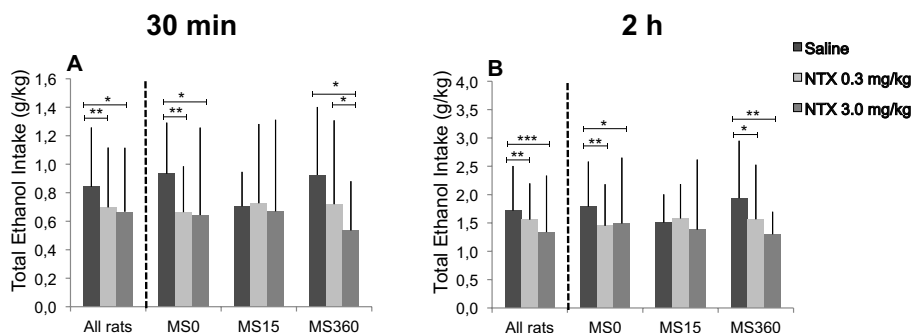
Figure 8. The total ethanol consumption, i.e., the combined intake of 5% and 20% ethanol (g absolute ethanol/kg body weight), after treatment with saline or naltrexone, 0.3 mg/kg or 3.0 mg/kg, respectively. On the left side of each figure, the naltrexone-induced effects in all rats are shown. On the right side of each figure, the specific effects in rats exposed to one of three rearing conditions are shown. The injections were made 30 min prior to ethanol access and the ethanol intake was measured at 30 min (A) and 2 h (B) after ethanol access. The data are shown as median ± interquartile range (IQR). MS = maternal separation, NTX = naltrexone. *$p ≤ 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Wilcoxon matched pairs test).
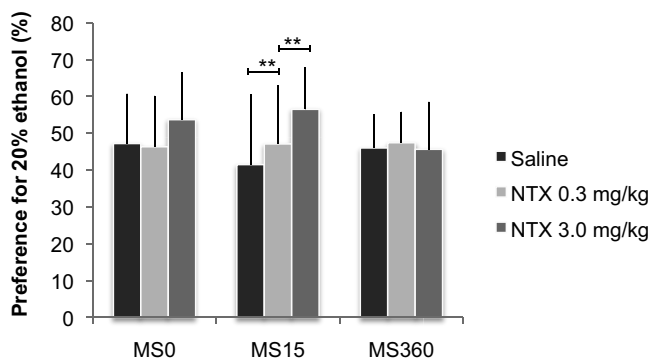
Figure 9. The preference (%) for 20% ethanol in relation to the total ethanol consumption (5% + 20% ethanol) after 2 h drinking in rats injected with saline or naltrexone, 0.3 mg/kg or 3.0 mg/kg, respectively. The injections were made 30 min prior to ethanol access. The data are shown as median ± interquartile range (IQR). **$p < 0.01$. (Wilcoxon matched pairs test).

Discussion

The primary and novel finding in the present study is that early environmental experiences determine the ability of NTX to reduce ethanol consumption in adult rats.

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Significant differences were observed in the efficacy of NTX to reduce ethanol intake in rats subjected to MS0 and MS360. The total ethanol consumption in the MS15 rats, which were exposed to the most natural and proposed beneficial early environment, was essentially unaffected by NTX. These findings suggest that individuals with a history of favourable rearing conditions will not benefit from treatment with NTX. In the MS15 animals, the intake of 5% ethanol decreased, whereas the preference for 20% ethanol increased with the higher dose of NTX. Interestingly, these results were consistent with the increased ethanol intake after NTX that was described in individuals with no family history of AUD (Krishnan-Sarin et al. 2007). In contrast to MS15 rats, the MS360 rats responded well to NTX. Naltrexone treatment clearly reduced ethanol intake and this was dose dependent at the concentrations studied. Previous studies have shown that rats subjected to MS360 have altered risk-assessment and risk-taking behaviour and a blunted stress-induced corticosterone response in adulthood (Roman et al. 2006). Furthermore, adult MS360 rats drink more ethanol (Roman and Nylander 2005) and prefer higher ethanol concentrations (Gustafsson and Nylander 2006). These findings suggest that MS360 conditions generate an environmentally-induced addiction-prone phenotype. The high efficacy of NTX in animals reared in a risk environment is of interest when comparing to human studies. Clinical reports have described better responses to NTX in certain subgroups of patients, such as risk-taking males, individual characterised by novelty-seeking and impaired impulse control, individuals with a family history of AUD, and early onset AUD (Gianoulakis 2004; Kiefer et al. 2008; King et al. 1997; Krishnan-Sarin et al. 2007). Thus, the present results indicate similarities between individuals with an inherent risk and animals exposed to environmental risk; they both respond better to NTX.

These results provide new insight in the controversy concerning the efficacy of NTX in clinical practice. Several studies have described genetic influence on the efficacy of NTX (Mague and Blendy 2010; Monterosso et al. 2001; Oslin et al. 2003) and highlighted the importance of identifying predictors for a robust response to NTX (Ray et al. 2010; Rubio et al. 2005; Tidey et al. 2008). In this study, we show the importance of considering not only genetic factors but also early environmental factors when identifying subgroups of AUD patients that respond well to NTX treatment.

Effects of MS on behavioural profiles in the offspring

Maternal separation is known to exert long-term effects on behaviour but most behavioural studies following MS are performed on adult Wistar rats (Kaneko et al. 1994; Ploj et al. 2002; Roman et al. 2006; Wigger and Neumann 1999) and few have investigated the behaviour of young Wistar rats following MS (Farkas et al. 2009; Marin and Planeta 2004; Ploj et al.

2002). Information about the MS-induced effects on behaviour during adolescence, and how behaviour develops over time, could provide important clues for the high ethanol drinking behaviour seen in MS15 rats during adolescence, but not during adulthood. Since traditional tests usually provide information related to a particular mental condition, e.g., anxiety-like behaviour, we here used the MCSF test that instead gives the opportunity for different environmental settings in a single test and may therefore show essential features related to a broader behavioural repertoire.

Results Paper II

Impact of rearing conditions on behaviour in young and adult rats
The trend analysis revealed an increase in risk-assessment behaviour with age in the MS15 group and a decrease in the MS360 group, showing an interaction effect between age and rearing condition in this functional category, Figure 10A. The trend analysis also showed that the young MS360 rats displayed more risk-assessment behaviour compared to the young MS15 and AFR rats, Figure 10A. Furthermore, the young MS360 rats displayed less shelter-seeking behaviour compared to the young AFR rats with a similar differences observed in the adult rats, Figure 10B.
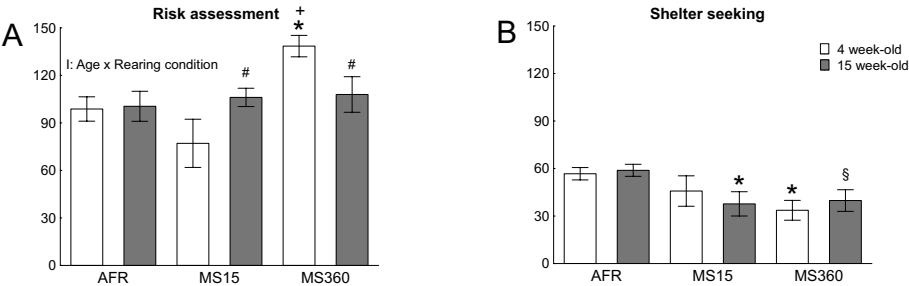
Figure 10. Trend analysis of the two MCSF tests, at 4 and 15 weeks of age respectively, in water-drinking animals from the three rearing groups: animal facility reared (AFR), maternal separation 15 min (MS15) and maternal separation 360 min (MS360). The graphs show A) risk-assessment and B) shelter seeking behaviours. [§] $p < 0.06$, * $p < 0.05$, compared to same age AFR, [+] $p < 0.05$ compared to same age MS15, [#] $p < 0.05$ compared to same rearing group at 4 weeks of age (repeated measures ANOVA followed by Fisher's LSD test).

Discussion

MS altered risk-assessment behaviour and shelter seeking behaviour at 4 weeks of age. The MS360 rats displayed higher risk-assessment than the MS15 rats. Furthermore, the young MS360 rats showed less shelter seeking behaviour than the young AFR rats. A similar trend was seen for the adult MS360 rats, i.e. less shelter seeking behaviour than the adult AFR rats. Moreover, the MS360 and AFR rats were more risk-taking over time, although these differences were not statistically significant. In a study investigating the behaviour of young Wistar rats after MS no differences were detected between MS rats and control groups in the OF test (Farkas et al. 2009) and this is in agreement with the current study, where there are no differences in the parameters that can be compared to OF parameters, i.e. the centre and central circle. In a study using the EPM the young MS360 rats had a lower number of open arm entries compared to AFR rats (Ploj et al. 2002). Comparable results were not observed in the current study, which may be explained by the fact that the design of the bridge is quite different from an open arm. However, the MCSF gives information about other types of risk-assessment and risk-taking behaviours and since differences were found in the risk-assessment measures, this supports the use of a more nuanced test, like the MCSF test.

The repeated testing showed that the AFR rats did not change their behaviour over time. With repeated testing in adulthood, a general finding is that rats decrease general activity and risk-taking behaviour, as shown by previous studies (Meyerson et al. 2006; Roman et al. 2007). On the other hand, when testing rats from adolescence to adulthood, but using different batches of animals for each age, activity in the OF and open arm exploration in the EPM increases with age (Lynn and Brown 2010). Herein, contrasting effects were observed between the MS15 and MS360 rats. The risk-assessment behaviour increased in the MS15 rats whereas it decreased in the MS360 rats over time, which shows that early-life conditions have consequences for the behavioural development. In fact, in the second behavioural test the groups were more similar to each other than in adolescence with the exception of shelter seeking, which was lower in MS15 and MS360 relative to AFR rats. It is possible that the effects of the MS360 procedure on adolescent behaviour are fading over time, but we assume that this is more likely due to single housing during adolescence since we have previously observed altered risk-assessment and risk-taking behaviour in adult MS360 rats (Roman et al. 2006).

Effects of MS on pup-retrieval strategies during MS and behaviour profiles of dams after weaning

The consequences of being exposed to MS are commonly assessed in the offspring. The MS-induced effects may be a consequence of maternal absence but may, at least in part, be mediated by altered maternal behaviour that in turn affects the pups. However, fewer studies address the consequences of repeated pup removal in the dam. It is therefore of interest to further study the consequences of dam-pup separations in the dam.

Results Paper IV

MS-induced effects on post weaning behaviour in dams

The results from the 20-min trial in the MCSF test revealed differences in risk-assessment behaviour between MS15, MS360, and AFR dams. The results in the trend analysis show that MS15 dams were found to be significantly less explorative than AFR dams, Figure 11. Furthermore, the MS15 dams showed significantly less risk-assessment behaviour compared to the MS360 dams. General activity, risk-taking and shelter seeking did not differ between the groups.
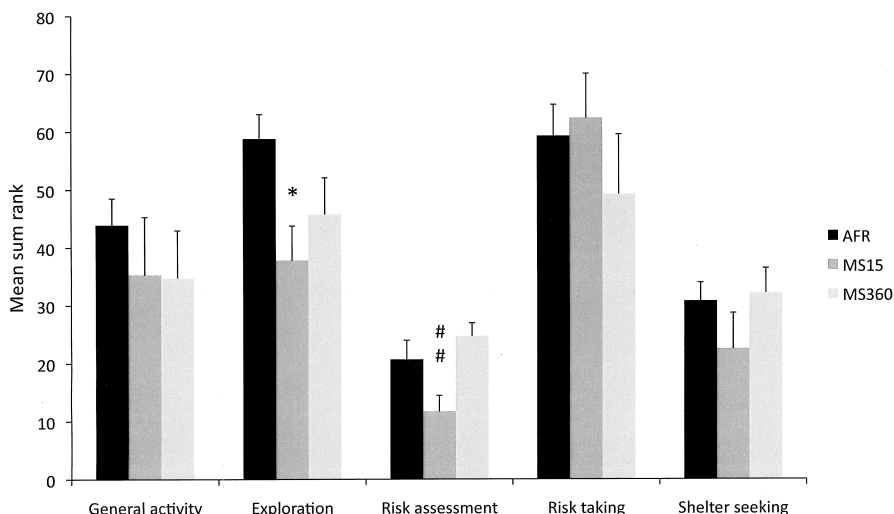
Figure 11. The trend analysis in AFR, MS15 and MS360 dams. * $p < 0.05$ compared to AFR dams; ## $p < 0.01$ compared to MS360 dams (Kruskal-Wallis, Mann–Whitney U-test).

Results Paper V

Pup-retrieval strategies during MS

The results from the first and second pup-retrieval tests are shown in Figure 12. In the first retrieval test, at PND 3, the MS360 dams had a shorter latency in first visiting the bridge compared to MS0 dams (Figure 12A-B). Large differences were observed in the individual retrieval strategies, both within the MS0 (Figure 12A) and the MS360 (Figure 12B) group. Both MS0 and MS360 dams had similar strategies in their pup-retrieval pattern from the bridge. In the second retrieval test at PND 5, there was a positive correlation between latency in first visiting the bridge and latency in first check of the pups in the MS0 rats, which was not seen for the MS360 rats.

Over time, there were differences in parameters of relevance for retrieval behaviour in the MS0 group. MS0 dams spent significantly shorter time and shorter time per visit on the bridge in the second relative to the first pup-retrieval test. In the second retrieval, the MS0 dams had a significantly shorter latency to the bridge and to the first check of the pups compared to first retrieval test (Figure 12A and C).

The MS0 dams that systematically retrieved the pups into the DCR also had shorter retrieval latencies in the second test while the dams that retrieved no pups in the first trial expressed the same behaviour in the second trial. In addition, the total amount of MS0 pups retrieved from bridge was almost the same (16 and 17 out of 28 pups) in both retrieval trials. On the other hand, the MS360 dams had a somewhat different pattern in the second trial; the systematic retrieval into the DCR was slower in two dams and more pups (13 out of 28 pups) were left on bridge compared to first trial (10 out of 28 pups).
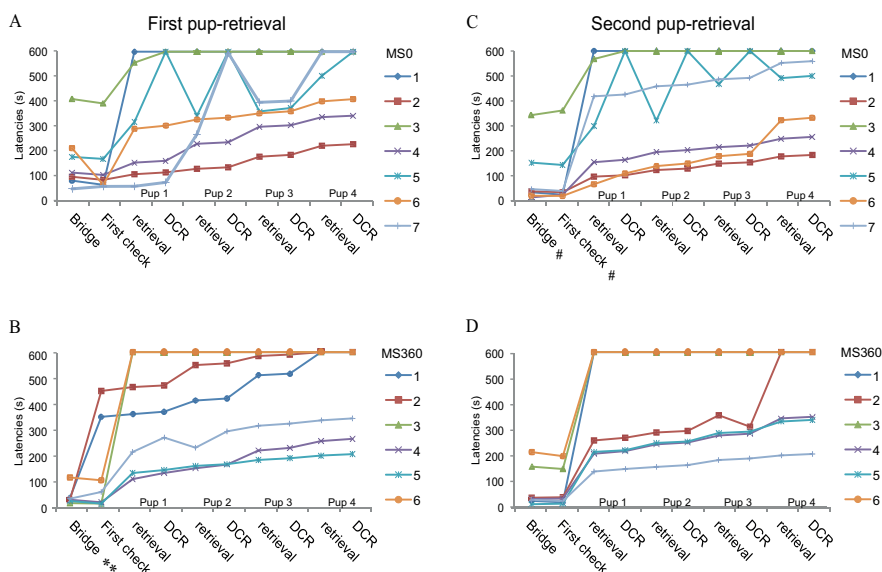
Figure 12. Individual pup-retrieval strategies in the first and second retrieval tests in the MS0 (A and C, respectively) and MS360 (B and D, respectively) dams, in the MCSF test. The figure depicts the latencies in first visiting the risk area (bridge), first check of the pups on the bridge, and to retrieve pup 1 – 4 from the bridge. The test session was 600 s and the latency is therefore set to 600 whenever the dams failed to retrieve a pup. ** $p < 0.01$ comparing MS360 and MS0 dams (Mann–Whitney U-test). # $p < 0.05$ compared to first pup-retrieval test (PND 3) (Wilcoxon signed rank test).

Discussion

The impact of different MS procedures on behaviour in dams during MS and after weaning are scarcely investigated and only few studies address the consequences of repeated pup removal in the dam (Boccia et al. 2007; Eklund et al. 2009; Kalinichev et al. 2000; Maniam and Morris 2010). In this thesis, we have focused on litter-wise MS360 to simulate an adverse environment for the offspring, but it is not well known how this environment affects the dam.

The study of the behavioural profiles after weaning shows increased risk-assessment behaviour in the MS360 dams. The risk-assessment behaviour is influenced by an exploratory drive and implies risk/benefit assessments (Blanchard and Blanchard 1988; Lima and Dill 1990). Risk-assessment and risk-taking behaviours are central traits in the behavioural repertoire of the rat. Impaired risk-assessment has some relevance for aspects of impulsive-like behaviour and can be a serious disadvantage to survival, but an over-expressed risk-assessment can also be detrimental (Lima and Dill 1990).

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Plausibly the higher risk-assessment behaviour seen in the MS360 dams immediately after weaning may have been, at least in part, transferred to the offspring since higher risk-assessment behaviour were also seen in male MS360 offspring after weaning (Paper II). Using the present MS-protocol, long-term neurochemical alterations (Nylander and Roman 2012) and effects on voluntary ethanol intake (Roman and Nylander 2005) and behaviour (Roman et al. 2006) following MS have been reported in male offspring while less or no effects were found in female offspring (Gustafsson et al. 2005; Roman et al. 2005; Roman et al. 2004). If the maternal behaviour were mediating the effects observed after MS, alone, one would expect similar outcomes in males and females. The effects observed in offspring subjected to MS may therefore be mediated, only in part, by transmission of information from the dam to her offspring (Macri and Wurbel 2006). However, the hypothesis that the maternal behaviour is mediating the effects observed after different environmental rearing conditions has already been questioned. Accumulating evidence indicates that many of the effects observed after different postnatal manipulations cannot be accounted for via altered maternal care (Millstein and Holmes 2007; Pryce and Feldon 2003).

In the retrieval study, there were two evident strategies in transposing the pups in the arena; either removing the pups out of potential danger (the bridge), which was more pronounced in the control group, or into safety (the DCR), which was more pronounced in the MS360 group. The MS360 dams did not change their strategies over time and left more pups on the risk area in both pup-retrieval tests, whereas the controls changed their strategies over time by being faster in retrieval of the pups in the second test. This implies different pup-retrieval strategies over time, depending on early-life conditions.

An advantage with this study is the assessment of retrieval strategies over time by repeated testing. Moreover, the USVs are important in dam-pup interactions and in retrieval behaviour as lactating dams will orient themselves toward neonatal ultrasounds and initiate retrieval of the pups (Allin and Banks 1972) and measures were therefore included in the study. Since our observations from the USV test on PND 4 show no difference between the groups, it may explain the similar behaviour expressed by both group of dams in reaching the pups during first retrieval test.

In the second retrieval test, the control dams were faster in reaching the bridge and had shorter latencies to first check of the pups and these latencies were positively correlated. This behaviour together with the shorter duration and duration per visit spent on the bridge during the second relative to first retrieval test points to a higher motivation in control dams to reach the pups and to remove them from the risk area. The higher USVs emitted by the control pups on PND 6 compared to PND 4 could have influenced this behaviour of the dams. Likewise, the unchanged latency to first check of the

pups by the MS360 dams in the second trial may relate to the unchanged USVs by the MS360 pups.

The pup-dam interactions in response to environmental disturbances is complex and partially under genetic control, and so far not fully understood. Approximately half of the number of dams within each experimental group had a fast systematic retrieval into the DCR. This may be explained by the general natural variation seen in maternal care (Champagne et al. 2003; Liu et al. 1997). The maternal behaviour may depend on the different repeated disturbances in maternal contact with the litter, i.e., the MS procedures, but further studies are necessary for a better understanding of how these processes relate to the MS paradigm, to the dam's behaviour after weaning, and the associated offspring phenotype.

General discussion

Environmental factors closely interact with genetic factors and may either add to or counteract the genetic influence and thus act as risk or protective factors. In this thesis, a rodent MS model was used to examine the impact of early-life environmental factors by manipulating early environmental conditions during the first postnatal weeks, and then examining the consequences at different ages later in life. The MS-induced effects on home-cage drinking in adult rats are generally consistent as evidenced by low adult voluntary ethanol intake following short periods of MS, whereas a higher intake has been observed after prolonged MS (Becker et al. 2011; Moffett et al. 2007; Roman and Nylander 2005).

In this thesis, we were able to confirm these results with the use of an intermittent ethanol access paradigm; the adult MS360 rats increased their voluntary ethanol intake and ethanol preference over time. A novel finding was that after early onset of ethanol drinking the voluntary consumption throughout adolescence was independent of previous rearing conditions. That is, the MS15 environment did not serve as a protective environment. An absence of a protective effect of MS15 has also been reported following individual MS, i.e. when the pup is separated from both dam and littermates (Oreland et al. 2011) and in female rats (Gustafsson et al. 2005; Roman et al. 2005; Roman et al. 2004). Taken together, differences between short and prolonged MS are only observed in adult male rats and in paradigms using litter-wise separations.

The adolescent rats had higher voluntary ethanol intake than the corresponding adult MS rats. The higher ethanol intake *per se* is not surprising and confirms results from previous studies (Doremus et al. 2005; Garcia-Burgos et al. 2009; Hargreaves et al. 2009; Vetter et al. 2007). A novel finding is that early environmental factors seem to be of minor importance in determining voluntary drinking in adolescent rats. Furthermore, it was shown that the ethanol-induced effects on opioid peptides were independent on previous rearing conditions. Higher ir dynorphin B levels in the hippocampus are of interest considering the involvement of dynorphins in learning and their putative role in cognitive deficits (Cai and Ratka 2012; Gallagher and Nicolle 1993). The finding of higher ir MEAP levels in the amygdala is in line with previous reports on the consequences of voluntary drinking in adult MS15 and MS360 rats (Gustafsson et al. 2007) and indicates that amygdala is an interesting brain area for further studies of adolescent ethanol

exposure. The previously described increased responses to ethanol in adult drinking MS360 rats as compared to MS15 rats in several brain areas (Gustafsson et al. 2007) were not seen after adolescent drinking. Furthermore, MS-induced effects on beta-endorphin were observed. The MS360 rats had lower ir beta-endorphin levels in the pituitary gland and PAG compared to the MS15 rats. Further studies on, for example, epigenetic modulation of opioid genes and gene expression of prohormones and receptors are warranted to elucidate the impact of adolescent ethanol drinking on endogenous opioids. Behavioural profiling after MS and before and after adolescent drinking showed distinct behavioural profiles. Young MS15 and MS360 rats differed significantly and changes in individual behaviour over time were observed that were dependent on MS condition but not affected by adolescent ethanol drinking. These new findings provide support for altered behavioural development in animals reared in different early-life environments.

Another interesting finding is that the response to naltrexone was dependent upon early rearing conditions. An adverse early environment was associated with a good response, i.e. a reduction in ethanol consumption, whereas the rats reared according to the protective environment, MS15, were unresponsive to naltrexone. The different effects of naltrexone could be explained by the enhanced ethanol-induced increase in MEAP in adult rats (Gustafsson et al. 2007). However, since adolescent drinking induced similar effects in MS15 and MS360 there may be alternative explanations, for example, MS-induced effects on opioid receptor function. Moreover, the behavioural differences between the adolescent MS15 and MS360 rat may indicate differences in their motivation to voluntarily drink ethanol and thereby different responses to naltrexone.

Control groups vary across MS studies (Jaworski et al. 2005; Lehmann and Feldon 2000; Pryce and Feldon 2003). AFR and NH rats are less suitable in studies on ethanol intake since the ethanol consumption in these rats is more similar to the separation group that mimics an aversive environment, i.e. MS180 or MS360 (Jaworski et al. 2005; Moffett et al. 2007; Roman and Nylander 2005). In this thesis, the MS0, MS15 or AFR groups have been used as control groups depending on the aim of each study. The MS0 rats were only briefly handled and used as controls for the experimental handling in Papers I, III and V. The MS0 rats in Paper I had an adult ethanol intake similar to that of the MS15 rats but different from MS360 rats. The MS15 group was used in all papers, except Paper V. The MS15 condition was considered the best choice as control for the MS360 condition in the thesis; the handling is the same, wild-life conditions include short periods of maternal absence and the difference in ethanol consumption between MS180/MS360 and MS15 is a robust finding (Becker et al. 2011; Holmes et al. 2005; Lehmann and Feldon 2000; Moffett et al. 2007; Nylander and Roman 2012; Pryce and Feldon 2003; Roman and Nylander 2005). However, AFR rats

were used in in paper II and IV since no previous information about behavioural profiles in adolescent rats and in dams after weaning was available.

The MCSF test revealed a lower risk-assessment and exploratory drive in MS15 dams relative to MS360 and AFR dams when tested just after weaning and support the notion that MS360 and AFR conditions are more similar to each other. The MCSF test was also shown to be useful for the assessment of pup-retrieval strategies. The pup-retrieval test revealed two main strategies in the MSCF arena; the dams that retrieved the pups from the risk area placed the pups either in a sheltered area or just away from the potential risk. However, these strategies were used by both MS15 and MS360 dams. Some differences were observed between MS15 and MS360 dams but with the small number of animals used these did not reach statistical significance. Thus, the question whether the MS-induced differences seen in the offspring are mediated by maternal care is still unanswered.

Taken together, the results in this thesis reveal new and valuable information about the use of animal models in studies on the impact of the early environment, including early-life manipulations of dam-pup interactions and adolescent ethanol drinking, on vulnerability for AUD. The results in this thesis shows that MS together with a combined neurobiological and behavioural approach to assess consequences in adulthood is useful for systematic studies of mechanisms underlying long-term effects of rearing environment, adolescent ethanol drinking and the interaction between these early-life factors.

Conclusions

- The present results confirm that the MS15 condition is protective whereas the MS360 setting is associated with risk with regard to the propensity for high ethanol intake in adulthood; adult MS15 rats display a low ethanol intake whereas adult MS360 rats increase their ethanol intake and preference over time. A novel finding is that the onset of ethanol drinking is critical for MS-induced effects on voluntary ethanol consumption. Access to ethanol during adolescence results in higher ethanol intake in all rats and no protective effect of MS15 is observed. These novel findings add to the previously known negative consequences of adolescent drinking.

- The results show that when free access to ethanol is given during adolescence, voluntary drinking elicits the same effects, i.e. higher dynorphin B in hippocampus and MEAP in amygdala, regardless of previous rearing conditions. The consequences of voluntary drinking and, in particular, adolescent drinking, on endogenous opioids are poorly described and here it was shown that opioids in the amygdala and hippocampus are interesting targets for future studies of the consequences of adolescent ethanol exposure.

- A novel finding is that the early-life environment affects later responses to naltrexone. Genetic factors are known to contribute to individual differences in the ability of naltrexone to reduce ethanol intake. Here, it is shown that ethanol-drinking rats with a history of adverse early environmental experiences responded well to naltrexone, whereas rats reared under simulated naturalistic conditions did not benefit from naltrexone treatment. These results highlight the importance of taking early environmental effects into account when assessing individual differences in treatment outcome.

- The MS-induced effects on behavioural profiles in young and adult rats revealed that the behaviour immediately following MS, and also the development of behavioural traits as assessed in adulthood, is dependent on rearing condition; MS360 rats displayed higher risk-assessment as compared to MS15 rats when measured just after weaning. When assessed again in adulthood a decreased risk-

assessment was observed in the MS360 rats whereas the MS15 rats displayed an increased risk-assessment. Ethanol consumption during adolescence had little impact on development of behavioural traits.

• Assessment of pup-retrieval strategies did not reveal direct evidence for or against the maternal impact on behaviour and neurobiology seen in offspring. The experiences during the separation period affected the behaviour in the dam after weaning. Taken together, the results provide new knowledge about dam-pup interactions during MS and also about effects of repeated MS on dam behavioural profiles following weaning.

Populärvetenskaplig sammanfattning

Alkoholmissbruk och -beroende orsakar stora problem i samhället och behovet av kunskap för att förebygga alkoholberoende samt förbättra behandlingen är stort. Sårbarheten för alkoholberoende bestäms av samverkan mellan genetiska faktorer och miljöfaktorer där miljön har störst inflytande då hjärnan utvecklas, dvs. under fostertiden och barn-/ungdomsperioden. Uppväxtmiljön kan utgöra såväl risk- som skyddsfaktor för alkoholberoende men de neurobiologiska mekanismerna bakom miljöfaktorernas inverkan är dock ofullständigt kända. Kroppsegna opioider studeras då de av flera orsaker är intressanta mål för uppväxtmiljön och även för individuella skillnader i alkoholintag och behandling av alkoholberoende. Kroppsegna opioider förmedlar belöningseffekter av såväl beroendeframkallande droger som naturliga belöningsstimuli och är mål för läkemedelsbehandling vid alkoholberoende. Det är dock inte klarlagt hur de medverkar i alkoholens verkningsmekanism.

Maternal separation är en djurexperimentell modell som utvecklats för att studera hur miljöfaktorer under uppväxten påverkar beteende och neurobiologi hos ungarna på kort sikt samt hos den vuxna individen på längre sikt. Den sociala interaktionen mellan mamma och ungar bryts genom olika långa separationer. Tidigare studier har visat att en lång daglig separation under de tre första levnadsveckorna simulerar en riskmiljö och resulterar i ett högt alkoholintag, medan en kort daglig separation resulterar i ett lågt frivilligt alkoholintag hos ungarna i vuxen ålder. En längre separation orsakar även långtidsförändringar i opioida peptidsystem och beteende, vilket kan vara en bidragande orsak till en hög alkoholkonsumtion i vuxen ålder.

Syftet med denna avhandling var att med hjälp av maternal separation studera konsekvenser av olika uppväxtmiljöer på tidigt alkoholintag, beteende och effekter av tidigt alkoholintag på beteendeutveckling, effekter av naltrexon på alkoholintag samt inverkan av mammans beteende för dessa miljöinducerade effekter.

Resultaten visar att det frivilliga alkoholintaget i vuxen ålder var beroende av uppväxtmiljön. Långtidsseparerade råttor ökade såväl alkoholintag som alkoholpreferens över tid vilket styrker att långtidsseparation i tidig ålder utgör en riskmiljö med avseende på alkoholintag i vuxen ålder. Alkoholintaget i unga individer var generellt högre jämfört med vuxna och konsumtionen var inte beroende av tidigare uppväxtmiljö vilket tyder på att

faktorer såsom hormonella, beteenderelaterade, neurobiologiska verkar ha större inflytande på alkoholintag i ung ålder.

De unga djurens beteende var påverkat av uppväxtmiljön och förändrades även olika över tid beroende på tidigare uppväxtmiljö. Vid alkoholintag i tidig ålder försvann dessa skillnader i beteendeutveckling. Opioiderna var också påverkade av miljön, men det fanns även alkoholinducerade effekter på dessa som var oberoende av uppväxtmiljön.

Naltrexon blockerar opioidreceptorer och används vid alkoholberoende för att reducera återfall. Naltrexon minskar alkoholkonsumtionen genom en förmodad utebliven belöningseffekt av alkohol. Naltrexonbehandlingen av alkoholdrickande råttor med olika uppväxtmiljöer visade att råttor från en miljö med korta separationer inte minskade sitt alkoholintag. Detta styrker betydelsen av att även beakta uppväxtmiljön som orsak till individuella skillnader i hur man svarar på naltrexonbehandling, och inte bara genetiska faktorer, i samband med alkoholberoende.

Resultaten från beteendestudierna visade att mammor till korttidsseparerade ungar hade ett mindre utforskande beteende och lägre grad av riskbedömning än mammor som upplevt långa separationer från kullen. När man studerade interaktionen mellan mamma och unge såg man att mammor som inte separerats från sin kull prioriterade att flytta ungarna från ett riskområde till en annan plats i en testarena, medan mammor som separerades längre tider från sin kull prioriterade att flytta ungarna till skyddsområdet eller lämnade kvar dem i riskområdet. Mammans beteende verkar vara beroende av hur länge ungarna separeras från mamman då ungarna är små. Eftersom mammans beteende påverkar ungarna tyder dessa resultat på att effekter som vi ser hos ungarna efter separation kan vara, till en del, orsakade av mammans beteende.

Sammantaget visar dessa resultat att tidiga miljöfaktorer såsom sociala interaktioner under uppväxtmiljön och tidig alkoholdebut, är av vikt för senare alkoholintag, beteendeutveckling och hur individen svarar på en läkemedelsbehandling.

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