Involvement of the Opioid System in High Alcohol Consumption

Environmental and Genetic Influences

BY

KAROLINA PLOJ

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2002
ABSTRACT


It is well accepted that both inherent and environmental factors influence the pathogenesis of alcohol dependence. This thesis investigates the role of the opioid system in the initiation and maintenance of high ethanol intake. Ethanol-preferring C57BL/6J mice differ from ethanol-avoiding DBA/2J mice in that they exhibit lower basal levels of the opioid peptides dynorphin B and Met-enkephalin-Arg⁶Phe⁷ (MEAP) in the nucleus accumbens, which may contribute to their divergent drug-taking behaviour. Chronic ethanol intake in C57BL/6J mice and repeated ethanol administration in Sprague-Dawley rats induce time-specific changes in dynorphin B and MEAP levels in regions, such as the nucleus accumbens and the ventral tegmental area, associated with reinforcing effects of drugs of abuse.

Daily neonatal handling for 15 min (H15) and maternal separation for 360 min (MS360) during postnatal day 1-21 were used as models for environmental manipulation early in life. H15 in male rats results in decreased anxiety-like behaviour, whereas MS360 increases anxiety-like behaviour. Both H15 and MS360 induce changes in dynorphin B and MEAP levels especially in regions related to the hypothalamic-pituitary-adrenal (HPA) axis. In female rats, regions related to the HPA axis are unaffected by H15. This suggests a gender-specific involvement of opioids in the HPA axis response to stress. More rats in the MS360 group initiate ethanol consumption and have a higher ethanol intake later in life than the H15 group. The H15 group has particularly low ethanol intake and also differs with regard to neurochemistry compared to both MS360 and control groups, suggesting that H15 can induce long-term changes, protective against high ethanol intake. Specific changes in opioid receptor density are observed after chronic ethanol consumption, such as an increased κ-receptor density in several brain areas, as well as changes in δ-receptor density in the frontal cortex and the nucleus accumbens. Altogether, these results suggest that the opioid system plays an important role in the mechanisms underlying the initiation and maintenance of high ethanol intake.

Key words: Opioids, ethanol, stress, maternal separation, neonatal handling.

Karolina Ploj, Division of Pharmacology, Department of Pharmaceutical Biosciences, Uppsala University, Box 591, S-751 24 Uppsala, Sweden

© Karolina Ploj 2002

ISSN 0282-7484
ISBN 91-554-5217-5

Printed in Sweden by Uppsala University, Tryck & Medier, Uppsala 2002
Mojí mami Mariji in v spomin na mojega očeta Gabriela
List of original papers

This thesis is based upon the papers listed below, which are referred to in the text by their Roman numerals I-V.


V. Ploj K, Roman E, Nylander I. Long-term effects of neonatal manipulation on ethanol intake and the brain opioid and dopamine systems in rats. *Manuscript*

Reprints of the original papers (I-IV) were made with permission from Elsevier Science and Harcourt Health Sciences
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>AA rats</td>
<td>Alko, Alcohol rats</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ANA rats</td>
<td>Alko, Non-Alcohol rats</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy-terminal</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide binding-protein</td>
</tr>
<tr>
<td>GABA</td>
<td>Gammaaminobutyric acid</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H15</td>
<td>15 min of neonatal handling during day 1-21</td>
</tr>
<tr>
<td>HAD rats</td>
<td>High alcohol-drinking rats</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.r.</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>LAD rats</td>
<td>Low alcohol-drinking rats</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>Leucine-enkephalin</td>
</tr>
<tr>
<td>MEAP</td>
<td>Met-enkephalin-Arg^6Phe^7</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>Methionine-enkephalin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS360</td>
<td>360 min of maternal separation during day 1-21</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte stimulating hormone</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adeninedinucleotide, oxidised form</td>
</tr>
<tr>
<td>N/OFQ</td>
<td>Nociceptin/orphanin FQ</td>
</tr>
<tr>
<td>NP rats</td>
<td>Nonpreferring rats</td>
</tr>
<tr>
<td>ORL1</td>
<td>Opioid receptor-like 1</td>
</tr>
<tr>
<td>P rats</td>
<td>Preferring rats</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomialanocortin</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TIQ</td>
<td>Tetrahydroisoquinoline</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
4 Results and discussion

4.1 Basal levels and alcohol-induced changes in opioid and N/OFQ
..... levels in alcohol preferring C57BL/6J mice (paper II) ......................... 31

4.2 Repeated ethanol administration in Sprague-Dawley rats alters
..... opioid peptide levels in the brain (paper III) ..................................... 34

4.3 Neonatal handling in male and female Sprague-Dawley rats
..... (papers I and IV) ................................................................................ 36

4.4 Neonatal manipulation on ethanol intake and the brain
..... opioid and dopamine systems in Wistar rats (paper V) ...................... 39

5 Summary and conclusions ............................................................... 50

6 Sammanfattning på svenska ............................................................. 52

7 Acknowledgements ........................................................................ 53

8 References ..................................................................................... 56
1 INTRODUCTION

1.1 Background

Every society has throughout history used drugs that alter mood, thoughts, and feelings. Alcohol is an example of a drug that has been central to social, religious and personal use all over the world. One of the earliest mentions of wine production is from an Egyptian papyrus dated 3,500 B.C. The industrial revolution caused an upsurge in the use of alcohol since many people considered alcohol drinking as the ideal way to escape the boredom and pain of their urban working lives. In an international perspective, Sweden is among those countries having the lowest total consumption of alcohol [101]. However, sporadic, binge-drinking practices outside mealtimes, which are common in traditional Swedish drinking culture, may produce as many negative, although qualitatively different, consequences as in countries with a much higher and more evenly distributed consumption [227]. Alcohol can cause considerable morbidity, mortality, and social problems. The medical and social consequences of high alcohol consumption are one of the biggest and most expensive health problems in today’s society. In Sweden, with a population of 8.9 million, approximately 300,000 people suffer from alcohol addiction or alcohol abuse. The financial costs caused by alcohol abuse have been estimated to a level over 100 billion Swedish Crowns every year [120].

The prevalence of alcoholism and alcohol-related problems contingents on age, gender, and culture, but also depends on the definitions and principles of classification employed. According to the definitions stated in the fourth revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [10], drug abuse refers to the harmful use of a drug leading to social and/or personal problems for the individual (i.e. recurrent use resulting in a failure to fulfill major role obligations at work, school or home, repeated substance-related legal problems, substance use in situations in which it is physiologically hazardous and/or continued use despite having problems caused or exacerbated by the effects of the drug). Substance dependence is diagnosed according to the following: A maladaptive pattern of substance use leading to clinical impairment or distress, as manifested by three (or more) of the following, occurring at any time in the same 12-month period:

1. Tolerance, as defined as either of the following: a) need for markedly increased amounts of the substance to achieve intoxication or desired effect b) markedly diminished effect with continued use of the same amount of the substance
2. Withdrawal, as manifested by either of the following: a) the characteristic withdrawal syndrome for the substance b) the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms
3. The substance is often taken in larger amounts or over a longer period that was intended
4. There is a persistent desire or unsuccessful efforts to cut down or control substance use
5. A great deal of time is spent in activities to obtain the substance, use the substance, or recover from its effects
6. Important social, occupational, or recreational activities are given up or reduced because of substance use
7. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Alcohol dependence may also be defined and diagnosed according to the tenth revision of International Classification of Diseases (ICD-10) stated by the World Health Organisation (WHO), 1993. In this thesis drug addiction will be equated with substance dependence as defined above. The aetiology of alcoholism is complex and alcoholism is a disorder that profoundly affects the biological, psychological, and social functioning of afflicted individuals. Treatment of alcoholism is difficult, due to the fact that the mechanisms underlying alcohol dependence are not yet fully investigated and understood. Identifying and studying risk factors for initiation of excessive alcohol intake is one important way to increase knowledge about alcohol dependence and abuse. Knowledge about the risk factors would allow a more efficient treatment schedule for each individual and it would be possible to generate more specific pharmacological therapies than those available today.

1.2 Factors involved in alcohol dependence

It is well known that, although a large number of people experiment with drugs for variable periods of time, only few of them develop addiction, i.e. a compulsive drug use that becomes the main goal-directed activity of the subject. This suggests that there are many factors, such as availability, genetics, gender, personality, history of drug use, stress, and other life events that contribute to the transition from drug use to drug addiction. In the theories behind the development of drug dependence it is suggested that the drugs ability to produce a sense of well being and euphoria is important in the initial drug-using phase. The time of initiation of alcohol use, environmental variables and/or individual characteristics are factors that can determine whether or not someone will become dependent on alcohol. [25,96,102,142,245]. A detailed knowledge about these factors is crucial to be able to define individual goals of alcohol addiction therapies.

1.2.1 Genetic factors

Substantial evidence exists for a genetic contribution to the vulnerability to develop alcoholism, but the underlying biological mechanisms are still largely unknown. Studies of twins from biological parents with a history of alcoholism, where the children have been adopted by non-alcoholic parents, have provided
strong evidence for an important genetic influence on the risk of becoming an alcoholic in both men and women [102]. Twin studies in Scandinavia and the United States have shown consistently higher rates of alcoholism in monozygotic compared to dizygotic twins of male alcoholics [109,122,138], and adoption studies have shown consistently higher rates of alcoholism in the adopted-away sons of alcoholic biological parents than in control adoptees [32,95]. Research strategies in which non-alcoholic offspring from families with a history of alcoholism are compared with offspring from families with no history of alcoholism on the basis of sensitivity to an alcohol challenge have been used. Differences in sensitivity to alcohol has been suggested, with sons of alcoholics showing higher tolerance to alcohol-induced effects [232]. Impulsivity and negative affectivity have also been identified as potential mediators of the genetic contribution to alcoholism [238].

In addition to the human twin studies, the knowledge about the role of heredity in alcohol abuse is acquired through animal studies. A number of models to evaluate different aspects of alcoholism have been established, including excessive drinking through the use of inbred strains and selective breeding programs [50,51]. These animal models can be used to identify heritable biological characteristics that are associated with, and may be causally related to, high ethanol (which is the alcohol found in alcoholic beverages) drinking. Furthermore, the efficacy of pharmacological, environmental, and behavioural agents that have the potential to alter ethanol-drinking behaviour can be tested with these models. Inbred strains represent populations of genetically similar individuals that have been produced by more than 20 generations of mating between closely related animals [21]. It has been established that various inbred strains of mice and rats widely differ in ethanol intake. For example, C57BL/6J mice voluntarily consume high amounts of ethanol, whereas DBA/2J mice prefer water over ethanol [169]. In addition, selective breeding, where selected individuals are intermated to produce offspring of a desired phenotype, such as high and low ethanol consumption, have been performed to study the genetics of ethanol drinking. This has resulted in the establishment of the AA (Alko, Alcohol), P (Preferring) as well as the HAD (high alcohol-drinking) rat lines that voluntarily consume high amounts of ethanol, and the ANA (Alko, Non-Alcohol), NP (Non-preferring) and LAD (low alcohol-drinking) lines that prefer water over ethanol [75,147,149]. These animals have been used in numerous investigations to determine genetic differences between the ethanol-preferring and non-preferring animals in a number of neurotransmitter and neuromodulator systems both under basal conditions and during ethanol intake and withdrawal [166].

1.2.2 Environmental factors

Both epidemiological and pre-clinical research has implicated adverse experiences early in life as a risk factor for the development of various physio- and psychopathological behavioural patterns [183,275]. Anxiety and depression are for example frequently co-morbid with alcoholism in humans [106,142] and there is evidence
that people with adverse early life experiences may have an increased risk of developing alcohol dependence [33,128,177,245]. In adults, clinical studies have implicated stress as an important factor contributing to relapse in abstinent alcohol addicts [29]. Studies of animals that have been bred for various degrees of anxiety show a strong correlation between degree of emotionality and ethanol preference [197,201].

Animal models of stress have been shown to induce an increase in the vulnerability to the addictive properties of drugs of abuse. There are many different types of stress that can be categorized into physical and/or psychological stress. Food restriction [200], immobilisation [236], witnessing stress [218], social isolation [26,231,277] and social interactions [100], all increase drug intake behaviour. Physical stressors, such as tail pinch [209] and electric foot shocks [74], as well as early life stress, such as prenatal [57] and postnatal stress [77,104,112,226] can also enhance the propensity to develop excessive drug taking behaviour. This suggests that various stressful experiences can increase the vulnerability of an individual for excessive drug intake and possibly to develop enhanced drug taking behaviour, although conflicting results have been reported [76,241]. Moreover, most of the previous studies have mainly focused on opioid and psychostimulant drugs, and less is known about the effects of different kinds of stressors on ethanol intake behaviour.

1.3 Animal models

1.3.1 Drug intake behaviour

Obvious ethical and methodological problems limit research with human subjects on alcohol abuse and dependence, regardless of whether it is conducted in healthy volunteers or alcoholics. The mechanisms by which particular drugs of abuse can create dependence in humans have remained elusive. Therefore, the development of animal models is critical for studies on ethanol-related health problems, in particular in studies of the neuropharmacological mechanisms involved in the development of addiction. Virtually all drugs that are rewarding in humans are also rewarding in laboratory animals [278]. Drug intake behaviour has successfully been studied in many species using different routes of administration: oral, intravenous, intramuscular, intraperitoneal (i.p.), intragastric, intracerebroventricular, pulmonary and intracranial routes. A wide range of species have been studied, including baboons, rhesus monkeys, squirrel monkeys, dogs, cats, rats and mice [172]. Operant paradigms include self-administration and intracranial self-stimulation where animals learn to perform a task in exchange for administration of a drug or electrical stimulation of a neural pathway, respectively. Conditioned place preference is a classical conditioning paradigm where animals learn to associate the experience of a drug with a particular context. In studies of voluntary oral drug intake the most commonly studied drug is ethanol and the two-bottle choice model, where the animal is
given a free choice between a bottle of tap water and a bottle containing an ethanol solution, is the most commonly used procedure.

1.3.2 Early life experiences

1.3.2.1 Maternal influences on development

Perhaps the most significant environmental factor during the early development of mammals, is the interaction between the infant and its mother. Newborn mammals naturally require nurturing care from the mother to survive. Infant rats normally spend at least their first week of life in a nest with their littermates. The dam suckles them almost continuously for the first day or two, then gradually increases the periods of absence [2]. Studies in experimental animals, across a variety of species, have demonstrated that the early relationship between mother and infant is critical for optimal development of the offspring and neonatal physiological homeostasis is, in large part, maintained by maternal stimuli. Studies, mostly in rodents, have demonstrated that the mother regulates physiological responses as diverse as heart rate, sleep/wake cycles, thermoregulation and growth hormone production in the infant [107]. During the second week of life the pups are developing fur, opening their eyes and ear canals, and beginning to show more physiologic autonomy [47,71]. Once the rat pups can hear and see, they explore their own environment more frequently and by weaning on days 20-24, pups are fully able to live independently on their own.

Most of the peripheral and central stress-responsive systems can be activated during early-life development. However, under conditions of normal dam-pup interactions, these responses are mostly suppressed by the dam’s behavioural interaction with the pups. In animals and humans, separation of an infant from its mother during the early developmental phase is a significant stressor that markedly and negatively affects the subsequent emotional development of the infant [36,107]. Separation of rat pups from their dams during the early postnatal period results in a variety of physiological changes, the nature of which are dependent on the specifics of the separation experience, the environmental conditions, and the duration of separation [107]. In addition to well-defined acute responses to such separation experiences, converging evidence indicates that there are robust behavioural and neurobiological effects following repeated separation that persist into adulthood [11,143]. These findings are supported by studies in non-human primates where absence of contact with mothers during infancy induce long-term behavioural and endocrine effects [48,77,104,105].

1.3.2.2 Neonatal handling and maternal separation

Interestingly, the nature of the separation of rat pups from the dams determines the direction of the long-term changes of separation. Experimental investigation of the early handling phenomenon in rats began when Bernstein (1952) [24] reported that laboratory rats which had been held in an experimenter’s hand for a few min-
utes daily, starting at weaning and continuing into adulthood, performed better in an behavioural test than non-handled animals. Shortly thereafter, Weininger (1953, 1956) [269,270] reported that a similar handling procedure made these rats less emotional and more robust than non-handled animals. Levine and Otis (1958) [145] found that handling before weaning was much more effective than handling after weaning. Hunt and Otis (1963) [111] demonstrated that it was unnecessary to actually hold the pups in the hand, as same effects could be achieved by simply removing the pups from their mother for a short while. Currently, the most commonly used method is to remove the dam from the nest cage, place the pups in small boxes for 3-15 min and replace them with the dam in the nest cage. This procedure is repeated daily during the first 2-3 weeks of life.

One of the best-characterised effects of handling in rats is the increased tolerance toward stress. The hypothalamic-pituitary-adrenal (HPA) axis is highly responsive to stress. Stress-induced activation of the HPA axis occurs when inputs from limbic, cortical and/or brainstem structures activate neurons of the medial parvocellular division of the hypothalamic paraventricular nucleus. These parvocellular neurons synthesise and release corticotropin-releasing factor (CRF) to enhance the synthesis and release of pro-opiomelanocortin (POMC) products from the pituitary corticotrophi cells. A major POMC product is the adrenocorticotropic hormone (ACTH), which is released into the general circulation, ultimately producing elevations in the synthesis and release of corticosterone (cortisol in humans) from cells of the adrenal cortex. Under resting conditions, ACTH and corticosterone levels are comparable in adult handled and control animals. However, handled rats have decreased mRNA and immunoreactive (ir) basal levels of CRF in the hypothalamus compared to control rats. In addition, handled rats respond to stressors with a more modest increase in corticosterone and ACTH and a faster return to basal plasma concentrations. Behavioural studies have shown that adult handled animals demonstrate decreased anxiety-like behaviour, enhanced learning performance and that handling protects against ageing-related cognitive deficits [11,143].

The underlying mechanisms for the reduction in stress-related functioning are unclear, but may involve the type of maternal behaviour that is displayed after the pups are returned to the mother. A brief removal of rat pups from the dam results in an increase in the amount of licking, grooming, and arched-back nursing that the mother lavishes upon the pups when they are returned. The total amount of time spent nursing and being with the offspring is not affected, but rather the quality of the interaction between mother and pup [151]. Pups born to mothers that naturally exhibit high levels of nurturance grow up to adults that display low anxiety-like behaviours [34]. Taken together, these findings suggest that increased nurturance by the mother can lead to a toned-down stress-responsive system in the offspring. In contrast, rat pups separated for longer periods do not experience increased but rather decreased maternal care [54].
In the mid-1970s a different but related procedure was introduced, namely that of prolonged maternal separation. Longer periods of maternal separation (>1 h) have generally been described as producing effects in adulthood contrary to those of handling, both with regard to neuroendocrine and behavioural effects. Although prolonged maternal separation procedures vary considerably across laboratories, this manipulation is widely considered as a neonatal stressor and has been reported to give rise to profound behavioural and neurochemical modifications in adult animals. Rat pups that are separated from the mother for 1 h or more show exaggerated HPA axis responses to stress, and increased stress-induced behaviours [11,99,141]. Increased anxiety-like behaviours and a long-lasting dysregulation of the CRF system has also been reported in non-human primates exposed to adverse rearing conditions during infancy [48,77,104,105]. These findings suggest that neonatal handling and maternal separation are useful animal models to study effects of early life events on adult physiology and behaviour.

1.4 The endogenous opioid system

Preparations of the opium poppy *papaver somniferum* (Figure 1) have been used for pain relief and pleasure longer than any other agent, except perhaps from alcohol. Writings that seem to refer to poppy extracts with opiate actions have been found in the area of Sumeria in the Middle East and judged to date from 4000 B.C. The ancient Greeks used opium for its recreational as well as its medicinal effects and the word opium is derived from “opos”, the Greek word for juice. The importance of opium in Roman culture is evident from the fact that Somnus, the Roman god of sleep, is often depicted carrying a container filled with the juice of the poppy plant.

![Figure 1. Opium poppies](Foto: Ulf Nyman)
Scientific insight into the chemical actions of opiates commenced with isolation of the active ingredient. In 1806, a 20-year-old German chemist named Friedrich Sertürner obtained pure morphine from the poppy plant [235]. He named the substance morphine after Morpheus, the Greek god of dreams. The high potency and specificity of morphine suggested that it could bind to specific receptors in the nervous system to induce its biological effects. In the early 1970s, several research groups identified specific binding sites for opiates in the rat brain and peripheral tissues in guinea-pig [205,240,254]. Endogenous ligands to the opioid receptors (Table 1), i.e. the enkephalins (from the Greek “in the head”), β-endorphin (from endogenous morphine) and dynorphins (dyn from Greek dynamis=power), were discovered shortly after the receptors were identified [93,110,153,255]. More recently, the endogenous peptides endomorphin I and II, which are structurally unrelated to the other endogenous opioid peptides, have been suggested to represent a new class of potent and selective µ-opioid receptor agonists [280]. The endomorphins may, however, also act as agonists on the κ-opioid receptor [92].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Structure</th>
<th>Receptor selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>δ</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
<td>δ</td>
</tr>
<tr>
<td>Arg⁴Phe⁵(MEAP)</td>
<td>Tyr-Gly-Gly-Phe-Met-Arg-Phe</td>
<td>δ</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln</td>
<td>κ</td>
</tr>
<tr>
<td>Dynorphin B</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Gln-Phe-Lys-Val-Val-Thr</td>
<td>κ</td>
</tr>
<tr>
<td>β-endorphin</td>
<td>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu</td>
<td>µ/δ</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>Tyr-Pro-Trp-Phe</td>
<td>µ</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Phe-Phe</td>
<td>µ</td>
</tr>
<tr>
<td>Nociceptin/Orphanin FQ</td>
<td>Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln</td>
<td>ORL1</td>
</tr>
</tbody>
</table>

Table 1. Sequences and preferred receptor(s) for the endogenous classical opioids and related peptides

To date, three opioid receptor subtypes have been cloned: the µ- [40,256], the δ-[129] and the κ-receptor [41,146,173]. In addition to the rodent opioid receptors, the human µ-, δ- and κ-receptors have been cloned [132,159,267]. Their amino acid sequences and their ligand selectivity are very similar to the rodent opioid receptors. Moreover, subtypes of the opioid receptors have been proposed (µ₁, µ₂, δ₁, δ₂, κ₁, κ₂, κ₃) [64]. In addition to these “classical” opioid receptors, some investigators have postulated the existence of sigma (σ) [161], epsilon (ε) [233], lambda (λ) [97], and zeta (ζ) [281] opioid receptors. The σ-receptor has subsequently been shown to be non-opioid [156]. Quantitative autoradiography studies have shown that µ-, δ- and κ-receptor binding sites are present in most regions of the central nervous system (CNS) and that they are heterogeneously distributed. The highest δ-receptor densities are present in the olfactory bulb, olfactory tubercle, neocortex, caudate putamen and nucleus accumbens. In the rat, κ-receptor binding sites appear to be
Involvement of the Opioid System in High Alcohol Consumption

relatively low (9%) compared to δ- (50%) and μ-receptor binding sites (41%) [158]. The highest densities of κ-receptors are observed in the nucleus accumbens, endopiriform nucleus, claustrum and interpeduncular nucleus. In contrast to δ- and κ-receptors, μ-receptors are widely distributed throughout the neuroaxis with a high density of binding sites in the caudate putamen, neocortex, thalamus, nucleus accumbens, hippocampus and amygdaloid nuclei [64]. In general, there is good correlation between the distribution of expressed receptor mRNA and their binding sites, indicating that opioid receptors are synthesised locally. However, in some cases a lack of correlation is found, which suggests that receptors are transported to their terminal regions [157]. The endogenous opioid peptides vary in their affinity to μ-, δ- and κ-receptors. β-endorphin is equiactive at μ- and δ-receptors with much lower affinity for κ-receptors [7,140], whereas Met- and Leu-enkephalin have high affinity for δ-receptors, ten-fold lower affinity for μ-receptors and negligible affinity for κ-receptors [49]. The opioid fragments of prodynorphin, particularly dynorphin A and dynorphin B, have high affinity for κ-receptors but also have significant affinity for μ- and δ-receptors [49].

All opioid receptors are G-protein coupled receptors with an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal structure. An activation of the receptors generally exerts neuronal inhibition by several mechanisms, like inhibition of cyclic adenosine monophosphate (cAMP) formation, membrane hyperpolarisation through opening of potassium channels and decreased transmitter release by inhibition of the opening of calcium channels [70]. In addition, opioid receptor activation may under certain conditions also exert stimulatory effects on neurotransmission [230,242].

The precursor proteins are post-translationally processed by proteolytic enzymes resulting in a unique mixture of products (Figure 2) with a high tissue specificity. Each of these precursors has an unique anatomical distribution throughout the CNS and in peripheral organs [6]. The endogenous opioids, with the N-terminal sequence Met- or Leu-enkephalin, are generated by enzymatic processing from three precursor molecules, POMC, proenkephalin, and prodynorphin [123,181,187]. The anterior pituitary lobe and the hypothalamus are major sites of POMC biosynthesis. Two distinct brain nuclei contain POMC neurons: the arcuate nucleus of the hypothalamus and the nucleus tractus solitarius. Widespread projections from these neurons are present throughout the brain. From POMC the opioid β-endorphin is generated, but also several non-opioid peptides, e.g., ACTH and melanocyte stimulating hormones (α-, β- and γ-MSH). Proenkephalin-containing neurons are widely distributed throughout the brain in both local circuits and long projection neurons. Peripherally, proenkephalin containing cells are found in the autonomic system, adrenal glands and the intestines. Enzymatic cleavage of proenkephalin generates Leu- and Met-enkephalin and several extended forms of these pentapeptides. Prodynorphin is synthesised in multiple cell groups throughout the brain, the ante-
rior pituitary lobe and in the gonadotrophs. Prodynorphin-containing neurons have both short and long projection pathways and can generate several opioid peptides, including α-neoendorphin, dynorphin A, and dynorphin B. In the brain, dynorphin peptides are present in striatonigral neurons suggesting a role in motor control, in neurons projecting to the nucleus accumbens suggesting effects on reward pathways and in mossy fibers of the hippocampus suggesting a role in learning processes [261].

**Figure 2. Opioid and nociceptin/orphanin FQ precursors and their products**

Many investigators have studied the physiological, pharmacological and behavioural effects of the opioids in attempts to elucidate their functional roles. The most well known effects of opioids are analgesia and the generation of euphoria. However, the opioid system is believed to play a role in a large number of other CNS functions, including stress, drug dependence, ingestive and reproductive activities, memory and learning, thermoregulation, and motor behaviour [196,260].

### 1.5 Nociceptin/Orphanin FQ

Molecular cloning of the µ-, δ-, and κ-opioid receptors led to the discovery of a novel receptor identified as opioid receptor-like 1 (ORL1) [30,84,176] whose putative ligand is the nociceptin [174] or orphanin FQ [221] (abbreviated here as N/OFQ) peptide. The ORL1 receptor has a primary sequence very similar to those of the opioid receptors, with approximately 60% homology [39].
N/OFQ, which is generated from a larger precursor peptide, pro-N/OFQ, is structurally related to the opioid peptide dynorphin A [188,220]. However, it does not bind to opioid receptors and opioid peptides do not activate the ORL1 receptor [219]. The localisation of N/OFQ, ORL1 receptor binding and mRNA levels are closely correlated. The ORL1 receptor is widely expressed in the CNS (cortical areas, olfactory regions, limbic structures, thalamus, central periaqueductal gray, substantia nigra, several sensory and motor nuclei and the spinal cord) and in peripheral tissues in rats [182]. The distribution of ORL1 receptors in human brain is similar to that of the rodents [204], which suggests the participation of the ORL1 receptor in numerous human physiological functions, such as emotive and cognitive processes, neuroendocrine and sensory regulation, and autonomic functions [175].

The role of N/OFQ in drug reward is not clear. Behavioural studies have shown that N/OFQ abolishes the rewarding properties of ethanol [45] and morphine [44,178] in the place conditioning paradigm. In addition, N/OFQ has been found to reduce ethanol consumption in ethanol-preferring rats [45] and to inhibit stress-induced ethanol-seeking behaviour, but not stress-induced cocaine-seeking behaviour [162]. Moreover, administration of N/OFQ fails to affect self-administration of heroin in the rat [265]. These findings suggest that drugs directed at central N/OFQ receptors may represent an interesting approach in finding new targets to the treatment of drug abuse. However, the findings are contradictory [162,265] and additional work will be required to elucidate the involvement of N/OFQ in reward mechanisms.

N/OFQ may also play an important role in stress- and anxiety-regulatory functions. It was recently shown that N/OFQ interacts with the neuroendocrine function of the HPA axis [62] and mice lacking N/OFQ show elevated basal corticosterone levels and display increased anxiety-like behaviour when exposed to a novel and threatening environment [139]. Furthermore, administration of ORL1 receptor agonists, attenuates behavioural responses to various stressors [118,119]. The site(s) mediating the anxiolytic effects of N/OFQ is/are not clear and additional work will be required to elucidate the underlying mechanisms of the anxiolytic effects of N/OFQ.

1.6 The brain reward system

Drugs that are abused by humans are considered rewarding, induce a desired effect, and reinforce drug-taking behaviour, and thereby often lead to further drug use. In an attempt to understand the mechanisms underlying drug dependence, research over the past decades has focused on the effects of drugs of abuse on the CNS. More than 40 years ago, Olds and Milner discovered that direct electrical stimulation of different brain areas can be rewarding and act as a reinforcer [195]. Since then, a large number of studies using several techniques have been conducted to identify the sites and substrates involved in the so-called brain reward system.
Methods to identify brain areas and transmitter systems involved in drug-taking behaviour in animals include administration of drugs of abuse into specific brain regions, systemic or intracranial treatment with agonists or antagonists, or neurochemical lesions of brain areas. In addition, biochemical studies, such as measurements of neurotransmitter levels in the brain and studies of receptor dynamics, have been used to clarify the role of neurobiological systems in the brain in drug-taking behaviour [137,276].

Dopamine, a catecholamine neurotransmitter, was discovered in the late 1950s as a major transmitter in the corpus striatum [37,38]. Among the various brain transmitter systems implicated in reinforcement and dependence, most attention has been devoted to the mesolimbic dopamine system. The cell bodies of the mesolimbic dopamine system are located in the VTA, originally described as the A10 group of catecholamine neurons [52] and the terminals are found in the nucleus accumbens. A dopaminergic negative feedback to the VTA from the nucleus accumbens has been suggested [217]. Cell bodies within the VTA send efferent dopaminergic projections to other forebrain structures as well, such as the amygdala, the medial prefrontal cortex, the olfactory tubercle and the septum [193]. The drug abused by humans are structurally diverse and produce different behavioural effects in the user. Drugs of abuse have the ability to modulate the mesolimbic dopamine system, a system fundamental to initiate and maintain behaviours crucial for survival. Rodents will self-administer a variety of drugs of abuse into the nucleus accumbens [167] and there is evidence that most drugs of abuse and also natural rewards such as food, water and sexual behaviour, have the ability to increase the activity in the VTA-nucleus accumbens dopaminergic pathway [66,206,207]. This action has been related to the reinforcing or dependence-creating properties of a drug. Thus, drugs of abuse affect neuronal networks that apparently evolved to reinforce behaviours that are necessary for survival and reproduction. These findings have led to the view that the VTA and nucleus accumbens are critical “brain reward regions” that mediate the reinforcing actions of many drugs of abuse. However, other components of the dopamine system, such as the medial prefrontal cortex [91], as well as non-dopaminergic pathways, have also been implicated in drug reward mechanisms [133]. A large body of evidence suggests that opioidergic, GABAergic (gammaaminobutyric acid), cholinergic, serotonergic and glutamatergic and other systems may play a relevant role in modulating the rewarding and reinforcing effects of drugs of abuse [135].

1.7 Interactions between dopamine reward processes and endogenous opioids

Behaviours in which reward plays an important role may be controlled or at least be modulated by endogenous opioid systems. Selective µ- and δ- receptor agonists are rewarding as defined by conditioned place preference [239] and intracranial self-administration [63] paradigms, whereas κ-receptor agonists produce aversive
Involvement of the Opioid System in High Alcohol Consumption

All three major classes of opioid receptors (µ-, δ- and κ-receptors) are present in the nucleus accumbens. Activation of κ-receptors possibly located on the dopaminergic nerve terminals in the nucleus accumbens [250], inhibits dopamine release in the nucleus accumbens whereas κ-receptor blockade increases dopamine release [61,66,247]. Instead, an activation of µ/δ- receptors within VTA results in an increase of dopamine release in the nucleus accumbens (Figure 3), which can be blocked by δ- and µ-receptor antagonists [61,247]. The exact mechanism by which δ/µ-opioid receptors in the VTA modulate the mesolimbic dopamine pathway is currently unknown. Activation of a β-endorphin pathway primarily originating from the nucleus arcuatus in the hypothalamus can increase dopamine release in the nucleus accumbens via disinhibition of the tonic inhibition of GABAergic neurons on dopamine cells in the VTA or by direct stimulation of dopamine cells in the nucleus accumbens [98,121,164]. Thus, the results presented so far, suggest that the opioid system is necessary for a basal dopamine release in the nucleus accumbens, which, in turn, might play a role in the motivational effects of different drugs of abuse.

**Figure 3.** A schematic drawing on the mesolimbic dopamine pathway (DA=dopamine)

It has been demonstrated that N/OFQ exerts an effect on the mesolimbic dopamine system involved in motivation and reward. Like dynorphin, N/OFQ inhibits basal dopamine release within the nucleus accumbens (Figure 3); however, unlike dynorphin, this effect is mediated by an enhanced GABAergic activity in the VTA [179]. N/OFQ has also been found to attenuate morphine-induced dopamine release in the nucleus accumbens [67].

1.8 Ethanol

Ethanol, which is the natural product of fruit or cereal fermentation, affects virtually all body organs, but it is consumed for its effects on the CNS. It is absorbed quickly and it reaches the blood stream within 5 to 10 minutes. A large amount of
ethanol is metabolised in the liver by first-pass metabolism into acetaldehyde. This process (Figure 4) is dependent on the availability of the enzyme alcohol dehydrogenase (ADH) and of the co-factor NAD$^+$. Acetaldehyde is enzymatically converted into acetic acid by aldehyde dehydrogenase (ALDH).

![Figure 4. The metabolism of ethanol](image)

The complexity of the ethanol effects paradoxically rely on the simplicity of its chemical structure (CH$_3$CH$_2$OH). The pharmacological effects of ethanol are non-selective in as much as it can affect membrane organisation, the function of membrane-bound enzymes, enzymes and proteins involved in signal transduction, ion channels, receptor-coupled ionophores, carrier proteins and gene expression [68,155,253]. Ethanol has been shown to affect a variety of different neurotransmitter systems. These include adenosine [14,46], glycine [5,163], acetylcholine [81,180], as well as monoamines and neuropeptides [130,185,257].

Ethanol is similar to other abused substances in that it increases dopamine release in the nucleus accumbens [66], whereas withdrawal of chronic ethanol treatment is associated with a profound reduction of the activity within the VTA-nucleus accumbens pathway [69]. Innate differences in central dopaminergic neurotransmission have been linked to high ethanol preference/excessive ethanol intake in selectively bred rodent lines [148]. In humans, some studies regarding the involvement of the dopaminergic system in alcoholism, have found an association between a divergent dopamine gene and alcoholism, whereas others have not [186]. In addition, an altered central dopamine function has been reported in alcoholics, where decreases in D$_2$ receptors and/or lower density of dopamine transporters have been found in the brains of alcoholics [222,258,263].

In 1970, a biochemical link was proposed between ethanol and the opioid system, based on the finding that beside several amines, the ethanol metabolites produced tetrahydroisoquinolines (TIQs). These TIQs seemed to have opioid-like effects and thus could interact with opioid receptors [53]. Evidence for an involvement of the endogenous opioid system in ethanol reinforcement and addiction has since then been demonstrated in numerous studies [87,103,262]. The non-selective opioid receptor antagonist, naltrexone (Revia®), reduces intake in both animals and humans and is used in the treatment of alcohol dependence in humans [192,264]. Antagonists selective for µ- and δ-opioid receptors decrease ethanol intake in animals and humans [80,83,192,249,264] and attenuate ethanol induced elevation of the extracellular dopamine concentration in the nucleus accumbens [1,23,94]. However, whereas reduced ethanol self-administration is found in µ-opioid receptor knockout mice [225], δ-opioid receptor knockout mice show increased ethanol self-
administration [224]. In addition, activation of κ-receptors, which mediates aversive effects, has recently been found to attenuate voluntary ethanol intake in animals [150,184].

There is also evidence that a genetic disposition towards alcohol drinking may be accompanied both by altered basal levels of opioid peptides in the brain and an increased sensitivity of the endogenous opioid system to ethanol. For example, the AA and ANA rat lines, selected for high and low voluntary ethanol drinking in a free-choice situation [75], have been shown to differ with respect to the amount of opioid peptides and mRNA levels in distinct brain areas [89,189]. An acute alcohol challenge produces larger opioidergic responses in the alcohol-preferring P rats and C57BL/6 mice compared to the non-preferring NP rats and DBA/2 mice [55,82]. Similarly, human subjects at a high risk of developing alcoholism have been reported to have lower basal plasma levels of \( \beta \)-endorphin and a higher response of the pituitary \( \beta \)-endorphin to ethanol than those at low risk [88]. Taken together, emerging data shows that the endogenous opioid system plays an important role in the mechanism of ethanol action and that an imbalance in opioid systems, inherent or acquired, may affect ethanol intake.

1.9 Interactions between stress and drug dependence

Stress has an established role in the initiation and maintenance of drug abuse and is a major determinant of relapse in abstinent individuals [210,213,214,237]. Stressful experiences, either early in life or during adulthood, may increase the vulnerability of an individual to develop drug dependence. These effects are complex and not completely understood and they may involve multiple neuronal systems.

An increase in the activity of the mesolimbic dopamine system may be one of the neural mechanisms through which stressful experiences enhance vulnerability to drugs of abuse. Both acute exposure to stress and drugs of abuse, increase extracellular levels of dopamine in the nucleus accumbens and related regions [31,59,125,199]. Stressed subjects have a largely enhanced drug-induced dopamine release in the nucleus accumbens [125]. The mesolimbic dopamine system is under tonic control of the opioid system [66,108] and it has been shown that inhibition of opioid receptors in the VTA prevents stress-induced activation of dopamine transmission in the prefrontal cortex and nucleus accumbens [124]. Repeated exposure to stress, like repeated exposure to most drugs of abuse, can result in locomotor sensitisation [73,125,194,244,282]. Moreover, stress and various drugs of abuse exhibit cross-sensitisation, and it has been suggested that the ability of stress to increase mesolimbic dopamine release results in sensitisation of the reward pathway to drugs of abuse [13,58,208,209]. Thus, repeated/chronic stress may enhance the sensitivity to the reinforcing effects of drugs of abuse, induced by an increased activity of the mesolimbic dopamine system.
In addition to actions on the mesolimbic dopamine system, stressful stimuli can activate the endogenous opioid system, and thereby induce responses at the neuroendocrine and behavioural level [251,260]. Endogenous opioid activity modulates the dynamics of the HPA axis. In humans, classical opiates and various opioid peptides have been found to suppress the activity in the HPA axis [251], whereas the opioid antagonist naloxone produce a rise in plasma cortisol levels [56]. However, in rodents a mixed picture of either inhibition or facilitation of the HPA axis has been suggested [35,90,115,212].

An interaction between the endogenous opioid system, the HPA axis and ethanol has been demonstrated. Ethanol activates both the mesolimbic dopamine system and the HPA axis and alcohol dependence appears to be associated with distinct patterns of HPA axis disturbances. Excessive cortisol secretion occurs during both chronic ethanol consumption and ethanol withdrawal [3,154,223]. Offsprings from families with a history of alcohol dependence have enhanced naloxone-induced HPA axis activation compared to offspring from families in which alcohol dependence is rare [266]. In addition, it is known that an acute administration of opioid antagonists activates the HPA axis, whereas chronic administration of the opioid antagonist naltrexone dampens ethanol-induced increase of ACTH in humans [168]. Since ethanol withdrawal is known to induce increased activity in the HPA axis, the dampening of the HPA axis may contribute to the therapeutic effectiveness of naltrexone in reducing alcohol craving and relapse in alcohol-dependent persons.

Together, these results suggest that interactions between stress and drug dependence originate from similar effects on the mesolimbic dopamine system and common adaptations after chronic exposure of drugs of abuse. The underlying mechanisms may also involve the endogenous opioid system that is activated by stress and that can modulate both the mesolimbic dopamine system and the HPA axis.
2 AIMS

2.1 General aim

The purpose of this thesis is to examine the role of opioids in mechanisms underlying individual differences in the propensity to develop high ethanol intake. Of particular interest is to examine the influence of the genetic background and environmental variables on the opioid systems and ethanol intake. The work can be divided into four parts with the following goals:

2.2 Specific aims

- To investigate 1) strain differences regarding the opioid and N/OFQ peptide systems in ethanol-preferring C57BL/6J mice and ethanol-avoiding DBA/2J mice, 2) time- and structure-specific changes in opioid and N/OFQ tissue levels after chronic voluntary ethanol consumption in C57BL/6J mice.

- To analyse short- and long-term effects of repeated ethanol administration in rats on opioid peptide levels in the pituitary gland and the brain.

- To study long-lasting effects of daily neonatal handling (15 min) during day 1-21 in female and male rats on opioid and N/OFQ peptide levels in the pituitary gland and the brain.

- To examine the long-term consequences of daily neonatal manipulation, that is neonatal handling (15 min) and maternal separation (360 min), until weaning on neurochemistry and behaviour, in particular the ethanol intake behaviour.
3 MATERIALS AND METHODS

3.1 Animals

In papers I, III and IV Sprague-Dawley rats (B&K Universal AB, Sweden) were used, while Wistar rats (B&K Universal AB, Sweden) were used in paper V. Alcohol-preferring C57BL/6J and alcohol-avoiding DBA/2J mice (MOB, Denmark) were used in paper II. The animals were allowed to habituate to the animal department housing room for at least one week before the start of any study. Animals were housed in controlled rooms with a 12:12 light-dark cycle (light on 6:00 h), at 22±2 °C and 50±5 % humidity. The rat pups in paper V were separated in a room with the same conditions, except for a higher temperature (25 °C). Standard laboratory chow (R36 Labfor, Lactamin, Vadstena, Sweden) and tap water were available at all times. The animals were sacrificed by either decapitation (rats) or dislocation (mice). All animal experiments were approved by the Local Animal Ethics Committee and performed in accordance with the Swedish Animal Protection Legislation.

3.2 Drugs and chemicals

Dynorphin A, dynorphin B, Met-enkephalin-Arg⁶Phe⁷ (MEAP) and N/OFQ peptides used for radioimmunoassays (paper I-V) were purchased from Bachem AG (Bubendorf, Switzerland). In the autoradiographic analysis (paper V), the radioligands [³H]CI-977 and [¹²⁵I]-iodosulpiride were obtained from Amersham (Little Chalfont, United Kingdom), whereas [¹²⁵I]-SCH 23982 and [³H]Ile⁵,⁶-deltorphin II were obtained from NEN Life Science Products (Boston, USA) and Izotop (Budapest, Hungary), respectively.

3.3 Neurochemical experiments

3.3.1 Radioimmunoassay, tissue extraction and purification

Radioimmunoassay is a highly sensitive and specific method based on the competition between an unlabelled antigen and the corresponding labelled antigen for a limited number of antibody binding sites. When the equilibrium is reached there will remain some free antigen and some will be bound in an antigen-antibody complex. Free antigen and the antigen-antibody complex are separated from each other and the radioactivity in the bound fraction is measured in a gamma counter. The percentage of labelled antigen that is bound decreases as the amount of unlabelled antigen in the sample is increased. The amount of unlabelled antigen in the sample is determined by a standard curve. Radioimmunoassay was used to measure ir dynorphin A (paper I), dynorphin B (papers I-V), MEAP (papers II-V) and N/OFQ (papers II and IV) peptide levels in the pituitary gland and in distinct brain areas (Figure 5). MEAP is exclusively cleaved from proenkephalin and was therefore measured as a marker for its precursor. Dynorphin A and dynorphin B were
measured as a marker for the prodynorphin system, whereas the structurally related peptide N/OFQ was analysed as a marker for the proN/OFQ system. Tissue extraction of peptides was performed with acetic acid. The samples were heated at 95°C for 5 min, cooled on ice and homogenised by sonication. The heating and cooling procedures were repeated once more, whereafter the samples were centrifuged for 15 min and the supernatant was collected. The tissue extracts were purified using a cation exchange procedure. The tissue extracts were added onto small cation exchange columns containing SP Sephadex C-25 gel (Pharmacia Diagnostics, Uppsala, Sweden). The peptides were eluted in separate fractions by stepwise elution using a series of buffers containing a mixture of pyridine and formic acid with increasing ionic strength. The fractions were dried in a vacuum centrifuge and stored at -20°C until use. Radioimmunoassay was performed with antisera produced in rabbits [43,211]. Samples subjected to MEAP assay were oxidised prior to the radioimmunoassay. The tracer peptides were labelled with $^{125}$I and the samples were incubated with antiserum and tracer peptide, both diluted in gelatine buffer. Following incubation, a sheep-antirabbit antiserum (N/OFQ, dynorphin A and dynorphin B assays; Pharmacia Decanting Suspension 3, Pharmacia Diagnostics, Uppsala, Sweden) or a charcoal (MEAP assay) suspension was added. The radioactivity of the bound fraction was counted in a gamma counter. Standard curves of known concentrations of opioids and N/OFQ were used for quantitative measurements. The values were expressed as fmol peptide/mg tissue (mean ± SEM, wet weight).

**Figure 5.** A schematic drawing illustrated the pituitary gland and various brain areas in the rat analysed using radioimmunoassay. 1=neurointermediate pituitary lobe; 2=anterior pituitary lobe; 3=hypothalamus; 4=frontal cortex; 5=medial prefrontal cortex; 6=nucleus accumbens; 7=striatum; 8=hippocampus; 9=amgdala; 10=substantia nigra; 11=ventral tegmental area; 12=periaqueductal gray; 13=medulla oblongata; 14=cingulate cortex
3.3.2 Receptor autoradiography

Autoradiography is a receptor binding technique where radioligands are used, which allows localisation of receptors in very small pieces of tissue. Radioligand binding sites are detected in cryostat-cut tissue sections by exposure to a photographic emulsion. Coronal brain sections were used for autoradiography to determine opioid (κ- and δ-receptors) and dopamine (D₁- and D₂-like receptors) receptor density (paper V). Intact brains were stored in isopentane at −20°C prior to sectioning. Coronal sections (20 µm) were prepared on a cryostat and thaw-mounted onto precleaned and gelatine-subbed ice-cold microscope slides and dried by using anhydrous CaSO₄ for 1 week at −20°C. Slides were preincubated in 50 mM Tris-HCl (containing 0.9% NaCl for opioid receptor determination), for 30 min before binding was carried out in 50 mM Tris-HCl (containing 100 mM NaCl for dopamine receptors) at room temperature for 1 h (opioid receptors), 2 h (D₁-like receptors) and 40 min (D₂-like receptors). A concentration of 8 nM of [³H]Ile⁵,⁶-deltorphin II (δ-receptors), 2.5 nM of [³H]Cl-977 (κ-receptors), 100 pM of [¹²⁵I]-SCH 23982 (D₁-like receptors) and 0.7 nM of [¹²⁵I]-Iodosulpiride were used for labelling. Non-specific binding was determined with 10 µM and 1 µM naloxone (δ-receptors and κ-receptors, respectively), 10 µM SCH 23390 (in addition with 50 nM ketanserin to inhibit ligand binding to 5-HT₁A and 5-HT₂A receptors for D₁-like receptors) and 30 µM apomorphine (D₂-like receptors). Slides were washed 2-3 times with ice-cold binding buffer and rapidly dried. Slides were then exposed to [³H]-Hyperfilm (Amersham) for a period of 8 weeks (κ-receptors), 4 weeks (δ-receptors), 3 days (D₁-like receptors) or 1 week (D₂-like receptors) along with [³H]- and [¹²⁵I]-microscales. The autoradiograms were digitised using a dia-scanner (DuoScan T1200, Agfa), and the optical densities were converted to fmol/mg wet weight based on the co-exposed standards using NIH Image software (NIH Image 1.62, NIMH, Bethesda, MD, USA). Structures were identified using the rat brain atlas of Paxinos and Watson (1997).

3.4 Behavioural experiments

3.4.1 Neonatal handling (15 min) and maternal separation (360 min)

Neonatal separation of rat pups from the dam for short and long periods was used to study effects of early life events on adult physiology and behaviour. Time-mated pregnant Sprague-Dawley (papers I and IV) or Wistar (paper V) rats were housed singly in standard macrolon cages. The litters were sexed and culled on day 0 (day of birth), and randomly assigned to one of the two treatments: animals subjected to 360 minutes separation (paper V) and/or 15 minutes handling (papers I, IV and V). Control animals were left with their mothers and were not disturbed until weaning, except for cage changes with clean bedding material once a week. First the mother and then the pups were removed from the nest. The pups were then put in individual plastic boxes at room temperature (22±2 °C, papers I and IV). In paper V
Involvement of the Opioid System in High Alcohol Consumption

Each litter was placed in a cage and moved to an adjacent room with a temperature of 25°C. After 15 min or 360 min, first the pups and then the mother were returned to their home cage. In the group exposed to 360 minutes of maternal separation (MS360) the dams were kept in their home cages during the separation period but taken out prior to the return of the litters. The separation was performed once a day for the first three postnatal weeks. All pups were weaned and separated by sex on postnatal day 22.

3.4.2 Elevated plus-maze test

The elevated plus-maze (Figure 6), in which rats are allowed to freely explore two elevated open and two elevated closed arms, is an ethologically derived behavioural model based on the conflict between the exploratory drive and the animal’s fear of open areas. Normally, rats prefer the closed arms of the maze. Either forced or voluntary passage onto the open arms of the plus-maze is associated with elevated plasma corticosterone concentrations, increased freezing, and production of faecal boli [79,202,203], hormonal and behavioural changes that are indicative of increased anxiety-like behaviour. The plus-maze test was used in papers I and V. To begin the test, each rat was placed in the centre of the maze, facing an open arm. To consider an animal being located within either the open, closed or the centre sections of the maze, all four of the animal’s paws had to be within one of these three defined sections. In paper I the number of entries into the open and closed arms as well as the total time spent in the open arms were recorded. In paper V additional parameters were recorded, namely total time spent in the arms, time spent in open arms expressed as the percentage of total time in the arms and the latency to the first open arm entry.

Figure 6. The elevated plus-maze model
3.4.3 Open field test

Like the elevated plus-maze test, the open field test (Figure 7) is an ethological test used to analyse behaviour that is based on natural conflict situations. In the open field test, it is the conflict between exploration of and aversion against open, bright areas that determines the animal’s behaviour [17,272]. Exploratory behaviours assessed in the open field test (paper I) were total numbers of locomotion and rearing counts during the test session in an open field arena. Locomotion activity was defined as horizontal traversing within the open field arena; rearing behaviour in the rats is defined as exploration of the open field arena by standing on the hind legs. The apparatus consisted of a plexiglas box (70 x 70 x 45 cm) equipped with two rows of infra-red light sensitive photocell beams on the outside of the box, allowing to locate the rat within the open field arena. The animal was placed in the centre of the arena at the start of the session and locomotion and rearing activity counts were assessed and recorded by automated apparatus.

![Figure 7. The open field test. Schematic picture of the equipment (left) with an outline of the relative position of photocells (right)](image)

3.4.4 Restraint stress

Restraint stress is probably most appropriately viewed as a psychological stressor, based on forced inability to escape potential threats, e.g. a predator. In paper V rats were subjected to restraint stress to evaluate the effects on ethanol intake. The rats were placed in a plexiglas tube for 1 h during 4 consecutive days. In experimental animals, restraint stress for 1 h has been shown to increase corticosterone levels, which are markers for stress, [16] and to cause anxiety-like behaviour as evaluated by the elevated plus-maze [165].

3.4.5 Ultrasonic call test

Ultrasonic vocalisations emitted by rodent pups are whistle-like sounds characterised by frequencies ranging between 30 and 90 kHz, with a duration of 10-200 ms and sound pressures of 60-100 dB [22,27,229]. These signals play an important communicative role in the mother-offspring interaction since they elicit a prompt response concerning caregiving behaviours in the dam. Rat pups emit ultrasonic
calls during brief episodes of separation from the mother. The number of ultrasonic calls emitted increases during the first days of life, reaches a maximum on postnatal days 6-8 and then gradually decrease [9]. The ultrasonic call test (Figure 8) was used in paper V to evaluate the effect of daily short (neonatal handling) and long periods of maternal separation on ultrasonic calls. Before the separation on day 5, the rat pups were placed singly in a circular recording chamber, made of aluminium with a diameter of 17 cm. One minute later the number of ultrasonic calls emitted within 1 min was recorded using a bat detector linked to an electronic counting device.

**Figure 8.** The ultrasonic call test

### 3.4.6 Voluntary ethanol consumption

To examine oral ethanol intake the two-bottle free choice procedure (Figure 9) was used, a common technique in studies of voluntary intake. In order to evaluate adaptive changes in dynorphin B and MEAP peptide systems after chronic ethanol intake in alcohol-preferring C57BL/6J mice the two-bottle choice procedure was used in paper II. Animals were housed individually in cages and were given access to two drinking bottles during 4 weeks, one containing tap water and the other containing a 10% ethanol solution (v/v), (Solveco Chemicals AB, Täby, Sweden). Control animals had access to two bottles filled only with water. After 4 weeks of ethanol or water consumption the first group of animals were sacrificed, whereas two groups of animals were sacrificed 5 and 21 days after the removal of the ethanol from the cages. In paper V, where the effects of neonatal manipulation on ethanol intake and brain opioid and dopamine systems were studied, rats were familiarised to ethanol during 28 days by giving them continuous access to a bottle containing an ethanol solution in addition to the water bottle. The ethanol concentration (v/v) was gradually increased (4 days with 2%, 4 days with 4%, 9 days with 6% and finally 11 days with 8%) over this period. In both paper II and V the position of the bottles was shifted daily to counteract development of side preference. Measurements of ethanol and water intake were performed daily; food intake and body weight was measured every third day.
3.5 Statistical analysis

For all data which followed criteria of parametric tests, Student’s paired or unpaired t-test, analysis of variance (one-way factorial or two-way repeated measures), were used, followed, when appropriate, by Fisher’s post-hoc test for comparisons between groups. For non-parametric analysis, the Kruskal-Wallis, Mann-Whitney U-test or Wilcoxon signed rank test was used. Differences were considered statistically significant at p<0.05.
4 RESULTS AND DISCUSSION

4.1 Basal levels and alcohol-induced changes in opioid and N/OFQ levels in alcohol-preferring C57BL/6J mice (paper II)

The C57BL/6J mice, which show an innate high preference to ethanol, are commonly used in experiments trying to identify neurochemical substrates associated with excessive drug intake. Since the opioid system has been shown to modulate the mesolimbic dopamine system [247], which is associated with the reinforcing effects of various drugs of abuse [66,279], we were interested in basal differences in ir dynorphin B and MEAP levels between the alcohol-preferring C57BL/6J mice and the alcohol-avoiding DBA/2J mice. In addition to opioid peptides, N/OFQ was investigated. Furthermore, in order to evaluate adaptive changes in dynorphin B, MEAP and N/OFQ peptide systems after 4 weeks of voluntary ethanol intake in C57BL/6J mice, peptides were measured the last day of the ethanol intake period as well as 5 and 21 days after ethanol was withdrawn.

Compared to DBA/2J mice, C57BL/6J mice have lower ir levels of dynorphin B and MEAP in the nucleus accumbens, the hippocampus, and the substantia nigra, lower ir dynorphin B levels in the striatum and lower ir MEAP levels in the frontal cortex. Higher ir dynorphin B levels were detected in the pituitary gland and in the PAG, and higher ir N/OFQ levels in the frontal cortex and in the hippocampus in C57BL/6J mice compared to DBA/2J mice (Table 2). N/OFQ has to our knowledge, not previously been analysed in drug-preferring animals. Our results regarding the brain opioid peptide system support a previous study where C57BL/6 mice had lower prodynorphin mRNA and ir dynorphin peptide levels in the nucleus accumbens and lower ir dynorphin levels in the striatum and the hippocampus than DBA/2J mice. In addition, C57BL/6 mice had lower κ-receptor density in the PAG [116]. Another study comparing ir MEAP levels in C57BL/6J and DBA/2J mice reported decreased ir MEAP levels in several brain areas, suggesting an enkephalin deficiency in the brain of the C57BL/6J mice compared to DBA/2J mice [117]. Moreover, a low endogenous dopamine function in the nucleus accumbens has been reported in C57BL/6J compared to DBA/2J mice [85]. Thus, an reduced functioning in dopamine and opioid neuronal networks within the nucleus accumbens may contribute to innate vulnerability to high ethanol-seeking behaviour in C57BL/6J mice. Since ethanol enhances the activity of the opioid and dopamine system, high risk subjects may consume high quantities of ethanol to compensate for this deficiency.

Other studies using animals with high voluntary ethanol intake, such as AA and Lewis rats, show differences in the opioid system in distinct brain areas. AA rats, for example, have lower ir MEAP and dynorphin peptide levels in the nucleus accumbens [189] and higher κ-receptor density in the striatum and the PAG as com-
pared to ANA rats [243]. In the Lewis rats, lower levels of prodynorphin derived peptides can be found in the nucleus accumbens, striatum and the substantia nigra [190] and lower proenkephalin mRNA levels in the nucleus accumbens and the striatum when compared to Fischer rats [160]. Altogether, these results demonstrate innate differences in the opioid system between ethanol-preferring and ethanol non-preferring rodents, which may underlie differences in ethanol consumption in these animals.

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J mice</th>
<th></th>
<th></th>
<th>DBA/2J mice</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dynorphin B</td>
<td>MEAP</td>
<td>N/OFQ</td>
<td>Dynorphin B</td>
<td>MEAP</td>
<td>N/OFQ</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>46.4±5.1**(9)</td>
<td>0.80±0.11(9)</td>
<td>n.d.</td>
<td>29.3±1.7(9)</td>
<td>0.83±0.08(7)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>8.61±0.31(9)</td>
<td>13.5±0.78(9)</td>
<td>9.89±0.51(11)</td>
<td>8.25±0.40(11)</td>
<td>16.4±1.57(9)</td>
<td>9.95±0.70(10)</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.92±0.10(11)</td>
<td>1.16±0.16*(6)</td>
<td>3.14±0.29**(11)</td>
<td>0.89±0.09(11)</td>
<td>1.81±0.23(6)</td>
<td>1.73±0.17(8)</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>14.4±1.3***(11)</td>
<td>16.15±1.69*(8)</td>
<td>4.12±0.65(9)</td>
<td>23.7±2.0(11)</td>
<td>24.8±3.22(8)</td>
<td>4.16±0.51(9)</td>
</tr>
<tr>
<td>Striatum</td>
<td>3.65±0.30****(11)</td>
<td>8.77±1.15(8)</td>
<td>1.53±0.14(11)</td>
<td>6.34±0.34(9)</td>
<td>11.6±1.93(9)</td>
<td>1.76±0.17(11)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.24±0.11***(11)</td>
<td>1.16±0.13**(1)</td>
<td>5.06±0.28**(9)</td>
<td>1.62±0.13(11)</td>
<td>2.85±0.29(9)</td>
<td>3.92±0.33(9)</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.25±0.14(11)</td>
<td>4.59±0.67(9)</td>
<td>8.73±0.68(9)</td>
<td>1.36±0.10(11)</td>
<td>5.99±0.62(9)</td>
<td>6.90±0.63(9)</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>21.1±0.95***(9)</td>
<td>2.73±0.20*(4)</td>
<td>4.74±0.65(8)</td>
<td>26.9±1.46(9)</td>
<td>4.05±0.37(6)</td>
<td>3.79±0.31(9)</td>
</tr>
<tr>
<td>VTA</td>
<td>2.03±0.36(9)</td>
<td>7.21±1.12(8)</td>
<td>4.91±0.36(10)</td>
<td>1.94±0.33(9)</td>
<td>7.93±0.70(6)</td>
<td>4.83±0.36(8)</td>
</tr>
<tr>
<td>PAG</td>
<td>1.63±0.12***(11)</td>
<td>7.07±0.96(9)</td>
<td>10.6±0.65(11)</td>
<td>1.25±0.05(11)</td>
<td>7.94±0.43(9)</td>
<td>9.55±0.41(11)</td>
</tr>
</tbody>
</table>

Table 2. Basal ir levels of N/OFQ, dynorphin B and MEAP in the pituitary gland and in brain areas of alcohol-preferring C57BL/6J mice and alcohol-avoiding DBA/2J mice, respectively. The values represent the mean ± SEM (n) and are expressed as fmol/mg tissue. *p<0.05, **p<0.01, ***p<0.001 (ANOVA, Fisher’s post-hoc test), n.d. = not detectable.

After 4 weeks of voluntary ethanol consumption, only minor changes in ir peptide levels were identified in the C57BL/6J mice. However, 5 and 21 days after ethanol was withdrawn, changes in ir peptide levels were found in several brain areas (Figure 10-12). Structures in which changes occurred include the VTA, the substantia nigra, the frontal cortex, the PAG, and the amygdala, regions that supposedly are involved in drug dependence and withdrawal [19,136]. Of particular interest were the time-dependent changes in ir peptide levels seen in the VTA, where all three peptides were reduced 5 days after cessation of the chronic ethanol drinking period. It has been proposed that the rewarding and reinforcing effects of ethanol may be mediated by stimulation of the mesolimbic dopamine system, which projects from the VTA to the nucleus accumbens [65,86,113,271]. The effects of ethanol on the mesolimbic dopamine system may, in turn, be dependent upon the effects of ethanol on the endogenous opioid system [247]. In addition to opioid peptides, the N/OFQ peptide has recently been shown to modulate the dopamine transmission in the nucleus accumbens. This was suggested to be mediated by an action within the VTA [179]. Our results suggest that the altered ir peptide levels in the VTA may contribute to changes in dopaminergic activity during and after periods of ethanol consumption.
Figure 10. Ir N/OFQ levels in various brain structures in C57BL/6J mice after 4 weeks of alcohol consumption and 5 and 21 days after the 4-week drinking period (n=11/group). Ir N/OFQ levels are shown as percent of levels found in control C57BL/6J mice (n=10/group) drinking water only. **p<0.01, ***p<0.001 (ANOVA, Fisher’s post-hoc test). Abbreviations: HT = hypothalamus; FCX = frontal cortex; STR = striatum; AMY = amygdala; VTA = ventral tegmental area; PAG = periaqueductal gray.

Figure 11. Ir dynorphin B levels in various brain regions and in the pituitary gland in C57BL/6J mice after 4 weeks of alcohol consumption and 5 and 21 days after the 4-week drinking period (n=11/group). Ir dynorphin B levels are shown as percent of levels found in control C57BL/6J mice drinking water only (n=10/group).*p<0.05, ***p<0.001 (ANOVA, Fisher’s post-hoc test). Abbreviations: PG = pituitary gland; HT = hypothalamus; FCX = frontal cortex; STR = striatum; NA = nucleus accumbens; HC = hippocampus; AMY = amygdala; SN = substantia nigra; VTA = ventral tegmental area; PAG = periaqueductal gray.
Figure 12. Ir MEAP levels in various brain regions and in the pituitary gland in C57BL/6J mice after 4 weeks of alcohol consumption and 5 and 21 days after the 4-week drinking period (n=11/group). Ir MEAP levels are shown as percent of levels found in control C57BL/6J mice drinking water only (n=10/group).***p<0.001 (ANOVA, Fisher’s post hoc test). Abbreviations: PG = pituitary gland; HT = hypothalamus; FCX = frontal cortex; STR = striatum; NA = nucleus accumbens; HC = hippocampus; AMY = amygdala; SN = substantia nigra; VTA = ventral tegmental area; PAG = periaqueductal gray.

4.2 Repeated ethanol administration in Sprague-Dawley rats alters opioid peptide levels in the brain (paper III)

The effects of repeated i.p. administration of ethanol (2 g/kg bw/dose, twice daily) for 13 consecutive days on ir opioid peptide levels were investigated in male Sprague-Dawley rats. In order to examine short- and long-term effects of ethanol administration in the pituitary gland and the brain, the ir MEAP and dynorphin levels were analysed 30 min, 5 and 21 days after the last dose on day 13. Decreased ir MEAP levels were found in the hippocampus, whereas increased ir levels were seen in the PAG 5 days after the last dose of ethanol as compared to controls (Figure 13). Ethanol administration induced an elevation of ir dynorphin B levels in the nucleus accumbens 30 min after the last injection, and a decrease in the cingulate cortex as compared to control rats (Figure 14). Five days after the last injection of ethanol, an increase in ir dynorphin B levels was found in the PAG. Rats treated repeatedly with ethanol followed by 21 days of recovery from treatment had increased ir dynorphin B levels in the nucleus accumbens compared to the control rats.

The enkephalin system was largely unaffected by repeated ethanol administration. However, 5 days after the last dose of ethanol, altered ir MEAP levels were found in the hippocampus, a brain area previously reported to have a role in ethanol dependence [12,234]. The PAG is formed by densely packed cells surrounding the
Involvement of the Opioid System in High Alcohol Consumption

aqueduct. It is involved in a broad variety of functions, including sensory, motor and automatic activity and it has been suggested that the PAG is implicated in several behaviours, including opioid withdrawal [28,42] and ethanol withdrawal seizures [78]. The increased ir dynorphin B and MEAP levels in the PAG 5 days after the last dose, seen in our study, suggest that PAG opioids also may be involved in ethanol withdrawal. Repeated ethanol administration induced an increase in ir dynorphin B levels in the nucleus accumbens 30 min and 21 days after the last ethanol dose, suggesting that the effect of ethanol on this system may be long-lasting. It has been suggested that the opioid-dopamine interaction in the mesolimbic system may be of importance to the mechanism of ethanol addiction [252,274]. Agonists to the µ- and δ-opioid receptors increase extracellular concentrations of dopamine which, in consequence, leads to reward responses. On the other hand, activation of κ-opioid receptors decrease the amount of dopamine released into the synapse, which leads to an aversive state [247]. Both repeated/chronic ethanol and morphine treatments increase prodynorphin mRNA and ir dynorphin peptide levels in the brain, including the nucleus accumbens in rodents during withdrawal [20,190,191,216,259]. The enhanced dynorphin B ir levels reported herein demonstrate a long-lasting upregulation of the dynorphin peptide system in the nucleus accumbens in response to repeated ethanol administration. In addition, chronic ethanol and cocaine treatment downregulated κ-receptor mRNA in the nucleus accumbens, which could be an adaptive response to an increased activity of the dynorphin peptide system after drugs of abuse [228]. Changes in ir dynorphin B levels were detected in the cingulate cortex of rats sacrificed 30 minutes after the last ethanol dose. It has previously been shown that the cingulate cortex is activated during acute and chronic administration of ethanol in both animals and humans [8,114,131]. Our results indicate that the dynorphin system in the cingulate cortex may be involved in these actions. These results show that repeated ethanol administration induces short- and long-term changes in ir opioid peptide levels, and especially altered ir dynorphin B levels were found in the nucleus accumbens, which may contribute in the development of alcohol dependence.
**Figure 13.** The effects of repeated ethanol (2 g/kg b.w./twice daily i.p.) or saline administration on ir MEAP levels in the pituitary gland (Pt); hypothalamus (HT), striatum (Str), nucleus accumbens (NAcc), ventral tegmental area (VTA), cingulate cortex (CC), hippocampus (HP) and the periaqueductal gray (PAG). The ir MEAP levels were measured at 30 min, 5 days or 21 days after the last dose of ethanol or saline. The values represent percent of the control values (mean ± SEM), where the controls are 100 percent. (ANOVA, Fisher’s post-hoc test, *p<0.05)

**Figure 14.** The effects of repeated ethanol (2 g/kg b.w./twice daily i.p.) or saline administration on ir dynorphin B levels in the pituitary gland (Pt); hypothalamus (HT), striatum (Str), nucleus accumbens (NAcc), ventral tegmental area (VTA), cingulate cortex (CC), hippocampus (HP) and the periaqueductal gray (PAG). The ir dynorphin B levels were measured at 30 min, 5 days or 21 days after the last dose of ethanol or saline. The values represent percent of the control values (mean ± SEM), where the controls are 100 percent. (ANOVA, Fisher’s post-hoc test, *p<0.05)

**4.3 Neonatal handling in male and female Sprague-Dawley rats (papers I and IV)**

In paper I and IV, Sprague-Dawley male and female rat pups were subjected to 15 min of daily neonatal handling (H15) between day 1-21. The purpose was to investigate if H15 could induce long-term behavioural effects, which were evalu-
Involvement of the Opioid System in High Alcohol Consumption

ated using the open field test and the elevated plus-maze test (paper I), and neurochemical changes in the dynorphin (papers I and IV), enkephalin (paper IV) and the N/OFQ (paper IV) systems. Observations in the open field and the elevated plus-maze tests, performed at 3 months of age, showed that male H15 rats exhibit attenuated fearfulness in novel environments as compared to non-handled male rats (Figure 15 and 16A-B). This is in agreement with previous studies demonstrating that handling induces long-term positive behavioural effects [11,144,171].

**Figure 15.** Effects of H15 on the time course of locomotion and rearing behaviours in the open field arena. Results are presented as mean ± SEM numbers of counts recorded during 30 min of open field observation (n = 10 in each group). ∆ p < 0.005 compared to non-handled group (ANOVA, Fisher’s post-hoc test).

**Figure 16A-B.** Mean ± SEM numbers of entries to open arms and total time spent in the open arms at the elevated plus-maze test (n = 10 in each group). **p<0.01 compared to non-handled group (Student’s t-test).

Neurochemical results in paper I indicated a persistent upregulation of the dynorphin system in the pituitary gland, the hypothalamus, the hippocampus, the striatum, the medulla oblongata and the midbrain in male H15 rats (Table 3). In paper IV using female rats, additional brain areas were analysed; the frontal cortex, the nucleus accumbens, the amygdala, the substantia nigra, the VTA and the PAG.
Female H15 rats had increased ir dynorphin B levels in the PAG and decreased ir levels of dynorphin B in the amygdala and the frontal cortex (Table 4). In contrast to male rats, areas related to the HPA axis were unaffected, which suggests that H15 induces different effects depending on the gender of the pup. The most prominent effects of H15 in female rats were found in the PAG, which plays a crucial role in the integration of an animal’s behavioural, somatic, and autonomic responses to threat, stress and pain [18]. In addition to dynorphin B, ir N/OFQ and MEAP levels were increased in H15 rats as well. Environmental manipulations early in life are of importance for developmental processes later on, both for humans and animals. Taken together, H15 induces persistent changes in neuropeptide systems in the pituitary gland and brain areas implicated in emotional processing. In addition, handled rats have reduced anxiety-like behaviour, which persist into adulthood. These results suggest that the opioid system is involved in the processes affected by environmental manipulations.

<table>
<thead>
<tr>
<th>DYNORPHIN A</th>
<th>DYNORPHIN B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Handled</strong></td>
<td><strong>Controls</strong></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>146.2 ±12.0**</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>581.8 ± 49.5***</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>44.2 ± 2.3</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Striatum</td>
<td>51.0 ± 3.0**</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Midbrain</td>
<td>21.9 ± 1.7</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Cortex</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
</tbody>
</table>

**Table 3.** Ir peptide levels in different brain regions in male rats 10 weeks after H15. Values (fmol/mg) represent mean ± SEM (n). *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test).
Involvement of the Opioid System in High Alcohol Consumption

Table 4. Ir peptide levels in different brain regions in female rats two months after H15. Values (fmol/mg tissue) represent mean ± SEM (n). *p<0.05, **p<0.01 (Student’s t-test). # The values are below detection limit in the radioimmunoassay.

<table>
<thead>
<tr>
<th>Region</th>
<th>N/OFQ</th>
<th>Dynorphin B</th>
<th>MEAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Handled</td>
<td>Non-handled</td>
<td>Handled</td>
</tr>
<tr>
<td>Anterior lobe</td>
<td>- #</td>
<td>3.42±0.41</td>
<td>(8) 2.60±0.30</td>
</tr>
<tr>
<td>Neuro intermediate lobe</td>
<td>- #</td>
<td>463.5±48.33</td>
<td>(8) 487.7±56.6</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>11.6±4.68</td>
<td>(10) 10.4±0.5</td>
<td>(10) 6.4±0.6</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>2.6±0.32</td>
<td>(9) 2.7±0.35</td>
<td>(8) 15.2±1.7</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>3.7±0.73</td>
<td>(8) 2.8±0.25</td>
<td>(7) 1.3±0.21</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.1±0.15</td>
<td>(8) 0.8±0.07</td>
<td>(10) 3.9±0.45</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>2.0±0.73</td>
<td>(8) 2.6±0.3</td>
<td>(9) 31.2±3.0</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1.1±0.20</td>
<td>(8) 1.4±0.2</td>
<td>(8) 0.4±0.04 **</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.3±0.21</td>
<td>(10) 2.1±0.3</td>
<td>(9) 5.0±0.77</td>
</tr>
<tr>
<td>Amygdala</td>
<td>2.2±0.20</td>
<td>(5) 1.9±0.1</td>
<td>(9) 1.8±0.15 *</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>5.3±0.45 *</td>
<td>(10) 3.9±0.45</td>
<td>(10) 2.3±0.28**</td>
</tr>
</tbody>
</table>

4.4 Neonatal manipulation on ethanol intake and the brain opioid and dopamine systems in Wistar rats (paper V)

In this study we examined behavioural and neurochemical effects of daily 6 h of maternal separation (MS360) or H15 during day 1-21 in male Wistar rat pups. In contrast to H15, prolonged periods (>1 h) of maternal separation during the first weeks of life result in animals with decreased ability to cope with stressors [11,99,141,143]. On day 5 the frequencies of ultrasonic calls were recorded prior to separation of the pups. During the separation procedure the rat pups were inspected daily to establish the day of eye opening. On postnatal day 22, the animals were weaned and their anxiety-like behaviour was tested in the elevated plus-maze. To investigate the long-term neurochemical effects of H15 and MS360, the opioid dynorphin B and MEAP peptides were analysed using radioimmunoassay in the pituitary gland and other brain areas 7 weeks after the neonatal manipulation procedure. In addition, density of the opioid κ- and δ-receptors and D1- and D2-like receptors were measured in the brain using quantitative autoradiography. The remaining rats (n=26-28/group) were tested for voluntary ethanol consumption during 4 weeks. The rats had a free choice between two bottles containing ethanol (2-8% ethanol concentration) and water during this period. After 3 weeks of access to ethanol and water, 8 rats in each group with the highest ethanol intake, were exposed to 1 h of restraint stress for 4 consecutive days to evaluate the effects on ethanol drinking. After 1 additional week of voluntary ethanol intake the rats were sacrificed by decapitation and the density of brain opioid and dopamine receptors were analysed.
Observations during the neonatal manipulation period (day 1-21) showed that MS360 rats opened their eyes later compared to both H15 and control rats (Figure 17 A), suggesting a delayed development in the MS360 rats. The ultrasonic call test on day 5 showed that MS360 rats vocalised more (Figure 17 B) and showed increased anxiety-related behaviour in the plus-maze test on day 22 (Figure 17 C-D) compared to H15 or control rats. This suggests an increased anxiety-like response in MS360 rats.

**Figure 17 A-D.** Effects of H15 and MS360 on different behavioural parameters during childhood. **A.** Time point for eye opening in the MS360, H15 and control group. The figure illustrates the percentage of animals in each group opening their eyes during postnatal days 12 to 17. **B.** Frequencies of ultrasonic calls during one minute on postnatal day 5 (n=12/group). **C.** Number of entries into the open arms (mean ± SEM) in the elevated plus-maze test at postnatal day 22 (n=12/group). **D.** Latency (s) to the first open arm entry (mean ± SEM) in the elevated plus-maze test on postnatal day 22 (n=12/group). *p<0.05 compared to control rats, #p<0.05 compared to H15 rats (Mann-Whitney U-test).

Neurochemical analysis 7 weeks after the neonatal manipulation (Table 5) revealed higher ir dynorphin B and MEAP levels in H15 rats in the hypothalamus compared to MS360 and/or control rats. In the neurointermediate pituitary lobe higher ir dynorphin B levels were fond in both H15 and MS360 compared to con-
Involvement of the Opioid System in High Alcohol Consumption

trol rats. Dynorphin [152] and enkephalin [215] peptides are colocalised with CRF in hypothalamic neurons, but their exact role in the regulation of the stress response is still not well understood. Our results demonstrate that H15 affects dynorphin peptides in areas directly related to the HPA-axis in a similar way in both Wistar and Sprague-Dawley male rats (paper I). In addition, besides the dynorphin system, also the enkephalin system may have an important role in the changed stress responsivity seen in these animals. Moreover, ir dynorphin B levels were affected in the amygdala, the substantia nigra and the PAG, areas related to emotional processing in the brain. Quantitative autoradiography of the opioid κ- and δ-receptors (Table 6 and 7) revealed minor changes in opioid receptor density after neonatal manipulation, suggesting that peptide levels were more easily affected. Taken together, these results suggest that early experiences, such as short (neonatal handling) and long periods of neonatal separation daily until weaning, induce long-lasting changes especially in the dynorphin B peptide system, and dysregulation in this system may contribute to the differences in behaviour between these rats as adults.

Table 5. Ir dynorphin B and MEAP levels in various brain regions and in the pituitary gland 7 weeks after H15 or MS360 and in control rats (n=7-10/group). The values represent the mean ± SEM and are expressed as fmol/mg tissue. ##p<0.01, ###p<0.001 comparison with H15, *p<0.05, **p<0.01, ***p<0.001 comparison with control rats (ANOVA, Fisher’s post-hoc test). n.d.=not detectable
**Karolina Ploj**

Table 6. Quantitative autoradiography of κ-receptor binding at 7 weeks after neonatal handling for 15 min (H15) or maternal separation for 360 min (MS360) daily during postnatal day 1-21 and control rats. Values represent mean specific binding of \(^3\)H]CI977 in fmol/mg brain tissue ± SEM (n=7-8 rats). Specific binding was >50% in brain regions with high binding. Regional measures were carried out at the bregma co-ordinates according to Paxinos and Watson (1997).

<table>
<thead>
<tr>
<th>Region</th>
<th>Bregma</th>
<th>H15</th>
<th>MS360</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial layers</td>
<td>5.20 mm</td>
<td>2.4±0.2</td>
<td>1.9±0.4</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Deep layers</td>
<td>5.20 mm</td>
<td>3.0±0.4</td>
<td>3.3±0.4</td>
<td>3.0±0.4</td>
</tr>
<tr>
<td>Cingulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial layers</td>
<td>1.60 mm</td>
<td>5.4±0.4</td>
<td>5.5±0.3</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Deep layers</td>
<td>1.60 mm</td>
<td>5.7±0.4</td>
<td>5.9±0.3</td>
<td>5.7±0.4</td>
</tr>
<tr>
<td>Parietal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial layers</td>
<td>-2.56 mm</td>
<td>2.6±0.2</td>
<td>2.5±0.1</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>Deep layers</td>
<td>-2.56 mm</td>
<td>1.8±0.2</td>
<td>2.0±0.1</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Occipital</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial layers</td>
<td>-5.30 mm</td>
<td>1.4±0.2</td>
<td>2.1±0.3</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Deep layers</td>
<td>-5.30 mm</td>
<td>1.0±0.3</td>
<td>1.3±0.3</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>Temporal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial layers</td>
<td>-5.30 mm</td>
<td>2.5±0.3</td>
<td>2.5±0.2</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>Deep layers</td>
<td>-5.30 mm</td>
<td>2.6±0.3</td>
<td>2.7±0.1</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>Claustrum</td>
<td>1.60 mm</td>
<td>15.6±0.8</td>
<td>14.1±0.8</td>
<td>14.7±0.7</td>
</tr>
<tr>
<td>Endopiriform nucleus</td>
<td>1.60 mm</td>
<td>15.7±1.1</td>
<td>16.0±0.5</td>
<td>16.5±1.0</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shell</td>
<td>1.60 mm</td>
<td>14.1±0.9</td>
<td>15.2±0.7</td>
<td>14.4±0.7</td>
</tr>
<tr>
<td>Core</td>
<td>1.60 mm</td>
<td>10.3±0.5</td>
<td>11.2±0.6</td>
<td>10.8±0.5</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>1.60 mm</td>
<td>6.7±0.3</td>
<td>6.8±0.3</td>
<td>6.4±0.3</td>
</tr>
<tr>
<td>Olfactory tubercle</td>
<td>1.60 mm</td>
<td>13.5±0.7</td>
<td>13.7±0.7</td>
<td>13.2±0.7</td>
</tr>
<tr>
<td>Lateral globus pallidus</td>
<td>-0.92 mm</td>
<td>1.4±0.2</td>
<td>1.6±0.2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Bed nucleus stria terminalis</td>
<td>-0.92 mm</td>
<td>9.6±0.6</td>
<td>8.9±1.0</td>
<td>10.2±0.8</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>-0.92 mm</td>
<td>11.5±0.9</td>
<td>11.7±0.8</td>
<td>11.4±1.4</td>
</tr>
<tr>
<td>Thalamus</td>
<td>-2.56 mm</td>
<td>1.6±0.1</td>
<td>1.6±0.1</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>-2.56 mm</td>
<td>7.8±0.5</td>
<td>8.2±0.5</td>
<td>7.8±0.6</td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial</td>
<td>-2.56 mm</td>
<td>7.1±0.8</td>
<td>6.7±0.5</td>
<td>6.4±0.4</td>
</tr>
<tr>
<td>Basomedial</td>
<td>-2.56 mm</td>
<td>6.3±0.4</td>
<td>6.8±0.5</td>
<td>7.1±0.3</td>
</tr>
<tr>
<td>Basolateral</td>
<td>-2.56 mm</td>
<td>5.2±0.2</td>
<td>5.8±0.4</td>
<td>5.8±0.2</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>-5.30 mm</td>
<td>1.9±0.3</td>
<td>2.1±0.2</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Subperficial gray of the superior colliculus</td>
<td>-5.80 mm</td>
<td>8.1±0.6</td>
<td>7.3±0.5</td>
<td>8.1±0.5</td>
</tr>
<tr>
<td>Intermediate gray of the superior colliculus</td>
<td>-5.80 mm</td>
<td>6.2±0.5</td>
<td>5.7±0.4</td>
<td>5.9±0.6</td>
</tr>
<tr>
<td>Periaqueductual gray</td>
<td>-5.80 mm</td>
<td>10.4±1.4</td>
<td>8.8±0.6</td>
<td>8.8±0.8</td>
</tr>
</tbody>
</table>
Table 7. Quantitative autoradiography of δ-receptor binding at 7 weeks after neonatal handling for 15 min (H15) or maternal separation for 360 min (MS360) daily during postnatal day 1-21 and control rats. Values represent mean specific binding of \[^3H\]Deltorphin II in fmol/mg brain tissue ± SEM (n=7-8 rats). Specific binding was >70% in brain regions with high binding. Regional measures were carried out at the bregma co-ordinates according to Paxinos and Watson (1997). **p<0.01 comparison with control rats (ANOVA, Fisher post-hoc test)
Table 8. Quantitative autoradiography of D₁-like receptor binding 7 weeks after neonatal handling for 15 min (H15) or maternal separation for 360 min (MS360) daily during postnatal day 1-21 and control rats. Values represent mean specific binding of [125I]SCH 23982 in fmol/mg brain tissue ± SEM (n=7-8 rats). Specific binding was >70-90 % in brain regions with high binding. Regional measures were carried out at the bregma co-ordinates according to Paxinos and Watson (1997). *p<0.05 comparison with control rats (ANOVA, Fisher's post-hoc test)
Involvement of the Opioid System in High Alcohol Consumption

Table 9. Quantitative autoradiography of $\text{D}_2$-like receptor binding 7 weeks after neonatal handling for 15 min (H15) or maternal separation for 360 min (MS360) daily during postnatal day 1-21 and control rats. Values represent mean specific binding of $[^{125}\text{I}]$Iodosulpiride in fmol/mg brain tissue ± SEM (n=7-8 rats). Specific binding was >90 % in brain regions with high binding. Regional measures were carried out at the bregma co-ordinates according to Paxinos and Watson (1997).

<table>
<thead>
<tr>
<th>Region</th>
<th>Bregma</th>
<th>H15</th>
<th>MS360</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limbic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep layers</td>
<td>3.20 mm</td>
<td>0.76±0.13</td>
<td>1.01±0.14</td>
<td>0.82±0.13</td>
</tr>
<tr>
<td>Cingulate</td>
<td>1.60 mm</td>
<td>0.45±0.07</td>
<td>0.54±0.12</td>
<td>0.31±0.05</td>
</tr>
<tr>
<td>Frontal-parietal</td>
<td>1.60 mm</td>
<td>0.56±0.10</td>
<td>0.86±0.15</td>
<td>0.54±0.08</td>
</tr>
<tr>
<td>Parietal</td>
<td>-2.56 mm</td>
<td>0.39±0.11</td>
<td>0.36±0.10</td>
<td>0.40±0.09</td>
</tr>
<tr>
<td>Offactory bulb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>External plexiform layer</td>
<td>5.20 mm</td>
<td>5.53±1.03</td>
<td>7.23±0.89</td>
<td>6.59±1.27</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shell</td>
<td>1.60 mm</td>
<td>15.03±1.17</td>
<td>16.43±1.32</td>
<td>16.29±1.21</td>
</tr>
<tr>
<td>Core</td>
<td>1.60 mm</td>
<td>15.30±0.92</td>
<td>16.84±1.10</td>
<td>16.83±0.84</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial</td>
<td>1.60 mm</td>
<td>22.33±1.16</td>
<td>23.48±1.07</td>
<td>24.07±1.01</td>
</tr>
<tr>
<td>Lateral</td>
<td>1.60 mm</td>
<td>23.99±1.15</td>
<td>25.00±1.07</td>
<td>26.00±1.16</td>
</tr>
<tr>
<td>Offactory tubercle</td>
<td>1.60 mm</td>
<td>11.07±0.86</td>
<td>13.29±0.63</td>
<td>12.15±0.98</td>
</tr>
<tr>
<td>Lateral globus pallidus</td>
<td>-0.92 mm</td>
<td>3.62±0.34</td>
<td>3.35±0.27</td>
<td>3.30±0.20</td>
</tr>
<tr>
<td>Preoptic area</td>
<td>-0.92 mm</td>
<td>2.33±0.31</td>
<td>2.09±0.30</td>
<td>2.25±0.28</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>-2.56 mm</td>
<td>1.16±0.12</td>
<td>1.14±0.14</td>
<td>1.22±0.14</td>
</tr>
<tr>
<td>Lateral hypothalamic area</td>
<td>-2.56 mm</td>
<td>1.89±0.28</td>
<td>1.81±0.24</td>
<td>2.10±0.25</td>
</tr>
<tr>
<td>Dorsal hypothalamic area</td>
<td>-2.56 mm</td>
<td>2.04±0.16</td>
<td>2.08±0.26</td>
<td>2.34±0.17</td>
</tr>
<tr>
<td>Zona incerta</td>
<td>-2.56 mm</td>
<td>1.28±0.18</td>
<td>1.31±0.18</td>
<td>1.17±0.15</td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>-2.56 mm</td>
<td>1.91±0.23</td>
<td>2.41±0.33</td>
<td>2.79±0.35</td>
</tr>
<tr>
<td>Thalamus</td>
<td>-2.56 mm</td>
<td>0.21±0.03</td>
<td>0.19±0.03</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>Laterodorsal thalamic nucleus</td>
<td>-2.56 mm</td>
<td>0.38±0.04</td>
<td>0.39±0.05</td>
<td>0.32±0.04</td>
</tr>
<tr>
<td>Lateral habenular nucleus</td>
<td>-2.56 mm</td>
<td>0.62±0.08</td>
<td>0.51±0.09</td>
<td>0.49±0.06</td>
</tr>
<tr>
<td>Endopiriform nucleus</td>
<td>-2.56 mm</td>
<td>0.66±0.10</td>
<td>0.62±0.10</td>
<td>0.75±0.08</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>-5.30 mm</td>
<td>7.95±0.20*</td>
<td>6.26±0.55#</td>
<td>6.49±0.49</td>
</tr>
<tr>
<td>Hippocampus, CA1 region</td>
<td>-5.30 mm</td>
<td>0.62±0.14</td>
<td>0.32±0.09</td>
<td>0.41±0.09</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>-5.80 mm</td>
<td>4.86±0.36</td>
<td>3.64±0.37</td>
<td>4.36±0.46</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>-5.80 mm</td>
<td>3.38±0.32</td>
<td>2.38±0.28#</td>
<td>2.67±0.18</td>
</tr>
<tr>
<td>Superficial gray of the superior colliculus</td>
<td>-5.80 mm</td>
<td>1.93±0.12</td>
<td>1.39±0.19</td>
<td>2.05±0.29</td>
</tr>
<tr>
<td>Intermediate gray of the superior colliculus</td>
<td>-5.80 mm</td>
<td>1.88±0.17</td>
<td>1.44±0.18</td>
<td>1.78±0.14</td>
</tr>
</tbody>
</table>

#p<0.05 comparison with H15, *p<0.05 comparison with control rats (ANOVA, Fisher’s post-hoc test).
A higher density of D₁–like receptors (Table 8) were found in the hippocampus in H15 rats compared to controls. The hippocampus is associated with memory and learning processes [4] and dopamine receptors in the hippocampus have been suggested to play a role in memory functions in the rat [198,273]. H15 rats show improved performance in various learning tasks [170,268], which might involve the hippocampal dopaminergic system. A higher density of D₂–like receptors (Table 9) were found in the PAG in H15 rats compared to MS360 rats and in the VTA compared to MS360 and control rats. The dopaminergic activity within the VTA is increased in response to acute stress [60,126] and, interestingly, decreased D₂–like receptor mRNA expression has previously been seen in the VTA after chronic stress [72]. Maternal separation has been reported to increase sensitivity to both stress and psychostimulantia later in life [127], which may involve decreased D₂–autoreceptor function in the VTA. These results suggest that adaptive changes in D₂–autoreceptor function in the VTA may be involved in the behavioural sensitisation seen after maternal separation.

Seven weeks after the neonatal manipulation procedure H15, MS360 and control rats were tested for voluntary ethanol intake behaviour. In humans, adverse early life experiences may increase the risk of developing ethanol dependence [33,128,177,245]. This study showed that rats exposed to MS360, as a model for adverse early life experiences, initiated ethanol drinking at a higher degree and consumed more of the highest concentrated ethanol than rats in the other two groups (Figure 18). At 8% ethanol, 23% of MS360 rats had an ethanol intake over 1 g/kg/day whereas only 4% of the H15 and control rats had an intake over 1 g/kg/day. The rats that initiated drinking and had the highest ethanol intake in each group (n=8 rats/group) were selected for further experiments. At 8% ethanol, H15 and MS360 rats had the lowest and highest ethanol intake and preference, respectively. Analysis of the ethanol preference at 8% ethanol solution showed that MS360 rats had a higher preference (19%) than H15 (5%), p<0.01, but not statistically significantly higher than control rats (11%). In contrast to MS360, H15 results in animals with decreased emotionality in response to stress persisting into adulthood [11,99,143]. Interestingly, this study shows a negative correlation between H15 and a high ethanol intake later in life, as fewer rats in this group initiated voluntary ethanol consumption. H15 rats were also found to consume less ethanol during 4 weeks of access than MS360 and control rats.

Restraint stress 3 weeks after first access to ethanol increased the ethanol intake in all three groups, although not statistically significant in the control group. This is in agreement with previous animal studies, which has shown that various stressful experiences can enhance intake of drugs of abuse [26,74,100,200,209,218,236]. Moreover, human studies indicate that people drink in response to different kinds of stress, such as economic stress, job stress, and marital problems, and that the more severe and chronic the stressor, the greater the ethanol consumption [214]. In addi-
Involvement of the Opioid System in High Alcohol Consumption

...stress has been implicated as an important factor contributing to relapse in abstinent alcohol addicts [29].

**Figure 18.** Mean ethanol intake (g/kg/day) in H15, MS360 and control rats (n=8/group) at 10-13 weeks of age, tested for two-choice selection of ethanol vs. water during 4 weeks. *p<0.05; **p<0.01 compared to control rats, +p<0.05; ++p<0.01 compared to MS360 rats (Mann-Whitney U-test).

Effects on brain opioid and dopamine receptor densities after the ethanol intake period are shown in Figure 19. The κ-system was affected by H15 as shown by altered peptide levels, which may relate to the lower ethanol intake in H15 rats. After ethanol drinking, effects could also be detected on receptors, with lower κ-receptor density in H15 rats. The frontal cortex sends projections to the nucleus accumbens, and can modulate its function and thereby response to drug-related stimuli [134]. MS360 and control rats had lower density of δ-receptors in the nucleus accumbens (trend level, p=0.065 in control rats compared to H15 rats) and higher density of δ-receptors in the frontal cortex after ethanol drinking compared to H15 rats. In addition to δ-receptors, lower density of D₁-like receptors were found in the nucleus accumbens (core and shell) in MS360 rats compared to H15 rats. Earlier studies have demonstrated a high dopaminergic activity in the nucleus accumbens in response to ethanol administration, which can be modulated by opioids [135,246,247]. Thus, the lower density of δ- and D₁-like receptors in the nucleus accumbens in MS360 rats could act to oppose the increased levels of dopamine and enkephalin as an adaptive response after ethanol drinking. Other brain areas where MS360 rats had lower density of D₁-like receptors compared to H15 and/or control rats were the ventral pallidum, the globus pallidus, the suprachiasmatic nucleus, the interpeduncular nucleus rostral and the superficial layer of the frontal-parietal cortex. These changes may be caused by adaptive mechanisms after ethanol consumption.
Figure 19. Quantitative autoradiography of receptor binding in H15, MS360 and control rats one month after access to ethanol. Values represent mean±SEM specific binding of A. [³H]Deltorphin II (δ-receptors), B. [³H]CI977 (κ-receptors), C. [¹²⁵I]SCH 23982 (D₁-like receptors) and D. [¹²⁵I]Iodosulpride (D₂-like receptors) expressed as fmol/mg brain tissue (n=7-8 rats). +p<0.05, ++p<0.01, +++p<0.001 comparison with MS360 rats, *p<0.05, **p<0.01 comparison with control rats (ANOVA, Fisher’s post-hoc test). Abbreviations: AcbC=Nucleus accumbens Core, AcbSh=Nucleus accumbens Shell, FrCx (sl)=Frontal cortex superficial layers, FrPaCx=Frontal-parietal cortex, FrPaCx (sl)=Frontal-parietal cortex superficial layers, Hi=Hippocampus, Hy=Hypothalamus, InG=Intermediate gray layer of the superior colliculus, LGP=Lateral globus pallidus, MeA=Medial amygdala, OcCx (dl)=Occipital cortex deep layers, PAG=Periaqueductal gray, SCh=Suprachiasmatic nucleus, SN=Substantia nigra, SuG=Superficial gray layer of the superior colliculus, Th=Thalamus, VP=Ventral pallidum, VTA=Ventral tegmental area

The H15 rats were found to differ from the other rats with higher density of D₂-like binding sites in the VTA after the neonatal manipulation procedure, both before and after access to ethanol. MS360 and control rats showed higher ethanol intake compared to H15 rats. A dysfunction in D₂-autoreceptors within the VTA may increase the sensitivity to ethanol with a higher release of dopamine in response to ethanol in these animals. A previous study has shown that chronic self-administration of psychostimulantia decreases D₂-autoreceptor levels in the VTA and D₁-like receptors in the shell of the nucleus accumbens [248]. These results suggest an important role for the VTA in the interaction of emotional stress, such as neonatal manipulation and ethanol intake behaviour.
Figure 20. Computer-enhanced colour autoradiograms of coronal sections of the rat brain. These illustrate differences in receptor density in the nucleus accumbens and the VTA in MS360, H15 or control rats one month after access to ethanol. For more detailed results see Figure 19. A. δ-opioid receptors labeled with \(^{3}H\)Deltorphin II, B. D\(_{1}\)-like receptors labeled with \(^{125}I\)SCH 23982, C. D\(_{2}\)-like receptors labeled with \(^{125}I\)Iodosulpride.
5 SUMMARY AND CONCLUSIONS

- The C57BL/6J mice, which show an innate high preference to ethanol, differ in basal ir opioid dynorphin B and MEAP levels in distinct brain areas compared to non-ethanol preferring DBA/2J mice. Nucleus accumbens, an area associated with reinforcing effects of drugs of abuse, was found to contain lower ir opioid levels in C57BL/6J mice. This provides further evidence that the opioid system is involved in mechanisms underlying the divergent ethanol-intake behaviours between ethanol-preferring and non-ethanol preferring animals. Five days after withdrawal of ethanol, which had been voluntarily consumed during one month, C57BL/6J mice were found to contain reduced ir opioid peptide levels in the brain compared to control C57BL/6J mice drinking water only. This was most prominent in the VTA, an area that also was found to contain reduced ir levels of N/OFQ. This suggests that ethanol consumption induces a dysregulation in the brain opioid and N/OFQ peptide systems. This dysregulation was found to be reversible. Twenty-one days after cessation of ethanol, the ir levels of opioids and N/OFQ were the same as in controls.

- Repeated i.p. administration of ethanol (2 g/kg bw/dose, twice daily) for 13 consecutive days in Sprague-Dawley rats, induces short- and long-term effects on opioid peptide levels in distinct brain regions. Increased ir dynorphin B levels 30 min after the last dose of ethanol in the nucleus accumbens, might be the result of a feedback response to ethanol-induced increase of dopamine. Increased ir dynorphin B levels were also present 21 days after the last dose of ethanol. Enhanced dynorphin actions could contribute to dysphoria associated with ethanol withdrawal.

- Neonatal handling can cause a reduced anxiety-like behaviour as adults. The opioid system may have an important role in these effects. H15 in male rats alters ir opioid peptide levels in several brain areas, however the most prominent long-lasting effects of H15 on the opioid peptide system were seen in the hypothalamus and the pituitary gland. This supports previous findings that environmental manipulations early in life have a pronounced effect in areas directly related to the HPA axis in both humans and animals [15]. H15 rats have been shown to exhibit a decreased HPA responsivity to stress. This suggests a close interaction between the opioid system and modulators of the HPA axis, such as the CRF and glucocorticoids. In female rats, opioid levels in the hypothalamus and the pituitary gland were unaffected by H15, suggesting a gender specific effects of H15. Environmental manipulations early in life are of importance for developmental processes later on, both for humans and animals. Here we present data that suggests that the opioid system is involved in the processes affected by environmental manipulations.

- Humans exposed to chronic stress during childhood may have an increased risk of developing psychopathology and/or alcohol dependence as adults [33,128,177,183,245,275]. Repeated maternal separation in rats, used as an animal model to study adverse early life events on physiology and behaviour later in life,
showed that MS360 induces a delayed normal development, and an increased anxiety-like behaviour. In addition, later in life MS360 rats initiated ethanol drinking at a higher degree and consumed more ethanol than especially the H15 rats, which consumed a low amount of ethanol. The neurochemical changes in the opioid and dopamine systems observed in H15 rats, may play a protective role against initiation of excessive use of ethanol late in life, whereas prolonged periods of maternal separation as in MS360 instead results in rats more susceptible to high ethanol consumption.
6 SAMMANFATTNING PÅ SVENSKA

Både arv och miljö har betydelse för utvecklandet av ett alkoholmissbruk. Syftet med denna avhandling är att undersöka det endogena opioida systemet i hjärnan och dess inverkan på initiering och upprätthållande av alkoholkonsumtion. Alkohol-prefererande C57BL/6J möss skiljer sig från DBA möss, som undviker alkohol, genom att ha lägre basala nivåer av de endogena opioida peptiderna dynorfin B och Met-enkefalin-Arg⁶Phe⁷ (MEAP) i nucleus accumbens, vilket kan vara en orsak till skillnaden i alkoholintaget mellan dessa djur. Kroniskt alkoholintag hos C57BL/6J möss och upprepad administrering av alkohol hos Sprague-Dawley råttor inducerar förändringar i dynorfin B och MEAP nivåer i nucleus accumbens och ventrala tegmentum vid specifika tidpunkter. Dessa hjärnregioner förknippas med belönande effekter av droger som missbrukas.

Som modell för miljömanipulering tidigt i livet användes daglig neonatal hantering i 15 min (H15) och separering från mamman i 360 min (MS360) under postnatal dag 1-21. Daglig 15 minuters neonatal hantering (H15) under dag 1-21 hos hanråttor resulterar i minskat ångestlikt beteende, medan daglig 360 minuters separering (MS360) från mamman istället ger ett ökat ångestlikt beteende. H15 och MS360 inducerar långtidsförändringar i dynorfin B och MEAP nivåer i hjärnan, speciellt i strukturer förknippade med hypotalamus-hypofys-binjureaxeln (HPA axeln). Hos honråttor är regioner kopplade till HPA axeln opåverkade efter H15. Detta tyder på att opioida peptider i hjärnan är involverade i HPA axelns svar på stress och att dessa effekter är könsrelaterade.

Fler individer i MS360 gruppen initierar ett alkoholintag och dricker även mer alkohol senare i livet än H15 gruppen. H15 som har ett väldigt lågt alkoholintag skiljer sig även med avseende på neurokemi jämfört med både MS360- och kontrollgruppen. Därför föreslås H15 kunna inducera långtidseffekter som verkar skyddande mot högt alkoholintag senare i livet. Specifika förändringar i densitet av opioida receptorer kan ses efter kroniskt alkoholintag, som tex. en högre densitet av κ-receptorer i flera hjärnregioner liksom förändringar i δ-receptordensitet i frontala cortex och nucleus accumbens. Sammanfattningsvis, tyder dessa resultat på att hjärnans opioida system har en viktig roll för mekanismer bakom initiering och upprätthållande av ett högt alkoholintag.
Involvement of the Opioid System in High Alcohol Consumption

7 ACKNOWLEDGEMENTS

Ett STORT TACK till:

Docent Ingrid Nylander, min handledare, för att just jag fick äran att vara din första doktorand, för all din kunskap du förmedlat, all hjälp på vägen, omtanke och förståelse, för att du alltid tagit dig tid och inte minst ditt lugn.

Erika, min fantastiska doktorandsyster – du är guld värd! Tack, för alla figurer, tabeller och granskande m m, m m, m m i min avhandling som du hjälpt till med allt ifrånt tidigt på morgonen till sent på natten. Utan dig hade det inte gått! Tack också för all hjälp under dessa år med labbande, djur och allt runt omkring. Som om det inte vore nog är du omtänksam, rolig, och en bra vän. En bättre rumskompis kan man inte ha! Hoppas bara att jag inte skrämmmer dig lika mycket idag, som den dag du kom.

Professor Bengt Meyerson och Marita Berg för alla kunskaper ni förmedlat om djurens beteende och all praktisk hjälp därtill.

I thank Professor Ian Kitchen for welcoming me into your laboratory at University of Surrey, Guildford, and for taking your time for introducing me to the marvellous world of autoradiography. I would also like to thank everyone in the Kitchen group for all your help during my visit. Especially, thanks to Dr Rob Goody for answering all my e-mails about autoradiography.

Docent Claudia Fahlke, för att du tog dig tid för att ge mig vetenskapliga tips och kommentarer som hjälpte mig på vägen i mitt avhandlingsskrivande.

Professor Fred Nyberg och Dr Lotta Arborelius för att ni ställde upp på min halvtidskontroll och gav mig värdefulla vetenskapliga råd.

Mina medförfattare i den här avhandlingen: Docent Abdul Mohammed, Dr Therese Pham, Docent Lena Bergström, Docent Bengt Henriksson, Docent Johan Franck och Lisa Gustavsson.

I thank Dr. Ants Kask for your dedication, all your efforts in our experiments and for fruitful collaboration.

Dr Annika Thorsell för lån av beteendeutrustning.

Min fantastiska mor för att du finns och för all hjälp under dessa år med att fylla min annars tomma frys, för pantning av tomflaskor, biltvätt, blomvård och allt annat pyssel och stöd. Du är alltid snäll och go’!
Karolina Ploj

Min kära storebror som alltid stöttat mig i livet och som tillsammans med Camilla ser till att mammas hemlagade mat och andra livsviktiga förnödenheter transporteras upp till mig i Uppsala

Bertil som alltid gör mig glad och får min mor att må bra & Christina och Robert som ser efter min mor!

Magnus Jansson för att du inte fått ett utbrott på mig för att jag inte kunde bestämma mig när avhandlingen skulle vara klar och framför allt för all hjälp med att designa till denna dära.

Min fd doktorandsyster, numera Dr Sara Lindholm, för all vetenskaplig hjälp och till Åsa Rosin för hjälp med vetenskapliga problem. Dessutom tack, Sara och Åsa för allt kuligt vi haft på konferenser och tjejsnacksmiddagarna. Fram för mer tjejsnack och middagar!

Pia Steensland, för all hjälp, roligt samarbete i doktorandrådets styrelse, Zug Spitze-vandringarna och för att du inte hoppade ut genom hotellfönstret i Garmisch-Partenkirchen i dina nattliga sömnvandringar och för att du är en så god vän på alla sätt och vis

Jonas Lindblom min dansante Hero, som förgyllt min tillvaro och gett mig kämpargläd genom att skicka mig en helt fantastisk Bowie-låt VARJE dag de senaste veckorna. Hoppas vi får svänga våra lurviga många gånger till på stället med billig öl, typ Baldis

Tina Kunz, som ser till att det blir lite tyska kommandotakter på BMC (auf, auf), för att du är en TOPPPPPPPEN tjej att leka med, snäll och god vän, för all språkgranskning och annan hjälp. Du bist eine Supermädel!

Skåning nr 1, Anna Kindlundh, BMC:s solstråle som tjohoar även klockan 6 på morgonen (till skillnad från mig) och skåning nr 2 Mathias Hallberg, min gamle kumpan, för att du har sport- och barnasinnet levande (=hockeykort, hockeytröja, hockeymugg, hockeylag, hockeyböcker, hockeyspel, mer?)

Alltid glada och goa Amanda Raine! Tack även för att du ställde upp som ”rörmokare” i mitt badrum på Golvfesten

Johan (Lizard King) Bylund som kämpat med att språkgranska min avhandling flera gånger om utom Acknowledgements!

Per-Anders (+ i kanten för västgöte), Qin, Chao, Svetlana, Barbro, Maria, Anne-Lie, Lena, Maija, Peteris, Pierre, Madeleine och alla andra BMC människor som
alla gjort att det varit ännu roligare att vara här på BMC

Annika Häger, Agneta Bergström, Erica Johansson och Agneta Hortlund för allt ni fixar och donar

Mårre Larsson & Helgi Schiöth för fantastiska förfester, huvudfester och efterfester. Tack också Helgi för att du en gång i tiden klev upp från badet hemma hos dig för att åka in till labbet och ta hand om mina prover när jag hade viktigare saker för mig.

 Alla studenter för allt labbande!

Marita & Kattis, stockholmstjejerna, som tar med mig på röj-röj

Marie, min Åsaka-Vrölhem-kompis som jag haft roligt tillsammans med i många, många, många år och som håller mig uppdaterad om vad som händer hemma i bygden. Tack även mina andra Trollis-kompisar, Malin, Anne-Lie och Corina för att ni alltid funnits där.

Lina 2 för att du en gång i tiden gav mig tak över huvudet som nyantagen doktorand, för alla trevliga pratstunder och att du kom när Totte-Linus behövde dig.

Apteket Björnen hemma i Trollhättan, för er positiva inställning till min forskarutbildning och för att jag någon gång fått komma ut i ”verkligheten” under min doktorandstid

Aptekarsocieteten för stipendier jag fått genom åren för att kunna besöka olika vetenskapliga konferenser

All personal på djuravdelningarna för att ni sköter om djuren

Alla andra trevliga vänner runtomkring mig-Malin & Roland, Jan, Freddy, Catta, Soffan och doktorander och andra vänner i och utanför Sverige
8 REFERENCES


Involvement of the Opioid System in High Alcohol Consumption


Involvement of the Opioid System in High Alcohol Consumption


Hanhinen, S., Nordic, Italian and German drinking habits, a comparison between surveys made since the 1980s, *Nordisk Alcoholtidskrift (English supplement)*, 12 (1995) 14-30.


[105] Higley, J.D., Suomi, S.J. and Linnoila, M., CSF monoamine metabolite concentrations vary according to age, rearing, and sex, and are influenced by the stressor of social separation in rhesus monkeys, *Psychopharmacology*, 103 (1991) 551-556.


Involvement of the Opioid System in High Alcohol Consumption


Involvement of the Opioid System in High Alcohol Consumption


Involvement of the Opioid System in High Alcohol Consumption


Involvement of the Opioid System in High Alcohol Consumption


Yokel, R.A., Methods of assessing the reinforcing properties of abused drugs. In M.A. Bozarth (Ed.), *Spring-Verlag*, 1987, pp. 1-34.


