Endogenous opioid peptides in drug addiction

Thesis

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Für H.-B. Schürmann

Abbreviations

5-HT	5-Hydroxytryptamine (serotonin)
AAV	Adeno-associated virus
aca	Anterior comissure, anterior
AcbC	Nucleus acumbens, core
AcbSh	Nucleus acumbens, shell
ACTH	Adrenocorticothropic hormone
ANOVA	Analysis of variance
BlA	Basolateral amygdala
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCP	1-Bromo-3-chloro-propane
cc	Corpus callosum
CDP-Star	Chemiluminescent substrate for alkaline phosphatase
CeA	Central amygdala
CPu	Caudate putamen
COGA	Collaborative study on the genetics of alcoholism
DAPI	4',6-Diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modifed Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual of mental disorders IV
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EIIa	Adenoviral promoter
ES cell	Embryonic stem cell
FCS	Fetal calf serum
FIAU	Fialuridine
FITC	Fluorescein isothiocyanate
g	Gravity
G418	Geneticin
GABA	γ-Aminobutyrc accid
γ-MSH	γ-Melanocyte stimulating hormone
GFP	Green fluorescent protein
GIRK	G-protein activated inwardly rectifying K^+ channel

GPCR	G-protein coupled receptor
HBSS	Hank's balanced salt solution
HET	Haus für experimentelle Theraphie
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
HWE	Hardy–Weinberg equilibrium
i.v.	Intravenous
ICD-10	International statistical classification of diseases and related health problems
IgG	Immunoglobulin G
kb	Kilobase
lacZ	Gene encoding ß-galactosidase
LD	Linkage disequilibrium
LIF	Leukemia inhibitory factor
LV	Lateral ventricle
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MEF	Mouse embryonic feeder
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
neo	Gene providing a neomycin resistance
NGFN	National genome research network
NMDA	N-methyl-D-aspartic acid
nor-BNI	*Nor*-binaltrophimine
ns	Not significant
NTP	Nucleoside triphosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDYN	Preprodynorphin
PENK	Preproenkephalin
PFA	Paraform aldehyde
Pgk-1	Phosphoglycerate kinase 1 promoter
POMC	Proopiomelanocortin
QTL	Quantitative trait locus
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Rotations per minute
SCID	Structured clinical interview for DSM disorders
SDS	Sodium dodecyl sulfate

SEM	Standard error of mean
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate
ß-LPH	ß-lipotripic hormone
SYBR	Cyanine dye to stain DNA
TE	Tris EDTA
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
vg	Virusgenome
VTA	Ventral tegmental area
WHO	World health organization
wt	Wild type
X-gal	Bromo-chloro-indolyl-galactopyranoside

Contents

Chapter 1

Introduction

Drug addiction is a disease causing major social and health problems in human society and many efforts have been made to understand the underlying biological mechanisms. It is commonly referred to as a chronically relapsing disorder, which is characterized by three major aspects: (A) the compulsion to seek and take the drug combined with (B) a loss of control in limiting intake and (C) the emergence of a negative emotional state, when access to the drug is prevented (KOOB AND LE MOAL, 2005). The behavioral changes that lead to addiction develop gradually and progressively due to repeated exposure to a drug of abuse and can persist for even long periods after abstinence (NESTLER, 2004B). A focus of research in this field is to understand the mechanisms underlying the transition from controlled occasional drug use to the uncontrolled intake characteristic for addiction (KOOB AND LE MOAL, 2008). The initial drive for drug consumption is its ability to produce pleasurable effects (FELTENSTEIN AND SEE, 2008). Such a stimulus that the brain interprets as intrinsically positive or as something to be attained is called reward (CAMÍ AND FARRÉ, 2003). Basically all drugs of abuse interact with the brain reward system (KOOB, 1992; FELTENSTEIN AND SEE, 2008). The brain reward system is defined as brain circuits involved in positive reinforcement in combination with positive hedonic valence (KOOB AND LE MOAL, 2008). Today, addiction theories assume that prolonged drug use leads to a state of dysregulation of the brain reward system resulting in compulsive drug use (FELTENSTEIN AND SEE, 2008; KOOB AND LE MOAL, 2008). The mesocorticolimbic dopamine system plays a key role in reward. Basically, all addictive drugs enhance mesocorticolimbic dopamine activity (KOOB, 1992; FELTENSTEIN AND SEE, 2008), although their interactions with this system occur at different levels (CAMÍ AND FARRÉ, 2003). The dopamine system consists of projections from cell bodies in the ventral tegmental area (VTA) to limbic structures (mesolimbic pathway) and to cortical areas (mesocortical pathway). These circuits operate in parallel, but may have somewhat different roles in the addiction process (CAMÍ AND FARRÉ, 2003). The mesolimbic pathway is involved in the acute reinforcing effects of drugs of abuse, for example rewarding stimuli lead to dopamine release in the

nucleus accumbens (NAc) (DI CHIARA, 2002; KOOB, 1992; FELTENSTEIN AND SEE, 2008). Besides dopamine, other transmitter systems have been identified as playing a role in acute reinforcement and reward, including the endogenous opioid system. Opioid signaling can modulate neurotransmission of γ-aminobutyric acid (GABA) and therefore influence the activation of the mesolimbic dopamine system (WILLIAMS ET AL., 2001; BERRENDERO ET AL., 2005). In addition, an important role for the opioid system independent of dopamine signalling has been proposed (KOOB, 1992) suggesting a general implication for this system in drug addiction.

1.1 The opioid system

Drugs derived from the poppy plant *Papaver somniferum* and structurally related compounds are collectively referred to as opiates. Their use for medical purposes has a long tradition, as opiates are well known for their pain relieving and rewarding properties. Early efforts to understand the endogenous targets of opiates led to the identification of the specific receptor sites in the brain and the subsequent cloning of μ-, δ- and κ-opioid receptors (PERT AND SNYDER, 1973; KIEFFER AND GAVERIAUX-RUFF, 2002). These receptors belong to the superfamily of G-protein-coupled receptors (GPCRs). Via coupling to G_i- or G_o-proteins, they can activate a class of inwardly rectifying potassium channels, inhibit voltage-sensitive calcium channels and adenylyl cyclase. In addition, they activate a number of kinase-mediated signaling cascades (VON ZASTROW ET AL., 2003). The term opioid refers to all drugs and endogenous substances that act via these receptors. Three types of endogenous neuropeptides bind to opioid receptors, namely enkephalins, dynorphins and endorphin (GIANOULAKIS, 2004; DREWS AND ZIMMER, 2009). These opioid peptides are produced through enzymatic processing of precursor proteins encoded by three different genes (*Penk1, Pdyn, Pomc*) (AKIL ET AL., 1984). The precursor proteins and the generated opioid peptides are illustrated in figure 1.1. The enkephalins, Met-enkephalin, Leu-enkephalin and two further extended peptides are derived from the preproenkephalin precursor (PENK) (WEISINGER, 1995; AKIL ET AL., 1984; GIANOULAKIS, 2004). Preprodynorphin (PDYN) is processed to dynorphin A (1-8), dynorphin A (1-17) and dynorphin B, whereas it might also yield in Leu-enkephalin through further cleavage of dynorphin (AKIL ET AL., 1984; GIANOULAKIS, 2004; DAY ET AL., 1998; ZAMIR ET AL., 1984). Proopiomelanocortin (POMC) is a multi-functional precursor protein that gives rise to ß-endorphin and other non-opioid peptides such as adrenocorticothrophic hormone (ACTH), γ-melanocyte stimulating hormone (γ-MSH) and ß-lipotropic hormone (ß-LPH) (AKIL ET AL., 1984; GIANOULAKIS, 2004). All opioid peptides share a common N-terminal Try-Gly-Gly-Phe-Met/Leu core (MANSOUR ET AL., 1995) that is indispensable for the activation of opioid receptors (AKIL ET AL., 1997). The opioid peptides differ in their opioid receptor binding affinity (Fig. 1.2). Enkephalins have a high affinity for the δ-opioid receptor and, to a lesser extent, for μ-opioid receptors. In case of the μ-receptor, Met-enkephalin has a higher affinity compared to Leu-enkephalin. ß-endorphin activates μ- and δ-receptors with similar affinities

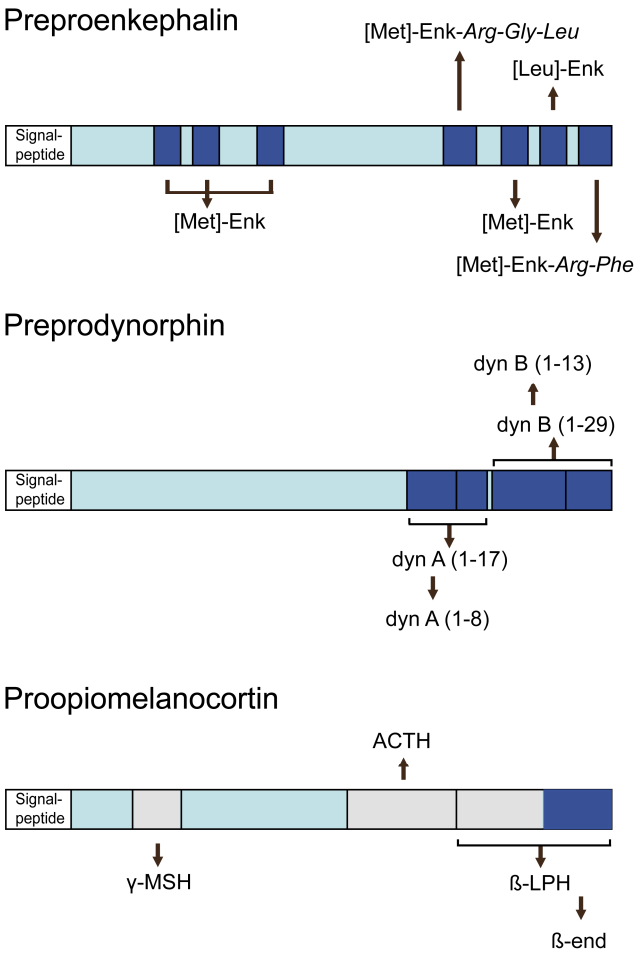

Fig. 1.1: Opioid precursor protein and their cleavage products. The opioid peptide precursor proteins preproenkephalin (top), preprodynorphin (center) and proopiomelanocortin (bottom) are illustrated. All precursor proteins contain a N-terminal signal peptide. Through enzymatic processing of preproenkephalin, one copy of Leu-enkephalin ([Leu]-Enk), four copies of Met-enkephalin ([Met]-Enk) and two further extended enkephalins can be derived. Cleavage of the preprodynorphin precursor yield dynorphin A (dyn A) and B (dyn B), two related peptides of different lengths. Processing of the proopiomelanocortin precursor yields in ß-endorphin (ß-end) and several non-opioid peptides such as ACTH, γ-MSH and ß-LPH.

and the κ-receptor is nearly exclusively activated by dynorphins (MANSOUR ET AL., 1995; CLARKE ET AL., 2003). An arginine residue in position 6 and a further extension of the dynorphin peptide sequence was shown to be important for κ-receptor binding (MANSOUR ET AL., 1995). However, it was reported that dynorphins can also bind to µ- and δ-opioid receptors with low affinities (CLARKE ET AL., 2003).

All stages of the addiction process can be monitored in animal models (see Box 1 for an example). The analysis of addiction-related behaviors in knockout mice for all components of the endogenous opioid system substantiated the role of this neuromodulatory transmitter system in drug addiction. Early studies focusing on the pharmacological effects of morphine have

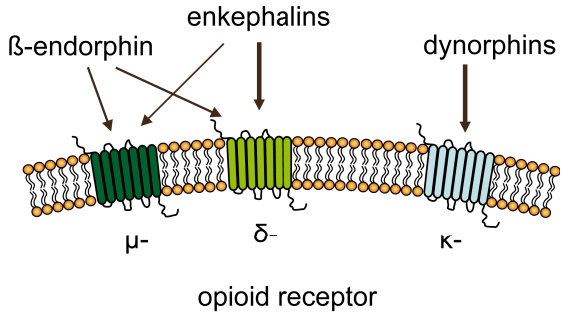

Fig. 1.2: Schematic view of the endogenous opioid receptors and their ligands. Enkephalins and ß-endorphin activate μ- and δ-opioid receptors, whereas enkephalins have a higher affinity for the δ-receptor. Dynorphins predominantly bind to κ-opioid receptors.

shown that in absence of μ-opioid receptors the rewarding properties were abolished. Additionally, the reinforcing properties of a wide range of other drugs, like nicotine, alcohol and cannabis were diminished in μ-opioid receptor knockout mice (CONTET ET AL., 2004). This indicates that μ-opioid receptor signaling influences a common molecular mechanism important for drug reward and reinforcement. The δ-opioid receptor mediates the emotional response to opioids (MATTHES ET AL., 1996; KIEFFER AND GAVERIAUX-RUFF, 2002). Additionally, results on dynorphin and κ-opioid receptor knockout mice suggested that dynorphins acting on κ-opioid receptors are involved in motivational circuits contributing to dysphoria (ZIMMER ET AL., 2001; GHOZLAND ET AL., 2002; GAVERIAUX-RUFF AND KIEFFER, 2002). The following sections describe insights into the role the endogenous opioid system plays in nicotine and alcohol addiction.

Box 1: Behavioral paradigms to study the rewarding properties of a drug:

Conditioned place-preference: Different locations are repeatedly paired with either administration of an addictive drug or administration of vehicle. In the test phase comparison of the time spent in the drug- or non-drug associated environment provides information about the rewarding properties of a drug.

Operant self-administration: In this model, the animal learns to self-administer a drug by an instrumental response contingently associated with delivery of the drug. Animals will learn this operant behavior depending on the rewarding properties of the drug.

(BERRENDERO ET AL., 2005; MARTÍN-GARCÍA ET AL., 2009; SANCHIS-SEGURA AND SPANAGEL, 2006)

1.2 The opioid system and nicotine addiction

Nicotine binds to the neuronal nicotinic acetylcholine receptors (nAChRs). These pentameric ligand-gated ion channels are expressed in many brain regions including the NAc and the VTA

(RAY ET AL., 2009; ROTH-DERI ET AL., 2008). Nicotine produces both rewarding and aversive responses by modulating mesolimbic dopaminergic transmission (BERRENDERO ET AL., 2005). Many studies demonstrated a role for the endogenous opioid system in mediating the rewarding properties of nicotine. For example, nicotine conditioned place-preference was blocked by naloxone, an unspecific opioid receptor antagonist (ZARRINDAST ET AL., 2003). A normally ineffective dose of morphine can produce place-preference by co-administration of an nAChR antagonist (REZAYOF ET AL., 2007).

Pharmacological studies revealed that acute and chronic nicotine administration alters opioid peptide levels and expression. A single injection of nicotine led to a dose-dependent increase in striatal preprodynorphin mRNA and dynorphin levels (ISOLA ET AL., 2009). Acute treatment also induced enkephalin release and increased the transcription of preproenkephalin mRNA (LIVETT ET AL., 1981; EIDEN ET AL., 1984; MACARTHUR ET AL., 1992; HÖLLT AND HORN, 1992; HOUDI ET AL., 1991; KIGUCHI ET AL., 2008). In contrast, POMC mRNA and ß-endorphin plasma levels remained unchanged after acute nicotine administration (OLIVE ET AL., 2003; WEWERS ET AL., 1994). These data indicate that acute nicotine treatment does not influence ß-endorphin, but that it modulates enkephalin and dynorphin transcription and release.

So far, μ-opioid receptor, enkephalin, dynorphin and ß-endorphin knockout mice have been studied in nicotine conditioned place-preference to assess the rewarding properties of nicotine. No change was observed in *Pdyn*$^{-/-}$ animals. However, these animals acquired nicotine self-administration with lower doses of nicotine compared to wild type mice, indicating that the reinforcing effects of nicotine are higher in the absence of dynorphin (GALEOTE ET AL., 2008). These findings underline the role of dynrophin in mediating dysphoria. The contribution of enkephalin and ß-endorphin acting on μ-receptors to reward was also approved. Nicotine was not rewarding in μ-opioid receptor, enkephalin and ß-endorphin knockout mice, as none of these mice exhibited a place-preference for nicotine (BERRENDERO ET AL., 2002, 2005; TRIGO ET AL., 2009). The contribution of enkephalin was further evaluated by microdialysis studies showing an attenuation of dopamine release in the NAc after nicotine injection. Hence, enkephalins seem to be essential for the activation of mesolimbic reward circuits by nicotine, presumably by acting on μ-receptors (BERRENDERO ET AL., 2005; DREWS AND ZIMMER, 2009).

To elucidate how enkephalin contributes to nicotine reward and reinforcement in detail, a closer analysis in specific brain regions is needed. In general, mutant mouse strains lacking genes of the endogenous opioid system are a useful tool to extend the research on this system. Nevertheless, in these mice the gene product is absent in all tissues and at all developmental stages. These mouse models are, therefore, inappropriate to elucidate the gene function in specific brain regions, cell types or during specific stages of a disease process. Conditional knockout mouse models (see Box 2), allowing the cell- or tissue-specific deletion of genes, would help to gain further insights.

Box 2: Generation of conditional knockout mice using the cre/lox technology:

LoxP sites are short phage-derived sequences that can be introduced into the mouse genome. The enzyme cre recombinase identifies these sequences and mediates recombination between two *loxP* sites. If the two *loxP* sites have the same orientation, the floxed region will be excised by cre recombinase-mediated recombination. The tissue-specific or temporary expression of cre recombinase therefore allows the specific knockout of floxed genes. (KUMAR ET AL., 2009)

1.3 The opioid system and alcohol addiction

In contrast to nicotine, ethanol acts on a variety of neurotransmitter receptors. Alcohol can inhibit *N*-methyl-*D*-aspartic acid (NMDA) receptors in a dose-dependent manner, enhance the function of $GABA_A$ and glycine receptors and potentiate 5-hydroxytryptamine-3 (5-HT, serotonin) and nACh receptor function (VENGELIENE ET AL., 2008; WANG ET AL., 1994). Ethanol exposure can influence endogenous opioid peptide expression and alter opioid peptide release in distinct brain regions associated with reward and reinforcement (GIANOULAKIS, 2004; KOOB ET AL., 1998; SPANAGEL ET AL., 1992). These changes largely depend on experimental procedures and the brain regions studied. For example, acute ethanol treatment increased PENK and POMC expression in limbic brain regions like the NAc and the hypothalamus (GIANOULAKIS, 1990; LI ET AL., 1998; RASMUSSEN ET AL., 1998). ß-endorphin release in the hypothalamus correlated with the ethanol concentration (WAELE ET AL., 1992; GIANOULAKIS, 1990). Results on enkephalin release were contradictory, some studies found increased levels after acute ethanol administration, while others did not (SCHULZ ET AL., 1980; SEIZINGER ET AL., 1983). Dynorphin levels and PDYN mRNA were changed in a region- and time dependent manner in ethanol-treated rats (GULYA ET AL., 1993; NYLANDER ET AL., 1994).

The observed expression changes suggest that ethanol exposure influences the activity of the endogenous opioid system. The endogenous opioid system might, therefore, mediate effects of ethanol (GIANOULAKIS, 2004; CRABBE ET AL., 2006; KIEFFER AND GAVERIAUX-RUFF, 2002). For example, administration of the nonspecific opioid receptor antagonists naloxone and naltrexone in rodents led to a dose-dependent decrease in ethanol consumption (ALTSHULER ET AL., 1980; FROEHLICH ET AL., 1990; GIANOULAKIS, 2001; MYERS ET AL., 1986; WEISS ET AL., 1990). Consistently, Nalmefene preferentially antagonizing µ-opioid receptor signaling suppressed ethanol self-administration in an operant model. This implicates that µ-opioid receptors play an important role in alcohol reward (JUNE, 2002). Studies on the δ-opioid receptor suggested a modulatory function as its inhibition attenuated ethanol intake (CICCOCIOPPO ET AL., 2002; HYYTIA AND KIIANMAA, 2001; KRISHNAN-SARIN ET AL., 1995A,B). Pharmacological evaluation of κ-opioid receptor signaling led to inconsistent results. Agonist treatment reduced ethanol preference in rats (SANDI ET AL., 1988; LINDHOLM

ET AL., 2001). In contrast, the antagonist nor-binaltrophimine (nor-BNI) selectively attenuated ethanol self-administration in alcohol-dependent rats (WALKER AND KOOB, 2008). All these findings demonstrate a general involvement of the opioid system in ethanol reward and addiction.

The analysis of μ-opioid receptor knockout mice in ethanol-related behaviors specified the importance of this receptor in mediating the rewarding properties of ethanol. The knockout mice showed a decreased ethanol consumption or did not self-administer ethanol at all (ROBERTS ET AL., 2000; HALL ET AL., 2001). In contrast, δ-opioid receptor knockout mice exhibited an increased ethanol preference (ROBERTS ET AL., 2001). This consumption was able to reverse the previously observed anxiogenic phenotype of these mice (ROBERTS ET AL., 2001; KIEFFER AND GAVERIAUX-RUFF, 2002). For κ-opioid receptors, a lower ethanol preference was reported (KOVACS ET AL., 2005). The deletion of preproenkephalin had no influence on voluntary ethanol consumption patterns or ethanol reward (KOENIG AND OLIVE, 2002; HAYWARD ET AL., 2004; RACZ ET AL., 2008). Opposing results were reported for ß-endorphin knockout mice. A recent study revealed a decrease in ethanol consumption and preference, perfectly matching the phenotype of μ-opioid receptor knockout animals (RACZ ET AL., 2008). Other groups observed comparable or increased ethanol consumption (GRAHAME ET AL., 2000; GRISEL ET AL., 1999; HAYWARD ET AL., 2004). It was suggested that different ambient stress levels led to these divergent results (RACZ ET AL., 2008). It is well known that stress affects ethanol consumption in mice, and ß-endorphin knockout and wild type mice differed significantly in their stress-induced ethanol consumption (RACZ ET AL., 2003, 2008). Female preprodynorphin knockout mice exhibited a significantly lower preference in a two-bottle choice paradigm, which is in line with the phenotype of κ-opioid receptor knockout mice (BLEDNOV ET AL., 2006). Since for both strains a reduced saccharine preference was reported, a more global effect on taste preference was suggested (KOVACS ET AL., 2005; BLEDNOV ET AL., 2006).

Results obtained in knockout mice provided further insights. δ-opioid receptors seem to influence alcohol intake via modulation of anxiety-related behaviors. The role for dynorphin acting on κ-opioid receptors might be due to a decreased orosensory reward of alcohol. The rewarding properties of ethanol are mostly mediated by ß-endorphin acting on μ-opioid receptors.

The contribution of genetic risk factors to addiction disorders, including alcoholism, is well established (REICH ET AL., 1999; CRABBE, 2002). The multiple genes contributing to the aetiology of alcoholism can be accessed by different approaches. One procedure is the analysis of genetic differences between groups of alcoholic patients and healthy controls. Data obtained in animals studies and information about biological pathways are merged to select possible candidate genes. In case of the endogenous opioid system, the knowledge gained in

animal experiments was already transferred to addiction treatment in humans. The non-specific opioid receptor antagonists naltrexone is already administered for the pharmacotherapy of alcohol addiction (KREEK ET AL., 2002; DREWS AND ZIMMER, 2009). Initial case-control studies of genetic variants mainly focused on the μ- and the δ-opioid receptor. In case of the μ-opioid receptor, the A118G coding variant, leading to an exchange of asparagin to aspartate at position 40, was of particular interest in association studies. Although some groups found higher frequencies for hetero- or homozygous A allele-carriers among alcoholics, the results remained inconclusive, as others reported negative findings (ROMMELSPACHER ET AL., 2001; TOWN ET AL., 1999; BERGEN ET AL., 1997; FRANKE ET AL., 2001; GELERNTER ET AL., 1999). A meta-analysis of this variant did not reveal evidence of an association with alcoholism (ARIAS ET AL., 2006). However, there were reports connecting the Asp40 form of the μ-opioid receptor with higher therapheutic effects of naltrexone and naloxone. This might be due to differences in antagonist-induced disinhibition of the hypothalamic-pituitary-adrenal (HPA) axis (KIM ET AL., 2009; CHONG ET AL., 2006; WAND ET AL., 2002). For other μ-opioid receptor coding variants association results were as well contradictory (XUEI ET AL., 2007). Concerning the δ-opioid receptor, two coding single nucleotide polymorphisms (SNPs) were studied. Neither the G80T nor the T921C variant showed association with alcoholism in alcohol-dependent Taiwanese Hans or Caucasians (LOH ET AL., 2004; FRANKE ET AL., 1999). Loh et al. also evaluated three SNPs (G36T, C459T, A843G) in the *OPRK1* gene encoding the κ-opioid receptor and found no association with alcoholism in Taiwanese Han subjects (LOH ET AL., 2004). Variants in endogenous opioid peptide genes were only analyzed by two groups. Geijer et al. did not find an association with a GC/AT base pair exchange located 301bp upstream of *PDYN* (GEIJER ET AL., 1997). In the study of Chan et al., a CA repeat in the 5' flanking region of *PENK* was not associated with alcohol dependence in populations of Asian, African-American and Caucasian origin (CHAN ET AL., 1994). To analyze a putative role of variants in opioid peptide genes a systematic evaluation of these gene loci is needed.

1.4 Aim of this work

Since the discovery of opioid receptors and the first pharmacological studies using morphine derivates, a tremendous amount of information has accumulated, indicating that the endogenous opioid system plays a central role in mediating addiction-related behaviors. Depending on the addictive drug, enkephalin and ß-endorphin vary in their contribution to reward. What is the specific contribution of these peptides? To gain further insights into different effects of opioid peptides in distinct brain regions, new tools are demanded. One aim of this work was therefore the generation of a new mouse model that enables the cell- or tissue-specific knockout of enkephalin. This will permit to further analyze the contribution of enkephalin for example to nicotine reward . Stereotactic surgery methods to assess relevant brain regions will be established for the subsequent analysis of this mouse model.

Data obtained in animal studies clearly demonstrate that the opioid system is involved in ethanol reward and addiction. Do variations in opioid peptide or receptor genes contribute to ethanol addiction in humans? Therefore the second aim of this work was to systematically analyze the contribution of variants in opioid peptide genes to human alcohol dependence. So far, variants in opioid peptide coding genes were not systematically studied in human case control samples. Findings on frequency differences between a group of cases and controls might allow conclusions if SNPs in opioid peptide genes are responsible for inter-individual differences in the susceptibility for alcohol addiction.

Chapter 2

Material

If not indicated differently, all applied chemicals are products from Invitrogen, Applichem, Sigma, Merck, Roche and Roth. In the appendix, contact information of the suppliers are given. The oligonucleotides were synthesized and delivered by Metabion. Information about primer sequences are provided in the appendix. TaqMan® gene expression or genotyping assays were purchased at Applied Biosystems. Chemicals specific for the mass spectrometry-based SNP genotyping were ordered at Sequenom, Inc. For sequencing, DNA was sent to Macrogen Inc. in Korea. An overview about the used databases is also given in the appendix.

2.1 Enzymes and antibodies

All restriction endonucleases were purchased from New England Biolabs (NEB). Reverse transcriptase was purchased from Qiagen. DNA polymerases were obtained from both companies.

Antigen	Species	Modification	Company
Antibodies			
GFP	rabbit		Abcam
Digoxigenin	sheep	alkaline phosphatase conjugated	Roche
rabbit IgG	donkey	biotinylated	Jackson
Antisera			
Met-enkephalin	rabbit		Bachem
Leu-enkephalin	rabbit		Bachem
rabbit IgG	donkey		Bachem

2.2 Kits

Dig RNA Labeling Kit	Roche
PCR Dig labeling mix	Roche
peqGOLD Gel Extraction Kit	Peqlab
peqGOLD Plasmid Miniprep Kit I	Peqlab
PureLinkTM HiPure Midi Plasmid Extraction Kit	Invitrogen
QIAquick Nucleotide Removal Kit	Qiagen
Peptide Radioimmunoassay Kits	Bachem
TSM^{TM}Fluorescin System	PerkinElmer
Avidin-Biotin Blocking Kit	VectorLaboratories
VECTASHIELD Mounting Medium with DAPI	VectorLaboratories

2.3 Bacteria, cells and plasmids

TOP10: An *E.coli* strain obtained from Invitrogen used for the transformation of recombinant plasmids (F-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lacZ*ΔM15, Δ*lac*X74, *rec*A1, *ara*D139, Δ(*araleu*)7697, *gal*U, *gal*K, *rps*L, (Str^R), *end*A1 and *nup*G).

MPI2 cells: An embryonic stem cell line generated from 129/Sv mice. These cells were established at the Max Planck Institute Göttingen in the laboratories of Prof. P. Gruss (VOSS ET AL., 1998).

pPNT-M2-Enk: Targeting vector for the mutation of the murine preproenkepahlin gene locus. This vector was generated by Monika König in the laboratories of Prof. A. Zimmer in Bethesda, Maryland (*Amp^R, Neo^R*).

Penk ribo: pBluscriptSK vector containing a part of the murine preproenkephalin exon 3. This vector was provided by Prof. B. Kieffer and used as template for the generation of the *in situ* probe (*Amp^R*).

AAV2-Cre: Recombinant adeno-associated virus serotype 2 expressing cre recombinase fused to GFP under the control of the CAG promotor. This vector was used for stereotactic injection and provided by Prof. B. Kieffer (concentration: 8.9 x 10^7 vg/µl).

AAV2-GFP: Recombinant adeno-associated virus serotype 2 expressing eGFP under the control of the CAG promotor. This vector was used for stereotactic injection and provided by Prof. B. Kieffer (concentration: 4 x 10^7 vg/µl).

2.4 Solutions

If not indicated differently all solutions were prepared using sterile deionized H_2O.

2.4.1 DNA isolation

Lysis buffer	
Tris/HCl, pH 8	100 mM
EDTA, pH 8	5 mM
NaCl	200 mM
SDS	2 % (w/v)

TE buffer	
Tris	10 mM
EDTA, pH 8	1 mM
adjusted to pH 7.4	

2.4.2 Agarose gel electrophoresis

6x loading	
Tris/HCl, pH 7	10 mM
EDTA, pH 8	50 mM
Bromphenol blue	0.03 % (w/v)
Xylene cyanol FF	0.03 % (w/v)
Orange G	0.4 % (w/v)
Ficoll	15 % (w/v)

TAE buffer	
Tris-Acetat	40 mM
EDTA, pH 8	0.5 mM

2.4.3 Cell culture experiments

ES cell medium	
DMEM	high glucose
FCS (ES cell aproved)	26 % (v/v)
Nonessential amino acids	1.2 % (v/v)
Sodium pyruvat	0.6 % (v/v)
β-mercaptoethanol	0.2 % (v/v)
LIF	2 ng/ml
Penicillin	3 U/l
Streptomycin	3 U/l

Selection medium	
DMEM	high glucose
FCS (ES cell aproved)	26 % (v/v)
Nonessential amino acids	1.2 % (v/v)
Penicillin streptomycin mix	0.6 % (v/v)
Sodium pyruvat	0.6 % (v/v)
β-mercaptoethanol	0.2 % (v/v)
LIF	2 ng/ml
G418	170 ng/ml
FIAU	130 nM

Fibroblast medium	
DMEM	high glucose
FCS	10 % (v/v)
Penicillin streptomycin mix	0.6 % (v/v)
Sodium pyruvat	0.5 % (v/v)

Freezing medium	
DMEM	
FCS	50 % (v/v)
DMSO	10 % (v/v)

Mitomycin solution	
Fibroblast medium	
Mitomycin C	10 µg/ml

2.4.4 *Southern* blot

Depurination	
HCl	250 mM

Denaturation	
NaOH	500 mM
NaCl	1.5 M

Neutralisation	
Tris/HCl	500 mM
NaCl	1.5 M

20x SSC	
NaCl	3 M
Na-citrat dihydrate	300 mM
adjusted to pH 7	

Maleic acid buffer	
Maleic acid	100 mM
NaCl	150 mM
adjusted to pH 7.5	

10x Blocking solution	
Maleic acid buffer	
Blocking reagent	10 % (w/v)
autoclaved at 121°C, 20 min	

Hybridization buffer	
20x SSC	25 % (v/v)
SDS	0.2 % (w/v)
10x blocking solution	10 % (v/v)
N-lauroylsarcosine	1 % (w/v)

Washing buffer	
Maleic acid buffer	
Tween 20	0.3 % (v/v)

Detection buffer	
Tris/HCl pH 9.5	100 mM
NaCl	50 mM
$MgCl_2$	25 mM

2.4.5 *In situ* hybridization

DEPC-water	
DEPC	0.1 % (v/v)
autoclaved at 121°C, 20 min	

10x DEPC-PBS	
NaCl	1.4 M
PO_4 buffer	100 mM
KCl	30 mM
DEPC	0.1 % (v/v)
autoclaved at 121°C, 20 min	

PFA/PBS	
10x DEPC-PBS	10 % (v/v)
Paraformaldehyde	4 % (w/v)

Triethanolamine solution	
Triethanolamine, pH 8	100 mM
Acetic anhydrid	0.2 % (v/v)

20x SSC	
NaCl	3 M
Na-citrat dihydrate	300 mM
adjusted to pH 7	
DEPC	0.1 % (v/v)
autoclaved at 121°C, 20 min	

10x Salts	
NaCl	3 M
Tris-HCL, pH 6.8	200 mM
EDTA	50 mM
NaH_2PO_4	54 mM
Na_2HPO_4	46 mM

50x Denhardt's solution	
Ficoll	1 % (w/v)
Polyvinylpyrrolidone	1 % (w/v)
Bovine serum albumine	1 % (w/v)

Hybridization mix	
Formamide	50 % (v/v)
Dextran sulfate	10 % (w/v)
10x salts	10 % (v/v)
50x Denhardt's solution	2 % (v/v)
tRNA	500 µg/ml

Washing buffer	
Formamide	50 % (v/v)
Tween20	0.1 % (v/v)
20x SSC	5 % (v/v)

Maleic acid buffer	
Maleic acid	100 mM
NaCl	150 mM
adjusted to pH 7.5	
autoclaved at 121°C, 20 min	

MABT	
Maleic acid buffer	
Tween20	0.1 % (v/v)

TBST	
NaCl	150 mM
Tris/HCl	100 mM
adjusted to pH 7.5	
autoclaved at 121°C, 20 min	
Tween20	0.1 %

NTMT	
NaCl	100 mM
Tris/HCl, pH 9.5	100 mM
$MgCl_2$	50 mM
Tween20	0.1 % (v/v)

Staining solution	
NTMT	
NBT (Roche)	0.45 % (v/v)
BCIP (Roche)	0.35 % (v/v)
Levamisole	100 µM

2.4.6 *LacZ* staining

Basic solution	
PBS	
$MgCl_2$	2 mM
EGTA, pH 8	5 mM

Glutaraldehyde solution	
Basic solution	
Glutaraldehyde	0.2 % (v/v)

Staining solution	
Basic solution	
Sodium doxycholat	0.1 % (w/v)
Nonidet P40	0.02 % (v/v)
$K_3[Fe(CN_6)]$	10 mM
$K_4[Fe(CN_6)]$	10 mM
X-gal	0.5 mg/ml

Washing buffer	
Basic solution	
Sodium doxycholat	0.1 % (w/v)
Nonidet P40	0.02 % (v/v)

2.4.7 Immunofluorescence staining

Peroxidase-neutralization reagent	
PBS	
H_2O_2	1 % (v/v)
NaN_3	0.1 % (v/v)

TNB	
Tris/HCl, pH 7.5	100 mM
NaCl	150 mM
Blocking reagent (TSA-Kit)	0.5 % (w/v)
stored at -20°C	

Fc-Block	
Anti-mouse CD 16/32 purified	1 % (v/v)
Normal rat serum	1 % (v/v)
Normal mouse serum	1 % (v/v)

2.4.8 Anesthesia

Ketamine/Xylazine	
NaCl	0.9 % (w/v)
Ketamine	0.5 % (w/v)
Xylazine	0.2 % (w/v)

Chapter 3

Methods

The methods used in this work are divided into six parts. In the first section, the methods relevant for the cultivation of mouse embryonic stem cells are stated. The second section deals with the molecular biological experiments conducted in the context of the newly developed mouse strain, whereas the methods used for the human case-control studies are stated in a separate section. The methods applied in the context of protein extraction, quantification and activity measurement are itemized in the fourth section, followed by the description of all animal experiments in section five. The chapter ends with an introduction of the statistical methods used.

3.1 Cell culture methods

In this section all methods relevant for the cultivation of mouse embryonic stem cells (ES cells) are stated. The experiments were conducted in the laboratories of the Institute of Molecular Psychiatry at the University Medical Center in Bonn.

3.1.1 Cultivation of cells

All cells were cultivated at 37°C in an incubator with 5% CO_2 and humid atmosphere on monolayers of mitotically inactivated mouse embryonic fibroblasts (MEF) as feeder cells. When ES cells reached 70% confluency, or, at the latest, every second day, cells were splitted at a ratio of 1:3 to 1:7. The ES cells were incubated in 25% of the culture volume trypsin/EDTA. The digestion was stopped by adding the same amount of ES cell medium. Subsequently, the cells were spun down (2 min, 1200 rpm). After gentle resuspension in medium, the cells were dispensed on fresh feeder plates.

3.1.2 Generation of embryonic mouse fibroblasts

Mouse embryonic fibroblasts were derived from E13.5 embryos. Matings of wild type mice and mice carrying a neomycin resistance gene were used. Mouse embryos from one or two pregnant mice were dissected. Subsequently the embryo's limbs were removed, internal organs were scooped out and discarded. The small pieces of the minced embryo were incubated in 30 ml Trypsin/EDTA for 15–20 minutes (37°C, 5% CO_2, 95% humidity). During this period the tube was shaken gently every third minute. After centrifugation (2 min at 1200 rpm) the pellet was resuspended in 25 ml fibroblast medium and transferred to a cell culture dish. After 3–4 days cells were grown to confluency and were subcultivated. Following an additional incubation time of 2–3 days, cells were prepared for long-term storage at – 80°C.

3.1.3 Storage and re-cultivation of cells

Cells were spun down, dispersed in freezing medium and stored in cryotubes at – 80°C. For long-term storage, the tubes were transferred to liquid nitrogen after 2–3 days.

For re-cultivation cells were thawed quickly, diluted in medium, spun down, resuspended in fresh medium and transferred to cell culture dishes.

3.1.4 Electroporation and isolation of recombinant ES cell clones

For electroporation, a culture of early-passage ES cells, grown on 15 cm cell culture dishes, was used. Per electroporation 2 x 10^7 cells were utilized. The medium was changed 1–2 hours before harvesting the cells. Subsequent to counting the cells in a hemacytometer they were spun down (2 min, 1200 rpm), washed and resuspended in HBSS. The suspension and 20 µl of prepared plasmid DNA (1 µg/µl) were transferred to an electroporation cuvette and a pulse of 250 V and 500 µF was applied. The cuvette was rinsed and cells were directly passed to a feeder plate. Selection was initiated 24 hours later by adding selection medium containing the antibiotic (G418) and FIAU. The medium was changed daily over a period of 7–9 days. Then, single colonies were transferred into a 24 well plate, each containing one drop of trypsin/EDTA. After dispersing the ES cells in 1 ml of ES cell medium, feeder cells were added and cultures were allowed to grow for 24 hours. The medium was renewed and cells were cultivated for 3–4 days. For storage, clones were harvested by trypsination and resuspended in 1 ml freezing medium, of which an aliquot of 750 µl was frozen. The remaining cells were cultivated further to obtain DNA for *Southern* blot analysis.

3.2 Molecular biology methods

The biomolecular experiments described in this section were also conducted in the laboratories of the Institute of Molecular Psychiatry. Standard methods, as agarose gel electrophoresis,

ethidium bromid staining or transformation and cultivation of *E.coli* were performed according to Molecular Cloning by Sambrook and Russel (SAMBROOK AND RUSSELL, 2001).

3.2.1 DNA preparation

Plasmid-DNA was amplified using *E.coli* cultures. The DNA was isolated using commercial kits. Depending on the required amount of DNA *Mini*-, *Midi*- or *Maxi*-preparation kits were used.

The material was incubated in lysis buffer at 56°C overnight with agitation (550 rpm) to isolate genomic DNA from cell lines or mouse tail biopsies. After spinning down non-soluble material, DNA was precipitated by adding isopropanol (1:1) to the supernatant. Subsequent to washing the pellet with 70 % ethanol the DNA was dried and eluted in TE-buffer.

3.2.2 Determination of nucleic acid concentration

The concentration of nucleic acids was determined using the spectrophotometer NanoDrop ND-1000. To calculate the nucleid acid concentrations (ng/µl), the following extinction coefficients were used:

$$\text{double-stranded DNA: } 50\ \frac{1}{cm*M}$$
$$\text{single-stranded RNA: } 40\ \frac{1}{cm*M}$$

3.2.3 Digestion of DNA

Sequence-dependent hydrolysation of DNA was performed using restriction endonucleases and the appropriate buffers. Preparative digestions were performed overnight using up to 10 µg DNA, whereas digestion for analytical purposes lasted one hour using up to 1 µg DNA.

3.2.4 Purification of DNA fragments

DNA fragments were purified using either commercial kits for nucleotide removal or phenol-chloroform extraction. For phenol-chloroform extraction the sample was adjusted to 700 µl and the same amount of phenol/chloroform/isoamylalcohol (25:24:1) was added. After a thorough mixing and centrifugation, the aqueous phase was transferred to a fresh tube and the procedure was repeated. The supernatant was mixed with chloroform, followed once again by phase separation. Finally the DNA was precipitated by adding 3 M sodiumacetat and ethanol. After washing with 70% ethanol, the pellet was dried and resolved in the appropriate amount of TE-buffer.

3.2.5 Reverse transcription

mRNA was transcribed into cDNA for expression analysis. For the generation of cDNA the *Omniscript Reverse Transcriptase* from Qiagen was used together with the RNase inhibitor

RNase OUT and *oligodT* primer (Invitrogen). In each individual reaction a maximum of 2 µg of RNA was transcribed.

3.2.6 Polymerase-chain reaction

Specific amplification of DNA fragments was achieved using polymerase-chain reaction (PCR). The reaction and temperature profiles were specifically adjusted according to the thermodynamic properties of the oligonucleotide primer and the lengths of the amplified PCR products.

PCR setup *Southern* probe

The *Southern* blot probe was marked using Digoxigenin labeled nucleotides.
PCR-setup (50 µl):

26.75 µl	sterile water
5 µl	10x PCR-buffer HotStarTaq (Qiagen)
10 µl	5x Q-solution HotStarTaq (Qiagen)
5 µl	Dig-labelling dNTP Mix
1 µl	5-Primer (10 µM)
1 µl	3-Primer (10 µM)
1 µl	*Wt* ES cell DNA ($\approx$ 400 ng/µl)
0.25 µl	HotStarTaq polymerase (5 U/µl)

Cycling parameters:

	I	Intitial denaturation	96°C	15 min
	II	Denaturation	94°C	15 sec
35 cycles	III	Annealing	55.5°C	45 sec
	IV	Elongation	72°C	60 sec
	V	Final elongation	72°C	10 min
	VI	Cooling	4°C	∞

Expression analysis using quantitative realtime PCR

SYBR green or TaqMan® expression assays were used to quantify the amount of a specific mRNA. The dye SYBR Green intercalates during cDNA amplification, whereupon a fluorescence signal is emitted. TaqMan® assays are based on fluorogenic probes. The fluorescence signal is transmitted after binding of this probe to a specific cDNA sequence during amplification. TaqMan® assays were applied to differentiate between splice variants of the *Penk1* gene. For both methods the intensity of the fluorescence signal was monitored using a 7900HT Fast Real-Time PCR System and intensity differences were quantified after normalization to a house

keeping gene (beta-actin) (LIVAK AND SCHMITTGEN, 2001). The TaqMan® assays were conducted as described by the manufacturer. For SYBR green-dependent detection the following set up was used:
PCR setup (20 µl):

6.5 µl	sterile water
10 µl	2x SYBR Green JumpStart Taq ReadyMix
1 µl	5-Primer (10 µM)
1 µl	3-Primer (10 µM)
1.5 µl	cDNA

Cycling parameters:

	I	Initial denaturation	94°C	2 min
40 cycles	II	Denaturation	94°C	15 sec
	III	Annealing	56°C	30 sec
	IV	Elongation	72°C	30 sec
	V	Fluorescence detection		
	VI	Melting curve measurement		

3.2.7 *In vitro* transcription

In vitro transcription was conducted to obtain Dig-labeled RNA probes for *in situ* hybridization. Briefly, 1 µg linearized purified template plasmid DNA was incubated for 2 hours at 37°C with SP6, T7 or T3 RNA polymerase (Roche). To remove template DNA 2 µl of DNaseI was added followed by a 15 min incubation at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8). The RNA was then precipitated by adding 72 µl of ammonium acetate together with 470 µl of absolute ethanol, incubated at – 80°C for 20 min and spun down at 13 000 rpm (20 min). The air-dried pellet was resolved in 22 µl DEPC-water and stored at – 80°C until further use. The quality of the probe was controlled by agarose gel electrophoresis.
Labeling reaction:

2 µl	10x NTP labeling mixture (Roche)
2 µl	10x transcription buffer (Roche)
1 µl	RNase inhibitor (Roche)
2 µl	RNA polymerase (Roche)
13 µl	Template DNA and DEPC-water

3.2.8 *Southern* blot

Screening of mouse embryonic stem cell clones for homologous recombination and analysis of mouse tail biopsies was done by *Southern* blot. Here, specific DNA fragments crosslinked to a

nylon membrane were detected using digoxigenin-labeled probes.

Digested DNA was separated in an agarose gel and the gel was prepared for blotting. The DNA was depurinated for 10 min followed by denaturation and neutralization (30 min each). Blotting was performed in 10x SSC for 1.5 hours at 5 bar. Via UV-crosslinking, the DNA was immobilized on the membrane before hybridization with denatured probes at 65°C overnight. Unspecific binding was avoided by prehybridisation with salmon sperm DNA.

The next day, the membrane was incubated twice for 10 min in 2x SSC/0.1% SDS at room temperature, followed by incubation in 0.2x SSC/0.1% SDS at 65°C (3 x 10 min). Subsequently, the membrane was equilibrated in maleic acid buffer. Prior to adding anti-digoxigenin antibody (1:20 000) for 40 min, an incubation in blocking solution (one hour) was conducted to prevent unspecific binding. Afterwards three washing steps (10 min in washing buffer) were performed. After equilibration for 5 min the detection was done with CDP-Star (Roche) diluted 1:100 in detection buffer. The results were monitored by exposure to a hyperfilm (Amersham Pharmacia) for approximately 35 min.

3.2.9 *In situ* hybridization

For qualitative expression analysis *in situ* hybridization was conducted. Cryosections (see subsection 3.5.2) of 10 µm were dried for 30 min prior to 10 min of fixation in PFA/PBS. After washing in PBS (2x 10 min), the slides were acetylated in a triethanolamin solution. The subsequent washing steps were performed in 2x SSC (10 min), followed by dehydration via incubation in ascending ethanol concentrations (60–100%). To remove lipids, immersion in chloroform was used. After incubation in descending ethanol concentration (100–80%), the slides were dried at room temperature for 30 min. For denaturation, the hybridization mix containing the *in situ* probe (2ng/µl) was preincubated at 70°C for 10 min. Next the sections were hybridized in a humid chamber at 65°C overnight, washed twice for 30 min in washing buffer and incubated in MABT (2x 30 min, room temperature). Blocking of unspecific binding was done for 1 hour in blocking solution followed by incubation with anti-digoxgenin antibody in a humind chamber at 4°C for either 2 hours or over night. To remove excess antibody the sections were washed 5 times for 20 min in TBST. The staining reaction was prepared via incubation in NTMT (2x 10 min). Freshly prepared staining solution was spread on the slides before overnight incubation in a humid chamber. The staining reaction was stopped by washing with TE-buffer. After drying the slides were embedded and covered for microscopic examination.

3.2.10 RNA isolation

Following tissue sample preparation (see subsection 3.5.1) RNA was isolated through homogenization in *TRIzol*® reagent. To gain high amounts of RNA, it is important that the tissue volume should not exceed 10% of the overall volume. The homogenate was pressed five times through a 21G needle using a syringe. After 5 min incubation the probe was spun down

(12 000x g 10 min, 4°C) to sediment nonsoluble ingredients. After adding BCP (10% of the inital amount *TRIzol*®) the probe was mixed for 30 s via vortexing. After an incubation time of three minutes, phase separation was obtained by centrifugation (12 000x g, 15 min, 4°C). The aqueous phase containing RNA was transferred into a fresh cap and the RNA was precipitated by adding isopropanol (50% of the initial *TRIzol*® volume) and spun down at 12 000x g, 4°C for 15 min. After washing with 70% ethanol, the pellet was dried and dissolved in DEPC-H_2O.

3.3 SNP genotyping

Frequency differences of polymorphic markers between two groups of human subjects were assessed by SNP genotyping. The genotyping using the MassARRAY platform Sequenom were performed in cooperation with Prof. Dr. M. Nöthen and Dr. A. Karpushova from the Institute of Human Genetics, University of Bonn. All other experiments were performed in the laboratories of the Institute of Molecular Psychiatry. The samples were provided by Prof. W. Maier (Department of Psychiatry, Bonn), Prof. M. Nöthen (Institute of Human Genetics, Bonn), Prof. L. Terenius (Karolinska Institute, Sweden) and Prof. M. Reuter (Institute of Psychology, Bonn).

3.3.1 Human subjects

An overview of all genotyped cohorts is given in figure 3.1. DNA of 154 patients with a definite current DSM-IV diagnosis of alcohol dependence (90 men, 64 women) and 206 control subjects consisting of German blood donors (154 males, 52 females) was analyzed for an initial exploratory genotyping. For an extensive genotyping the cases group was extended and the control sample was changed. DNA of 247 patients with a definite current DSM-IV diagnosis of alcohol dependence (180 men, mean age $= 42.0 \pm 9.0$; 67 women, mean age $= 44.3 \pm 8.5$) was available and 247 population-based control subjects were selected with regard to gender and age (180 men, mean age $= 41.7 \pm 8.9$; 67 women, mean age = 44.2 ± 8.4). Further, the sample size of the female subgroup was increased for two markers (rs934778 and rs3769671) to 83 (67+16) female patients (mean age $= 44.4 \pm 8.6$) and 140 (67+73) female control subjects (mean age $= 45.8 \pm 9.6$).

Substance abuse and dependence (including alcohol and illicit drugs) as well as psychiatric disorders were assessed with the whole Structured Clinical Interview for DSM-IV Axis I Disorders (SCID). An experienced psychiatrist was responsible for the DSM-IV diagnosis of alcoholism on the basis of the SCID-interview and all available clinical information and records. Subjects that additionally met DSM-IV criteria for dependence on an illicit drug, or had suffered recently (within 1 year) from any major psychiatric disorder, were excluded. History of psychiatric disorders such as a depressive episode did not lead to exclusion. Patients were re-

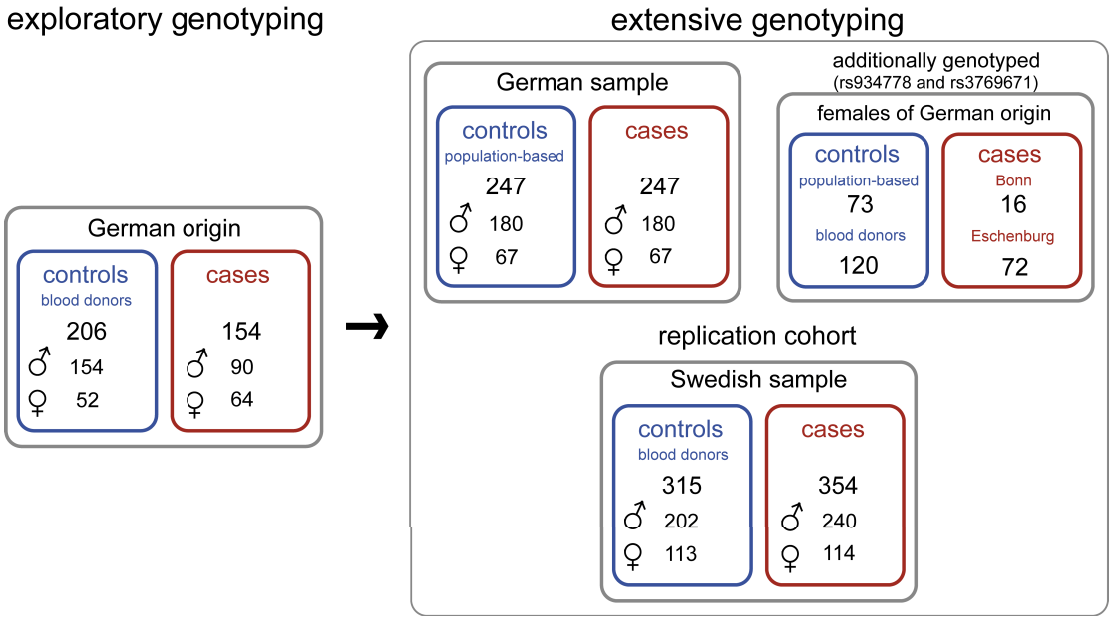

Fig. 3.1: Illustration of the genotyped samples. First an exploratory genotyping was performed using a small number of cases and controls. For an extensive genotyping the number of cases was increased and a population-based control sample was selected (German sample). For two markers the number of female cases and controls was further increased (rs934778 and rs3769671). A cohort of Scandinavian ancestry served as a replication cohort (Swedish sample).

cruited at the Department of Psychiatry (University of Bonn) or by collaborating psychiatric hospitals in Düsseldorf, Mainz, and Essen.

The population-based control sample of Caucasians of German origin was collected with the support of the local Census Bureau of the City of Bonn. The sample was established within the framework of the German National Genome Research Network I (NGFN I) of the Federal Ministry of Education and Research between 2000 and 2003 and serves as an epidemiological control sample for complex genetic studies within the NGFN.

To verify findings, a group of 120 healthy women (mean age $= 29.5 \pm 10.1$) and a group of 72 female alcoholic subjects (mean age $= 47.3 \pm 10.6$) were additionally genotyped for two markers (rs934778 and rs3769671). The female patients were recruited in the Clinic Eschenburg, a hospital specialized in the treatment of alcohol abuse and alcoholism. They met the ICD-10 criteria for alcohol dependence (F10.25) of the World Health Organization (WHO) and were committed to the rehabilitation clinic after alcohol detoxification. They participated in the study directly after admission to the Clinic Eschenburg. The individuals of this group had no clinical diagnosis of dependence on illicit drugs. All patients and control subjects were Caucasians of German origin.

Alcoholic patients (240 men, mean age = 47.25 ± 10.65; 114 women, mean age = 46.09 ± 10.39) and control subjects (202 men, 113 women) of Scandinavian ancestry served as a replication cohort. The affected individuals were collected within the Swedish Alcoholism in

Siblings Study. Alcoholism was defined according to DSM-IV criteria after a structural psychiatric interview with the Schedules for Clinical Assessment in Neuropsychiatry. The comorbidity with any other psychiatric disorder was below 1%. The group of healthy control individuals consisted of Swedish blood donors (202 men, 113 women). For this group no information concerning the age of the control subjects was available.

3.3.2 SNP selection and genotyping

To select appropriate markers representing genes of interest, data of linkage disequilibrium (LD) is taken into account. On the website of the international HapMap project information on LD in reference populations can be retrieved. Using the program haploview, haplotype blocks (highly linked regions) and tagging markers, which cover most of the genomic variance in the region of interest, can be calculated. In the initial phase of the present dissertation project this information was not available for genes encoding endogenous opioid peptides. Therefore markers evenly distributed across the gene loci of interest were chosen. A second criteria for SNP selection was the availability of appropriate genotyping assays.

For the extensive genotyping HapMap Phase II data were available and the LD information was taken into account. According to HapMap project data for Caucasians, the predicted tagging SNPs for *PENK* and *PDYN* were analyzed. In contrast, markers of the human *POMC* gene locus showed weak LD. Therefore SNPs were selected randomly with an average spacing of 3 kb.

The initial and the additional genotyping (for rs934778 and rs3769671) were realized using Assays-On-Demand SNP Genotyping products. For the extensive genotyping matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry-based SNP genotyping was performed using the MassARRAY System according to the manufacturer's specifications. Genotyping was done using Sequenom iPLEX chemistry, which is based on single base pair extension utilizing modified nucleotides.

3.4 Protein biochemical methods

The different methods used to detect peptides and proteins are described below. All experiments were conducted in the laboratories of the Institute of Molecular Psychiatry.

3.4.1 Radioimmunoassay

Radioimmunoassay (RIA) protocols for Met-enkephalin and Leu-enkephalin were established to assess the amount of the main enkephalin peptides in the brain. Therefore, mouse brains were homogenized in 3 ml of 2 N acetic acid at 4°C and clarified at 15 000x *g* for 30 min at 4°C. Initially duplicate aliquots of 10, 50, and 100 µl of the homogenate were dried under

vacuum in polystyrene tubes, resuspended in 100 µl of RIA buffer, and assayed following the protocol for the S-2118 Leu-Enkephalin and S-2119 Met-Enkephalin Radioimmunoassay kit. Later, duplicate aliquots of 100 µl sample were assayed. The standard curve for these kits ranged from 0.1 to 64 pg.

3.4.2 *LacZ* staining

The bacterial ß-galactosidase gene (*lacZ*) is frequently used as a reporter gene. The expression of transgenic constructs can be monitored by histochemistry with the chromogenic substrate X-gal. It enables precise cellular localization of gene activity. Tissue sections were prepared as described in subsection 3.5.2. Slides were fixed in glutaraldehyde solution for 5 min. Afterwards the sections were incubated twice for 10 min in washing buffer, followed by an incubation in freshly prepared staining solution overnight at 37°C. On the next day the staining reaction was stopped by rinsing the slides in tap water. Counterstaining was performed using nuclear fast red. After drying, the slides were embedded and covered for microscopic examination.

3.4.3 Immunofluorescence staining

For visualization of eGFP expression in tissue sections, immunofluorescence staining was conducted. First the slides were incubated in peroxidase-neutralization reagent (1 hour) and subsequently washed two times in PBS. Next, unspecific immune complex binding to immunoglobulin Fc receptors was prevented by incubation in Fc-Block for 25 minutes followed by Avidin-block (20 min) and Biotin-block (20 min). For specific antigen detection, anti-GFP antibody (1:2000 in TNB) was added and the sections were kept at 4°C overnight. The secondary biotinilated antibody was incubated (1:500 in TNB) for 30 min at room temperature. Afterwards the secondary antibody was bound by Streptavidin-HRP (1:200 in TNB). For antibody detection FITC-tyramide diluted in amplification buffer (1:100) was added and incubated in the dark (8 min). After each incubation the slides were rinsed two times in PBS. At the end the sections were mounted using VECTASHIELD mounting medium containing DAPI and kept at 4°C until microscopic examination.

3.5 Animal experiments

Behavioral experiments were conducted at the animal facility of the University Medical Center. The microinjection service of this facility was utilized to obtain chimeric mice. The S1 (Sicherheitsstufe 1, §§4-7 GenTSV) animal facility provided standardized climatic conditions. For behavioral testing all animals were housed under reversed light-dark conditions (lights on at 7:00 p.m. and lights off at 7:00 a.m.). Animal care and experiments described in this work were approved by legal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW).

All procedures were in compliance with national regulations (Tierschutzgesetz v. 18.5.2006 (BGBl. I S. 1206, 1313), g v. 18.12.2007 (BGBl. I S. 3001; 2008, 47)) and institutional guidelines. Cage allocation conformed the prescribed number of animals. Generally, mice are housed in groups of up to six animals. Mice were housed individually if this was necessary for the respective experiment. The animals had free access to food and drinking water.

The nicotine self-administration experiments were conducted by Dr. J.M. Trigo in the laboratory of Prof. R. Maldonado in Barcelona, Spain.

3.5.1 Tissue preparation

Mice were sacrificed via cervical dislocation, the required tissue was removed rapidly and immediately shock-frozen in isopentan bedded on dry ice. If a mouse tissue was utilized for immunofluorescence staining, mice were anesthetized using a ketamine/xylazine mixture and perfused using PBS. Subsequent to preparation the tissue was transferred to 4% PFA solution and fixed for up to 6 hours. Cryprotection was achieved by incubation of the tissue in 20% sucrose solution. Tissue samples were stored at – 80°C until further use.

3.5.2 Preparation of tissue sections

Prepared tissue samples were put on dry ice, embedded in Tissue Tec and sectioned to a thickness of 10-20 µm using a cryostat (cm 3050 S, Leica) at a temperature of – 24 to – 18°C. The sections were dried quickly at room temperature and either directly fixed or stored at – 80°C.

3.5.3 Generation of chimeric mice

Chimeric mice were obtained by blastocyst injection. Therefore cells of a targeted ES cell line were injected into blastocysts harvested from superovulated 4-6 week old C57BL/6J mice (Charles River). Blastocysts were then retransfered to pseudo-pregnant foster animals. The quality of chimeric animals was evaluated by coat color and the transmission to their progeny. Chimeric animals were backcrossed to the C57BL/6J mouse strain.

3.5.4 Deletion of the neomycin gene

Mice carrying the floxed preproenkephalin gene locus were bred to mice expressing cre recombinase under the control of an early acting promoter. Two different cre recombinase-expressing strains were used to delete the neomycin (*neo*) gene. First *Pgk-cre* mice, expressing cre recombinase under the Pgk1 promoter (LALLEMAND ET AL., 1998), second *EIIa-cre* mice, expressing cre recombinase under EIIa promoter, were mated to *Penk1*$^{wt/lox}$ animals. The later mouse strain is known to generate a mosaic pattern of deletion due to incomplete cre recombinase-mediated recombination (LAKSO ET AL., 1996).

3.5.5 Anxiety measurement

The zero maze apparatus used for anxiety measurements consisted of an annular white platform (inner diameter of 46 cm, 5.6 cm width) elevated 40 cm above the ground level (Fig. 3.2). It was divided into four equal quadrants, whereas non-transparent walls enclosed two opposite quadrants. Each mouse was placed into the open area of the maze. The animals' behavior was videotaped by a camera fixed above the maze and analyzed using a video-tracking system (VideoMot; TSE Systems). Between subjects the apparatus was cleaned with a 70% ethanol solution. Distance traveled in the open parts of the maze in relation to the total distance, the time spent in the open area and the latency to enter the closed area were evaluated as parameters for anxiety.

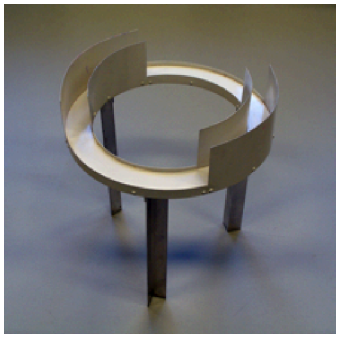

Fig. 3.2: Zero maze. The maze is divided into four equal quadrants, whereas non-transparent walls enclose the two opposite quadrants. At the beginning of the test the mouse is placed into the open area of the maze.

3.5.6 Stereotactic injection

Stereotactic surgery is a minimally invasive form of surgical intervention. It makes use of a three-dimensional coordinate system to locate small targets inside the organism and to perform localized actions such as injection, stimulation and implantation. For orientation within the mouse brain, the mouse stereotactic brain atlas (PAXINOS AND FRANKLIN, 2001) was used. Bregma, the craniometric point at the junction of the sagittal and coronal sutures at the top of the cranium, served as a reference point. Stereotactic injections were performed to target specific regions of the mouse brain in order to obtain regional deletion of the *Penk1* locus. For this purpose cre recombinase expressing adeno-associated viruses – or GFP expressing AAVs as a control – were injected into the brain region of interest. The targeting of a specific region using the virus approach works best if the infusion rate is not higher than 0.05-1 µl/min. Therefore, a specific setup needed to be established (Fig. 3.3). Anesthesia was induced via inhalation of isofluran. Subsequently, mice were placed in a stereotactic frame and received a constant stream of an oxygen/isoflurane mixture to maintain anesthesia. This guaranteed that mice stayed narcotized for the long-lasting surgery. After midline incision of the scalp, the

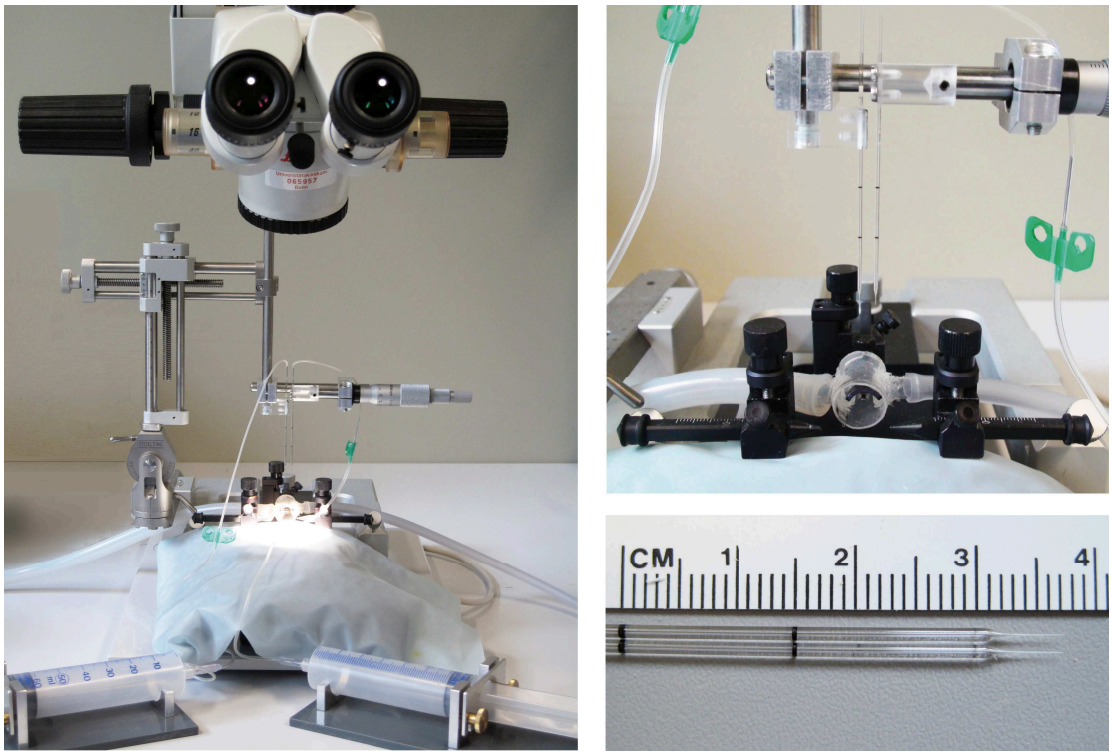

Fig. 3.3: Set up for sterotactic injection. To allow simultaneous bilateral assessment of a brain region a specific device was added to the stereotactic frame. For virus injection two glass capillaries with tip resistance of 2–4 MΩ attached to a 50 ml syringe via small flexible tubes were used. During surgery the scull of the mouse was fixed by two earbars and a nose clamp. To maintain anesthesia mice received a constant flow stream of oxygen/isoflurane via a mask around the nose clamp.

bregma area was exposed and two small openings were made with a dental drill, at the appropriate coordinates for the brain region of interest. To allow simultaneous bilateral assessment – also to decrease the duration of surgery – a specific device was added to the stereotactic frame as shown in figure 3.3. To inject the virus two glass capillaries with tip resistance of 2–4 MΩ attached to a 50 ml syringe via small flexible tubes were used. They were inserted stereotactically through the holes ventral to the dura into the brain region that was targeted. By pushing the syringe, pressure was built up for a slow injection of the virus. To target the nucleus accumbens 2 µl virus were injected. Mice quickly recovered from surgery and further experiments were conducted after two weeks.

3.5.7 Nicotine self-administration

For the analysis of nicotine self-administration a permanent catheter was implanted intravenously to allow self application of nicotine via nose-poking in an operant model. Briefly, mice were narcotized with a ketamine/xylazine mixture and then implanted with indwelling

i.v. silastic catheters as previously described (SORIA ET AL., 2005). The self-administration experiments were conducted in mouse operant chambers equipped with two holes, one randomly selected as the active sensor and the other as the inactive sensor. Nicotine was infused via a syringe that was mounted on a microinfusion pump and attached to the mouse intravenous catheter. The whole procedure is described in detail by Martin-Garcia and co-workers (MARTÍN-GARCÍA ET AL., 2009).

3.6 Statistical methods

Data obtained from mouse experiments were analyzed using the methods named in the subsection general statistics, whereas the statistical methods for the analysis of case-control data is described in a separate subsection. P-values < 0.05 were considered to be significant.

3.6.1 General statistic

In the case of mouse experiments, differences between genotypes were analyzed using one-way ANOVA followed by *Bonferroni* post hoc test. If raw data did not follow a normal distribution, data were transformed by calculating the $\log_{10}$-values followed by statistical analysis. Data of the nicotine self-administration experiment were analyzed by repeated measure ANOVA. The software Graph Pad Prism 4.0 was used for statistics and generation of graphs.

3.6.2 Case control study

Statistical analysis was performed with the program FAMHAP version 17. To calculate unadjusted p-values for association of single markers the option *singlecc* was used. Using this option the program calculates three different p-values for each marker (allelic, global and *Armitage* p-value) to detect significant differences in allele frequencies or genotype distributions between cases and controls. Significant differences of allele frequencies among cases and controls were calculated via a χ^2- based test (allelic p-value). The global p-value for differences in genotype distributions was computed in the same way. Additionally the *Armitage* trend test p-value was calculated to analyze significant differences in genotype distributions. When groups are ordered the *Armitage* trend test is typically used in categorical data analysis. In case of the genotype distribution groups are ordered by the dosage of a specific allele that means they are classified in non-carriers and in carriers of one or two alleles. By considering this ordered categories the *Armitage* test detects the significant difference in genotype distributions between cases and controls and whether the distributions show a significant trend depending on the allele dosage (ARMITAGE, 1955). This makes the *Armitage* trend test more robust against

stratification effects that might lead to false positive results (ARMITAGE, 1966). For haplotype analysis, FAMHAP estimates haplotype frequencies with the expectation-maximization (EM)-algorithm. P-values were computed by a permutational χ^2- based test (BECKER AND KNAPP, 2004). Pair-wise LD and haplotype block structure based on the control data was visualized using the HAPLOVIEW program v.3.32 (BARRETT ET AL., 2005). Block definition was done according to criteria proposed by Gabriel and co-workers (GABRIEL ET AL., 2002). For single marker analysis a *Bonferroni* correction for multiple testing was applied.

Chapter 4

Results

Two different approaches were used to enlighten the role of endogenous opioid peptides in drug addiction. One approach was the development of a new mouse model with a floxed preproenkephalin locus to elucidate the function of enkephalin in specific brain regions or cell types in the context of nicotine addiction. The introduction of *loxP* sites into the genome allows the subsequent excision of the embedded region via cre recombinase-mediated recombination. The excision depends on cre recombinase activity which enables the tissue or cell-specific deletion of the corresponding gene region. The second approach included the analysis of polymorphisms in human opioid peptide genes. Single nucleotide polymorphisms were studied for frequency differences between alcoholic patients and corresponding control groups.

4.1 Generation of floxed preproenkephalin mice

In this section all experiments concerning the generation of the floxed preproenkephalin mouse line are described.

4.1.1 Targeting strategy

The targeting vector pPNT-M2-enk is depicted in figure 4.1. It was derived from the pPNT-M2 vector by introducing a 5.7 kb fragment containing all three exons of the murine preproenkephalin locus. This 5.7 kb fragment served as 5' homology. Directly upstream of exon 2, a *loxP* site was added. As a selectable marker for ES cell culture a neomycin resistance gene flanked by two *loxP* sites was inserted downstream of exon 3. A *lacZ* gene was introduced to function as a reporter gene after cre recombinase-mediated excision of the coding exons 2 and 3. Therefore, the 3' part of the vector contained the *Penk1* exon boundary of intron 1/exon 2 (including the splice acceptor site) fused to the open reading frame of the *lacZ* coding sequence. The 3' end of the *lacZ* open reading frame was fused to a 1.5 kb fragment containing the downstream region of the preproenkephalin gene. This also served as 3' homology for

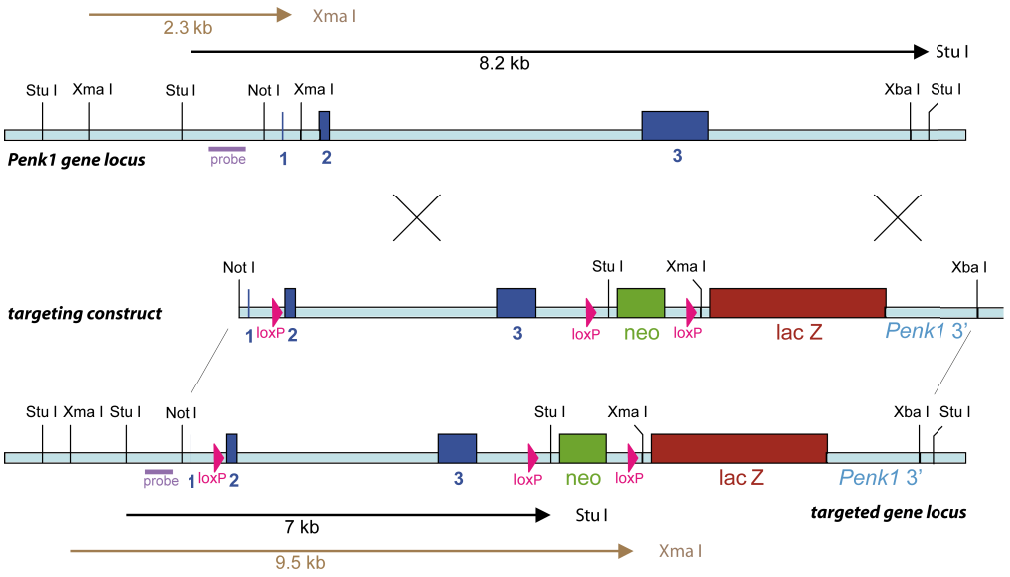

Fig. 4.1: Targeted mutagenesis of the murine preproenkephalin locus. Depicted are the wild type preproenkephalin locus (top) containing three exons, the targeting construct (center) and the targeted gene locus (bottom). A *neo* gene (green) flanked by two *loxP* sites (pink triangle) and the *lacZ* coding sequence (red) were inserted 3' of the *Penk1* wild type locus. A small fragment containing the splice acceptor site of exon 2 was fused to the *lacZ* sequence. Upstream of exon 2 another *loxP* site was inserted. For electroporation in ES cells the targeting construct was linearized via *NotI* digestion. Restriction sites used for *Southern* blot analysis plus the detectable fragments (black *StuI*, brown *XmaI*) using the *Southern* blot probe (purple) are indicated.

the recombination event in ES cells. Verification and amplification of this targeting construct was done in previous experiments in the context of the diploma thesis "Untersuchungen am endogenen Opioidsystem" (SCHÜRMANN, 2004).

The vector was linearized using *NotI* digestion prior to electroporation in ES cells. Within the scope of this work four electroporations were performed resulting in $\sim 2.1\%$ of homologous recombinant ES cell clones out of 475 harvested cells in total. The success of recombination was verified by means of *Southern* blot analysis using *StuI* digestion. Since the 5' *loxP* site was only maintained if recombination occured distal to this *loxP* site, positive ES cell clones were checked by *XmaI* digestion and *Southern* blot analysis for presence or abscense of this *loxP* site. The expected fragments are depicted in figure 4.1. The existence of all three *loxP* sites was demonstrated in one ES cell clone (H32). *Southern* blot results for two ES cell clones after *StuI* and *XmaI* digestion are illustrated in figure 4.2. In clone H32, recombination occured for the complete 5' homolgy region demonstrated by the expected restriction patterns. These patterns are presence of a 9.5 kb fragment (after *XmaI* digestion) and a 7 kb fragment (after *StuI* digestion). Analysis of clone G17 also showed the 7 kb *StuI* fragment indicating a homologous recombination event. However, in contrast to clone H32, in clone G17 only the wild type (wt) fragment of 2.3 kb was detected after *XmaI* digestion.

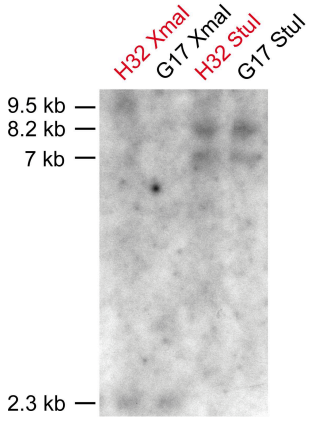

Fig. 4.2: Homologous recombinant ES cell clones. *Southern* blot analysis of ES cell DNA to verify homologous recombination. Results of clone H32 and G17 are shown exemplary after *StuI* and *XmaI* digestion. Both clones showed homologous recombination indicated by presence of the 7 kb fragment after *StuI* digestion. Only in H32 recombination occurred distal to the 5' *loxP* site as the 9.5 kb fragment was detected after *XmaI* digestion. For G17 only the *XmaI* wild type fragment of 2.3 kb was present.

4.1.2 Generation of conditional knockout mice

Injection of the homologous recombinant ES cell clone H32 into blastocysts derived from C57BL/6J mice lead to highly chimeric mice that were predominantly males. The degree of chimerism in these mice was evaluated by coat color, because the ES cell line used was derived from agouti 129/Sv mice and the blastocyst from non-agouti C57BL/6J mice. Chimeric mice were backcrossed to C57BL/6J females. The coat color revealed germline transmission, indicated by agouti pups (Fig. 4.3A). In parallel, *Southern* blot analysis of mouse tail DNA proved that the mutation was transmitted to the progeny, as digestion with *StuI* lead to the expected

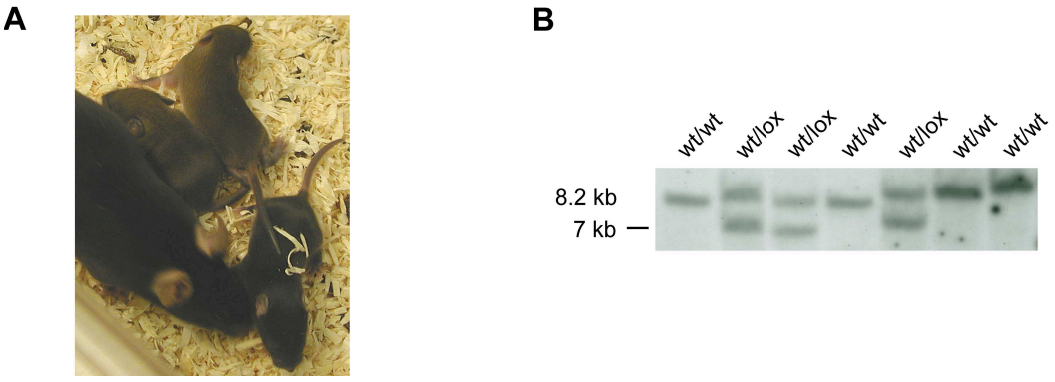

Fig. 4.3: Germline transmission of clone H32. Offspring of chimeric mice generated by injection of the homologous recombinant ES cell clone H32 in blastocysts. The coat color (A) as well as *Southern* blot analysis after *StuI* digestion (B) revealed that the mutation was transmitted to progeny. The alleles detected by *Southern* blot analysis are indicated (B). wt, wild type; lox, mutated gene locus.

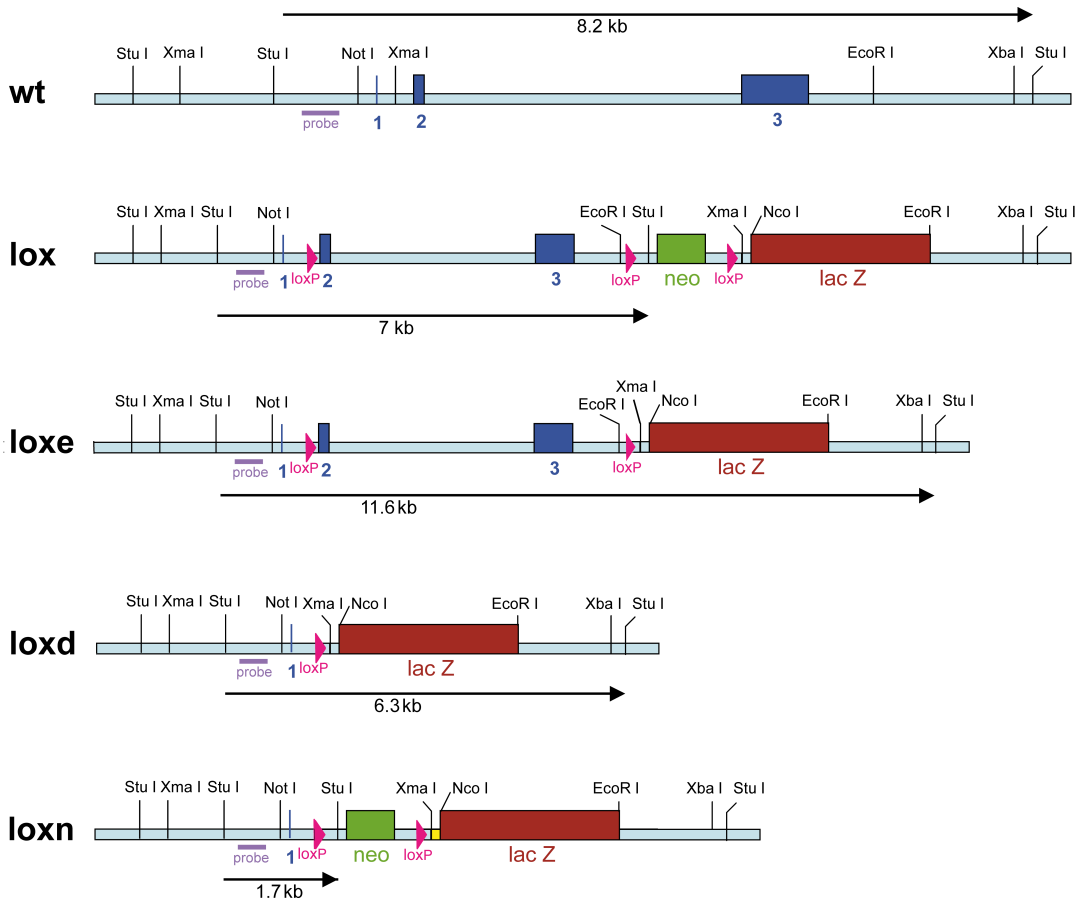

Fig. 4.4: Description of the mutated *Penk1* gene locus. All possible allele combinations are depicted. The *Penk1* wild type locus (wt) consists of 3 exons. This locus was modified by the introduction of 3 *loxP* sites (pink triangle), a *lacZ* (red) and a *neo* gene (green) named lox allele. Cre recombinase-mediated recombination could result in different alleles depending on where recombination occurs. The null mutant allele carrying *lacZ* as a reporter gene is named loxd, whereas the modified locus after excision of the *neo* gene is designated as loxe. If only the *neo* cassette and the *lacZ* reporter remained after cutting out the coding exons the allele is indicated as loxn.

fragments of 7 and 8.2 kb (Fig. 4.3B).

The resulting mouse line carried a floxed preproenkephalin locus as well as a floxed *neo* gene on a mixed 129/Sv x C57BL/6J background. In these mice cre recombinase-mediated recombination could occur between all 3 *loxP* sites. Depending on the strength of cre recombinase expression, this might lead to a mosaic pattern of cells carrying the different alleles illustrated in figure 4.4 (loxe, loxd, loxn). For example recombination between the 1st and the 2nd *loxP* site would lead to deletion of the enkephalin coding exons, but the floxed *neo* gene would be present (loxn allele). In contrast, recombination between the 1st and the 3rd *loxP* site would result in a knockout of exon 2 and 3 and excision of the *neo* gene. This directs *lacZ* expression under the control of the endogenous enkephalin promoter (loxd allele). A third possible allele could occur after excision of the *neo* gene subsequent to recombination between the 2nd and

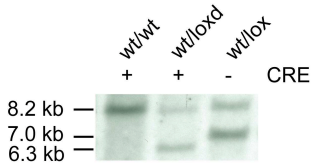

Fig. 4.5: Analysis of the offspring from *Pgk-cre*$^{+/-}$ x *Penk1*$^{wt/lox}$ matings. Exemplary *Southern* blot results of *StuI* digested mouse tail DNA are shown. Presence of cre recombinase expression (+) and the mutated *Penk1* allele resulted in complete deletion of the floxed gene locus as the loxd allele was present.

the 3rd *loxP* site (loxe allele). As the *neo* gene might additionally influence the stability and expression of nearby genes, its excision is advisable.

The possibility of different recombination events was used to excise the *neo* gene. During cre-mediated recombination varying alleles can occur simultaneously, also leading to the required loxe allele. Therefore mice carrying the lox allele were mated to *Pgk-cre* mice that are known to produce ubiquitous expression of cre recombinase (LALLEMAND ET AL., 1998). In this strain, cre recombinase expression is driven by the Pgk-1 promoter which is active during early embryogenesis. The offspring of these matings were screened by *Southern* blot analysis because *StuI* digestion would result in diverse fragments for all possible alleles (Fig. 4.4). Exemplary outcome is depicted in figure 4.5. The presence of the cre recombinase transgene

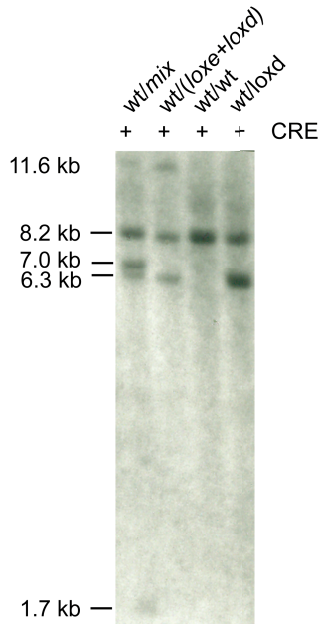

Fig. 4.6: Analysis of the offspring from *EIIa-cre*$^{+/+}$x *Penk1*$^{wt/lox}$ matings. Exemplary *Southern* blot results of *StuI*-digested mouse tail DNA are shown. Availability of cre recombinase expression and the mutated *Penk1* allele resulted in the presence of different allele combinations of the mutated gene locus.

always led to deletion of the entire floxed gene locus, indicated by the presence of the loxd allele.

As mating of *Pgk-cre*$^{+/-}$ and *Penk1*$^{wt/lox}$ did not provide the expected results, another cre recombinase expressing mouse strain was used. *EIIa-cre* mice carry the *cre* transgene under the control of the adenovirus EIIa promoter. This promoter also targets expression of cre recombinase to early mouse development (LAKSO ET AL., 1996). Analysis of the progeny of *EIIa-cre*$^{+/+}$ x *Penk1*$^{wt/lox}$ matings revealed that multiple alleles were present in a single mouse (Fig. 4.6). One mouse labeled wt/*mix* in figure 4.6 even showed all possible fragments after *StuI* digestion, indicating presence of all allele combinations (wt 8.2 kb, lox 7 kb, loxe 11.6 kb, loxd 6 kb, loxn 1.7 kb).

This *Penk1*$^{wt/mix}$ mouse was mated to C57BL/6J mice to obtain animals carrying either the loxe or the loxd allele. These animals were further backcrossed to C57BL/6J animals to establish two mouse lines that can be used in parallel. Namely, one constitutive knockout strain *Penk1*$^{loxd/loxd}$ expressing ß-galactosidase under the control of the endogenous enkephalin promoter and a second strain carrying a floxed preproenkephalin gene locus (*Penk1*$^{loxe/loxe}$). Mice of both strains were backcrossed to C57BL/6J animals to obtain congenic mice. In general a congenic background is established after mating for 10 generations. During this thesis the mice were backcrossed for 9 (*Penk1*$^{loxe/loxe}$) or for 8 (*Penk1*$^{loxd/loxd}$) generations.

4.1.3 Quantitative expression analysis

At first, expression levels of *Penk1*$^{lox/lox}$ mice were evaluated by quantitative real time PCR. Analysis of expression data from striatal mRNA preparations revealed a significant genotype effect depending on the lox allele [$F_{2,14}$ = 4.38, p = 0.038] (Fig. 4.7A). In mice homozygous

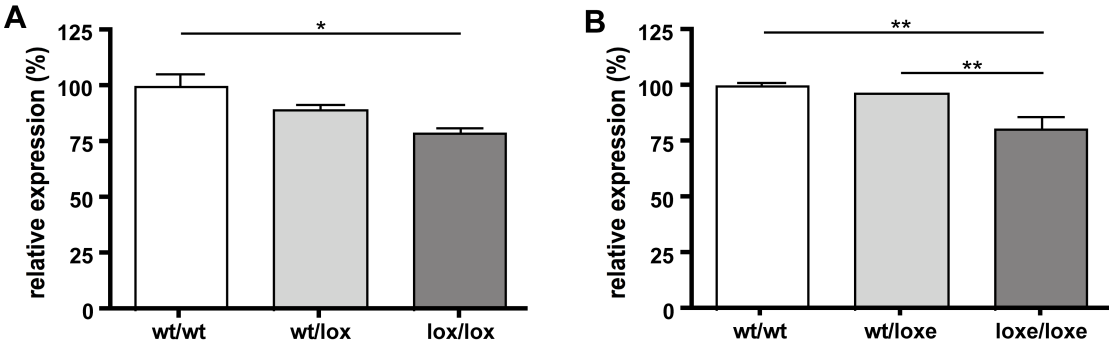

Fig. 4.7: Expression of enkephalin in the striatum. Preproenkephalin mRNA levels normalized to ß-actin were compared between either wt, heterozygous, or mice homozygous for the mutated *Penk1* allele. (A) In *Penk1*$^{lox/lox}$ mice (n=3) post hoc comparison revealed a significant reduction in RNA levels compared to their wild type counterparts (n=3). (B) A significant difference was also detected between *Penk1*$^{loxe/loxe}$ (n=4), *Penk1*$^{wt/loxe}$(n=6) or *Penk1*$^{wt/wt}$ mice (n=4). Values represent mean ± SEM whereas the mean of wild type mice was set to 100%. Significance thresholds are *p $<$ 0.5, **p $<$ 0.01 (*Bonferroni* post hoc test).

for the lox allele, mRNA levels were significantly reduced by 20% compared to their wild type littermates. Thus, the genetic modification had an impact on enkephalin expression. There was no significant difference in the direct comparison of heterozygous *Penk1*$^{wt/lox}$ and homozygous *Penk1*$^{lox/lox}$ or *Penk1*$^{wt/wt}$ animals. After establishing the *Penk1*$^{loxe/loxe}$ strain by deletion of the *neo* gene (see subsection 4.1.2), the mRNA levels were measured in these mice. The loxe allele also influenced preproenkephalin mRNA levels [$F_{2,13}$ = 13.65, p = 0.001]. There was a significant reduction of 20% between *Penk1*$^{wt/wt}$ and *Penk1*$^{loxe/loxe}$ and a reduction of 16% in *Penk1*$^{loxe/loxe}$ compared to *Penk1*$^{wt/loxe}$ (Fig. 4.7).

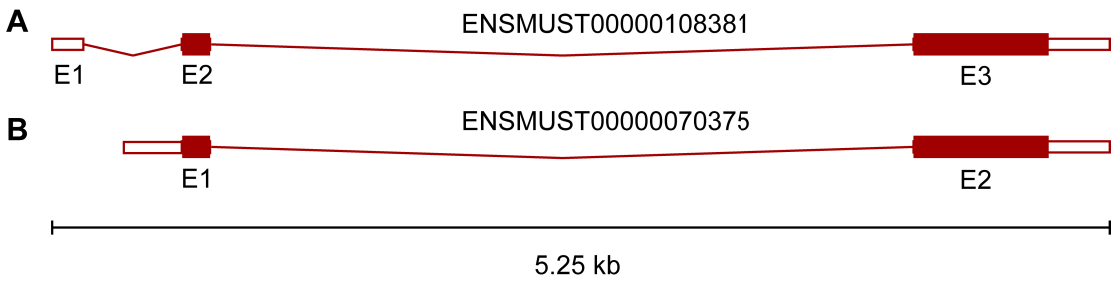

Fig. 4.8: Different splice variants of the preproenkephalin gene. The alternative splicing of preproenkephalin RNA can lead to two different transcripts. One transcript contained three exons (A) and the other contained two exons (B). The coding region (indicated by red boxes) did not differ between these transcript, but the 5'UTR was changed.

A second transcript of *Penk1* consisting of two exons instead of three was recently annotated in public databases (Fig. 4.8). In this second transcript (ENSMUST00000070375) exon 2 is identical to exon 3 of ENSMUST00000108381, whereas the first coding exon differs. Thereby the 5' *loxP* site introduced into the *Penk1* locus is located in the 5' UTR of the new annotated transcript. It was evaluated if the genetic modification introduced into the *Penk1* locus influences the expression or the stability of the different transcripts. Therefore transcript-specific assays were used. For transcript ENSMUST00000070375, mRNA levels were significantly decreased in mice carrying one or two copies of the loxe allele [$F_{2,13}$ = 24.70, p $<$ 0.0001], whereby mice homozygous for loxe showed a reduction of 72% compared to their wild type littermates (Fig. 4.9A). Unexpectedly, a similar result was observed for transcript ENSMUST00000108381 in which the *loxP* site is located upstream of exon 2 [$F_{2,13}$ = 18.00, p = 0.003] (Fig. 4.9C). The total level of *Penk1* mRNA is reduced by 70% in *Penk1*$^{loxe/loxe}$ animals. In *Penk1*$^{wt/loxd}$ mice enkephalin mRNA levels were reduced to 50%, meaning that homozygous loxe mice had less mRNA than heterozygous knockout animals (Fig. 4.9B,D). None of the two transcripts was detected in *Penk1*$^{loxd/loxd}$ animals and for both transcripts a significant genotype effect depending on the loxd allele was observed (ENSMUST00000070375 [$F_{2,11}$ = 138.4, p $<$ 0.0001], ENSMUST00000108381 [$F_{2,11}$ = 32.84, p $<$ 0.0001]).
Quantitative PCR was also performed for preprodynorphin mRNA in order to check if reduction

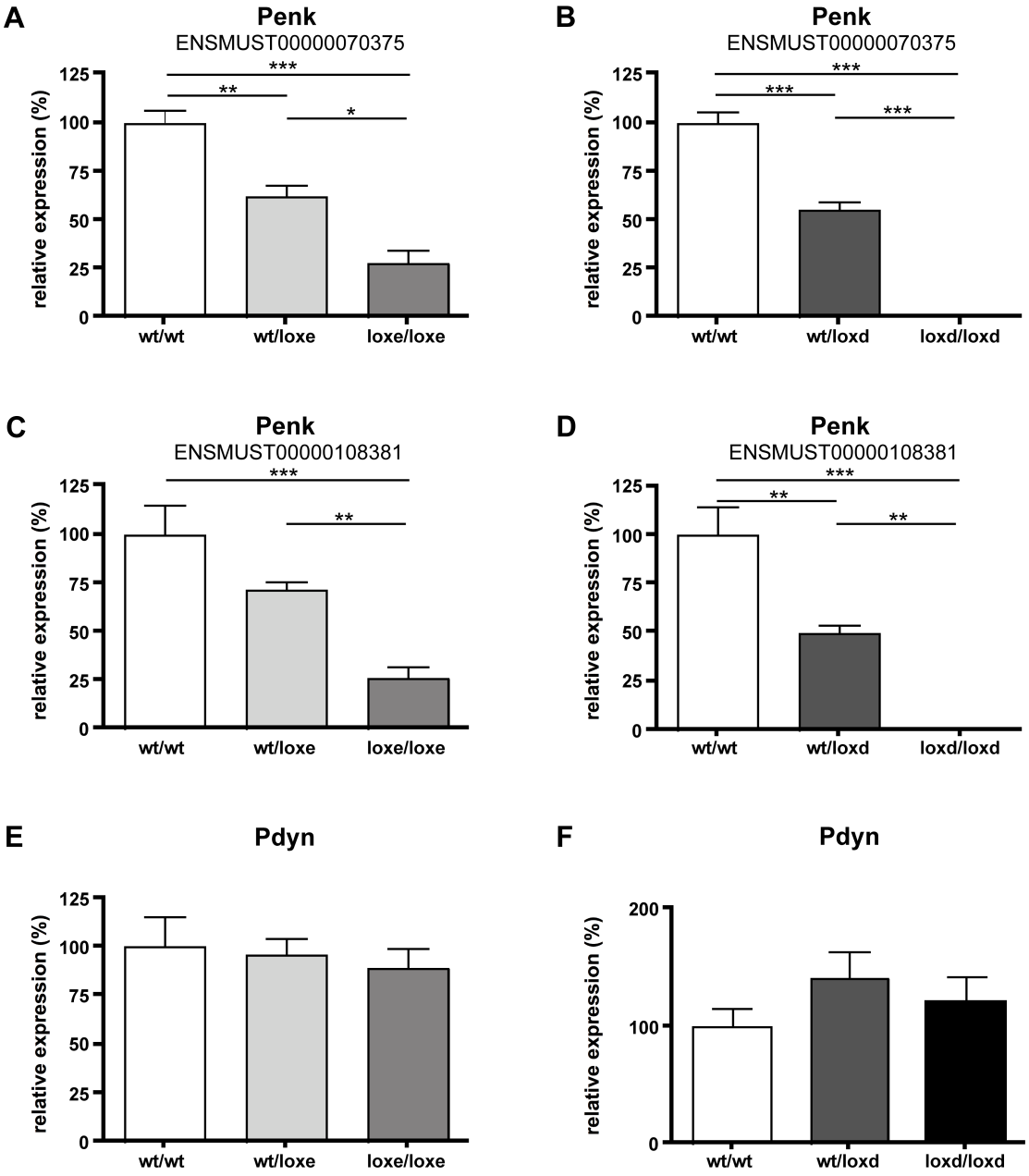

Fig. 4.9: Expression of all enkephalin coding transcripts in the striatum. mRNA levels of transcript ENSMUST00000070375 were altered depending on the *Penk1* genotype. The loxe allele led to a significant reduction in expression levels (A), and after deletion of the coding exons there was no preproenkephalin detectable (B). Similar results were observed for transcript ENSMUST00000108381 for the loxe (C) or loxd (D) allele. Transcript levels of Pdyn RNA that can give rise to Leu-enkephalin were independent of the allelic status of the *Penk1* gene locus (E),(F). Values represent mean $\pm$ SEM, whereas the mean of wild type mice was set as 100%. Significance thresholds are *p < 0.5, **p < 0.01, ***p < 0.001 (*Bonferroni* post hoc test). *Penk1*$^{wt/wt}$ n = 4, *Penk1*$^{wt/loxe}$ n = 6, *Penk1*$^{loxe/loxe}$ n = 4, *Penk1*$^{wt/loxd}$ n = 4, *Penk1*$^{loxd/loxd}$ n = 4.

in enkephalin mRNA causes a compensatory up-regulation, as it was suggested that one copy of Leu-enkephalin is derived from the dynorphin precursor protein (DAY ET AL., 1998). No significant genotype effect was observed for the loxe [$F_{2,13}$ = 0.20, p = ns] or for the loxd allele [$F_{2,11}$ = 0.99, p = ns] (Fig.4.9), not indicating compensatory up-regulation of preprodynorphin.

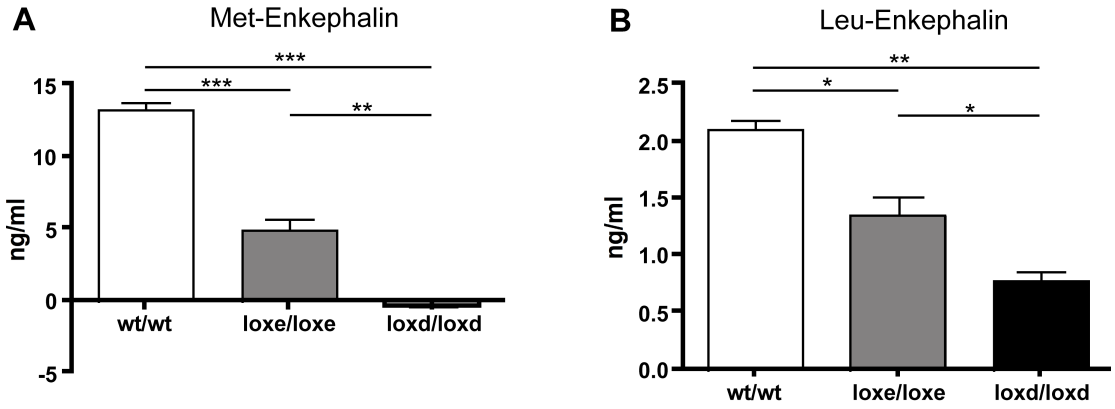

Fig. 4.10: Met- and Leu-enkephalin levels in mice carrying different *Penk1* alleles. Met- (A) and Leu-enkephalin (B) levels were changed as a function of the genotype. Amounts of both enkephalins were significantly reduced in *Penk1*$^{loxe/loxe}$ versus *Penk1*$^{wt/wt}$ mice. However, the enkephalin levels in *Penk1*$^{loxe/loxe}$ mice still differed significantly compared to *Penk1*$^{loxd/loxd}$ animals. In these mice, Met- enkephalin was absent, while $<$ 50% of the Leu-enkephalin was detected. Values represent mean $\pm$ SEM. Significance thresholds are *p $<$ 0.5, **p $<$ 0.01, ***p $<$ 0.001 (*Bonferroni* post hoc test). *Penk1*$^{wt/wt}$ n = 2, *Penk1*$^{loxe/loxe}$ n = 3, *Penk1*$^{loxd/loxd}$ n = 3.

Radioimmunoassays for Met- and Leu-enkephalin were performed to reveal if the differences in expression levels are reflected by the amount of enkephalin peptides. Significant genotype effects were observed for Met-enkephalin [$F_{2,7}$ = 173.5, p $<$ 0.0001] as well as for Leu-enkephalin [$F_{2,7}$ = 27.67, p = 0.002]. In *Penk1*$^{loxe/loxe}$ mice, only $1/3$ of the Met-enkephalin peptide of wild type littermates was found (Fig. 4.10A). As expected, Met-enkephalin was absent in *Penk1*$^{loxd/loxd}$ animals, whereas approximately half of the Leu-enkephalin was left (Fig. 4.10B). This was probably derived from the preprodynorphin precursor protein. The reduction of enkephalin levels in homozygous loxe mice thus mirrored the reduced amount of mRNA. The concentration of Met-enkephalin was higher compared to Leu-enkephalin, except for the homozygous knockout mice (Fig. 4.10A,B).

4.1.4 Qualitative expression analysis

In situ hybridization was conducted to detect preproenkephalin mRNA on brain slices. A pBluescript SK vector containing a DNA fragment with exon 3 of the *Penk1* gene was used as template for production of the *in situ*-probe. Because the antisense probe was directed against the sequence of exon 3, both possible transcripts were detected. There was pronounced expression of enkephalin in the striatum of *Penk1*$^{lox/lox}$ mice (Fig. 4.11). In contrast, no enkephalin

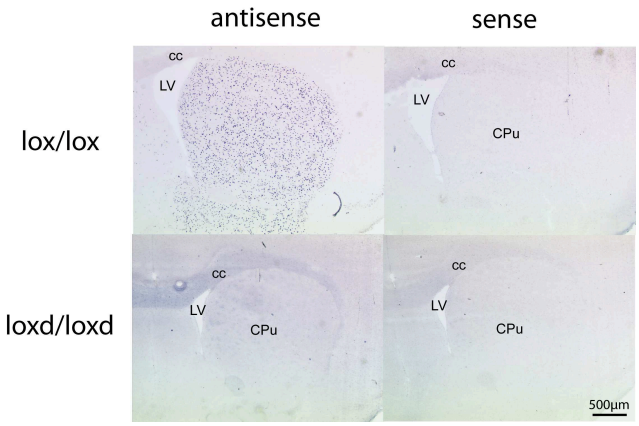

Fig. 4.11: Preproenkephalin expression in the striatum. *In situ* hybridization revealed marked expression of enkephalin in the striatum (CPu) of *Penk1*$^{lox/lox}$ mice, whereas expression was missing after deletion of the coding exons (*Penk1*$^{loxd/loxd}$). The absence of signal after application of the sense probe proved its specificity. cc, corpus callosum; CPu, caudate putamen; LV, lateral ventricle.

mRNA was detectable in the null allele mice (*Penk1*$^{loxd/loxd}$), which is in line with the quantitative expression data. This result, together with the absence of signal using the sense probe, also proved the high specificity of the *in situ* probe.

The *lacZ* gene was introduced in the mutated gene locus to function as a reporter gene. Subsequent to the deletion of the enkephalin coding region it should be expressed under the control of the endogenous enkephalin promoter. X-gal staining on brain slides of *Penk1*$^{loxd/loxd}$ mice was performed to assess ß-galactosidase activity. Staining was detected in all brain regions that normally express preproenkephalin. Two prominent regions of enkephalin expression are the striatum, especially the nucleus accumbens (NAc), and the central amygdala (CeA). Exemplary results of staining in these regions are illustrated in figure 4.12.

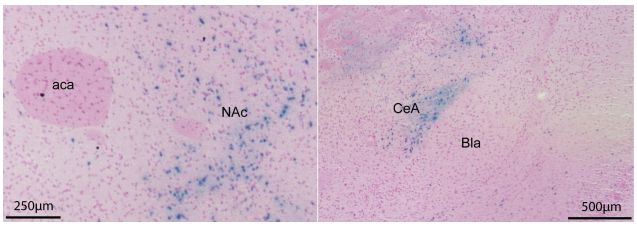

Fig. 4.12: ß-galactosidase activity in *Penk1*$^{loxd/loxd}$ mice. An exemplary X-gal staining in *Penk1*$^{loxd/loxd}$ mice is shown. Deletion of the enkephalin coding exons led to ß-galactosidase expression and activity in regions of former enkephalin expression like the nucleus accumbens (NAc) and the central amygdala (CeA). aca, anterior comissure,anterior; Bla, basolateral amygdala; CeA, central amygdala; NAc, nucleus accumbens.

4.1.5 Behavioral phenotyping

As described in subsection 4.1.3, there is a reduction in enkephalin expression as well as in Met- and Leu-enkephalin levels in mice homozygous for the loxe allele. To evaluate possible behavioral consequences these mice were studied in the zero maze test, which is an animal model for anxiety. The anxiety phenotype of constitutive *Penk1* knockout was very robust in this test.

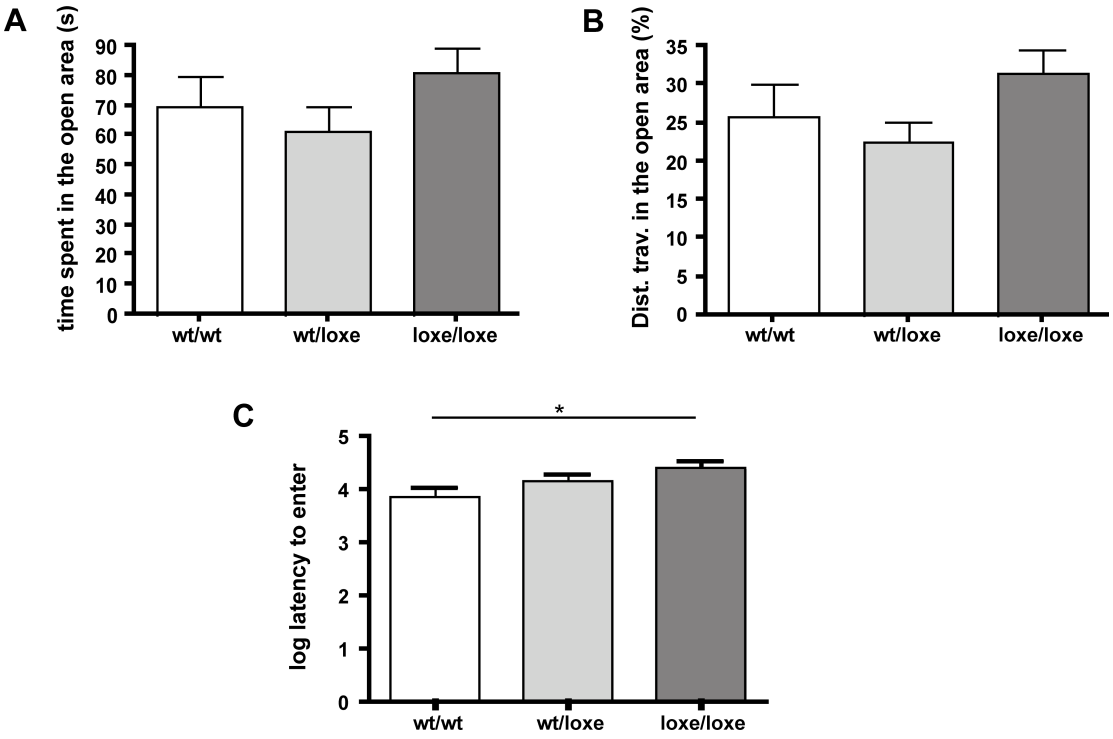

Fig. 4.13: Zero maze behavior. Anxiety levels depending on the *Penk1* allele were assessed by time spent in the open area (A), distance traveled in the open area (B) and latency to enter the closed area of the maze (C). Only the latency values differed significantly between *Penk1*$^{wt/wt}$ and *Penk1*$^{loxe/loxe}$ animals. Values represent mean $\pm$ SEM. Significance thresholds are *p < 0.5 (*Bonferroni* post hoc test). *Penk1*$^{wt/wt}$ n = 11, *Penk1*$^{wt/loxe}$ n = 9, *Penk1*$^{loxe/loxe}$ n = 11.

There was no genotype effect on the time spent in the open area [$F_{2,30}$ = 1.15, p = ns] nor on the distance traveled in the open part of the maze [$F_{2,30}$ = 1.90, p = ns]. However, the latency to enter the closed area differed significantly dependent on the genotype [$F_{2,26}$ = 4.19], p = 0.027]. In *Penk1*$^{loxe/loxe}$ mice the latency was increased, which was significant in direct comparison to *Penk1*$^{wt/wt}$ animals (Fig. 4.13).

4.1.6 Viral injection into the nucleus accumbens

The floxed preproenkephalin mice were generated to provide a tool that enables a tissue- or region-specific knockout of enkephalin. A region-specific knockout can be achieved by the

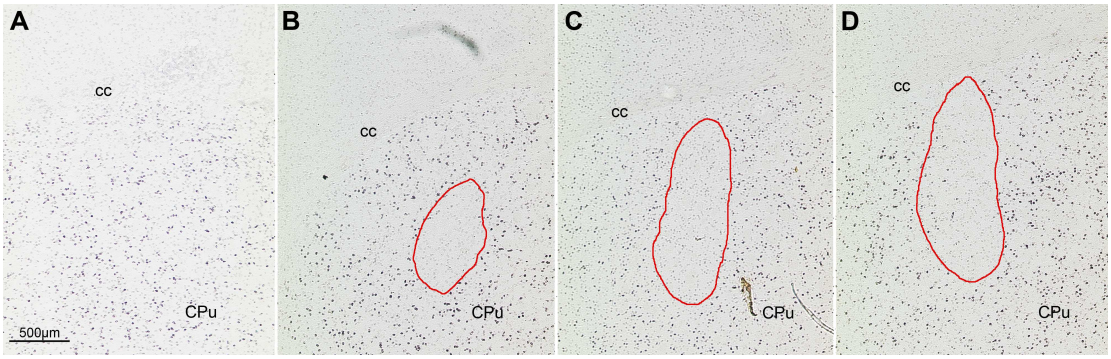

Fig. 4.14: Knockout of enkephalin after viral injection. *In situ* hybridization results of consecutive brain sections are presented. Signals for preproenkephalin mRNA were absent subsequent to injection of an adeno-associated virus expressing cre recombinase of *Penk1*$^{lox/lox}$ mice (red circles; B,C,D). The knockout of enkephalin was restricted to the injection site. In contrast, injection of adeno-associated viruses expressing GFP did not influence preproenkephalin mRNA (A). cc, corpus callosum; CPu, caudate putamen.

injection of cre recombinase-expressing viruses into distinct brain regions. To elucidate the efficacy of virus-mediated knockout, a cre recombinase-expressing adeno-associated virus was injected into the striatum of *Penk1*$^{lox/lox}$ mice. The results were evaluated by *in situ* hybridization. The lack of preproenkephalin mRNA at the injection site indicated a definite knockout of enkephalin after viral injection (Fig. 4.14).

It was a major focus of the present work to investigate the role of endogenous opioid peptides in drug addiction. Since dopamine signaling in the NAc is a key regulator of addiction

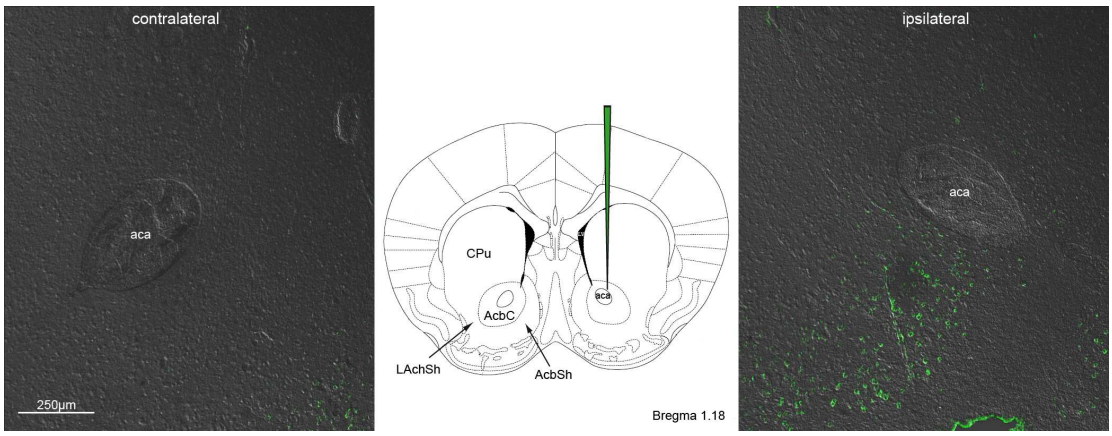

Fig. 4.15: Viral injection in the nucleus accumbens. Confocal images of brain slides are shown. Mice received a single injection of eGFP-expressing adeno-associated viruses (AAVs) at 1.2 mm (anteroposterior), 1.0 mm (mediolateral), 3.6 mm (dorsoventral) according to bregma to target the nucleus accumbens. GFP staining was observed at the injection site. GFP was not expressed contralateral to the injection site. aca, anterior commissure anterior; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; CPu, caudate putamen; LAcbSh, lateral accumbens shell; LV, lateral ventricle.

processes, this region was the first target for the regional deletion of enkephalin. To illustrate the targeting of the nucleus accumbens, eGFP-expressing AAV2 viruses were injected into one brain hemisphere of wild type mice. As shown in figure 4.15, a specific eGFP staining in the NAc was detectable only at the injection site.

Like many other drugs of abuse, nicotine administration leads to an increase in dopamine levels in the NAc. This nicotine-dependent increase was attenuated in constitutive *Penk1*$^{-/-}$ mice. Additionally, these mice exhibited no nicotine-induced conditioned place preference (BERRENDERO ET AL., 2005), suggesting that nicotine is not rewarding for them. One mechanism underlying this phenotype might be the modulation of dopamine release via enkephalinergic projections from the NAc to the VTA. Specific knockout of enkephalin in the NAc was used to elucidate the role of enkephalin in this process. Therefore GFP expressing AAVs (control) or cre recombinase expressing AAVs were injected into the NAc of *Penk1*$^{loxe/loxe}$ mice. These mice were then analyzed for nicotine self-administration in the laboratory of Prof. R. Maldonado (Barcelona, Spain).

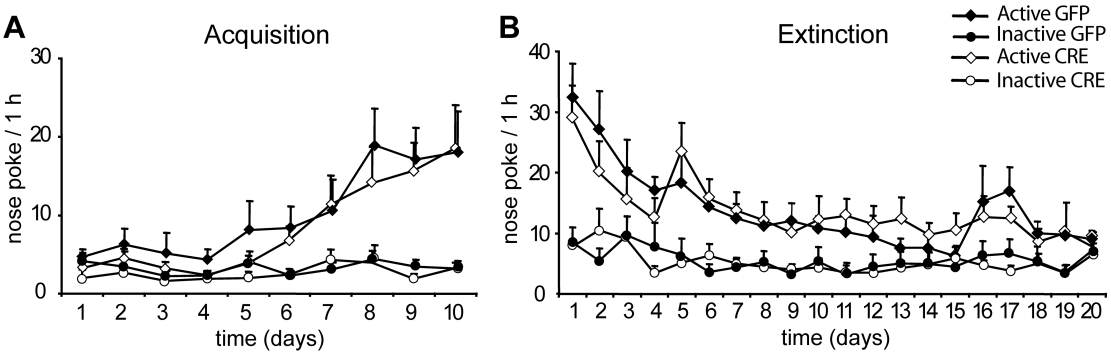

Fig. 4.16: Nicotine self-administration. Subsequent to surgery, mice were analyzed in an operant model to examine nicotine self-administration. Mice received injections of either cre recombinase- or eGFP-expressing adeno-associated viruses to target the nucleus accumbens. Illustrated are the nose pokes per 1h for the active and the inactive sensor. No difference was observed either in the acquisition (A) or in the extinction (B) phase. Acquisition CRE n = 8, GFP n = 8; extinction CRE n = 8, GFP n = 7.

In a first experiment, 18 mice (10 females, 8 males) were injected with eGFP-AAV and 19 mice (9 females, 10 males) with Cre-AAV. The results of the subsequent nicotine self-administration experiment are depicted in figure 4.16. The time needed to achieve the self-administration acquisition criteria did not differ between the groups. All mice discriminated between the active and inactive holes and an increase in the number of active nose-poking responses was seen independent of the injected virus (Fig. 4.16A). Repeated-measures two way ANOVA revealed a significant effect for factor discrimination, time and the interaction term [$F_{1,26}$ = 22.25, p $<$ 0.0001 for factor *discrimination*; $F_{9,234}$ = 10.44, p $<$ 0.00001 for factor *time*; $F_{9,234}$ = 7.79, p $<$ 0.00001 for *discrimination x time*]. No genoytpe effect was detected [$F_{1,26}$ = 2.22, p = ns].

During the extinction phase, no significant difference was observed between Cre-AAV or GFP-AAV-injected animals [$F_{1,16}$ = 0.28, p = ns]. Time to achieve the extinction criteria and the number of active or inactive nose-poking were similar in both groups. During the first extinction session, all mice showed an enhancement in the number of responses (active sensor) compared to the last day of self-administration training. At the end of the extinction phase discrimination between the holes vanished [$F_{19,304}$ = 139.95, $p < 0.00001$ for *discrimination x time*].

4.2 SNP genotyping

In this section, the results of the human association studies are described. Polymorphisms in human genes coding for preproenkephalin, proopiomelanocortin as well as preprodynorphin were evaluated for frequency differences between controls and alcohol-dependent subjects. This analysis was conducted to investigate the contribution of variants in genes coding for opioid peptides to alcohol dependence in humans.

4.2.1 Exploratory analysis

When the initial genotyping was started, no linkage disequillibrium data for the genes coding for endogenous opioid peptides were available. Markers were chosen to cover a region of around 20 kb surrounding the corresponding gene locus. For the human preproenkepahlin gene locus, which maps to chromosome 8q23-q24, five markers were selected, whereas one marker was located downstream, one was intronic and three markers were located upstream of the coding region (Fig. 4.17). The human gene coding for proopiomelanocortin maps to chromosome 2p23.3. Here, initially three SNPs were selected, later one had to be excluded due to inadequate clustering of the genotypes. For all gene loci, locations of the finally genotyped markers are shown in figure 4.17. One marker lay upstream and one downstream of the transcribed region.

Tab. 4.1: SNPs major allele frequency and association with alcohol dependence for the exploratory genotyping.

Gene	Marker	SNP	Controls	Cases	p (Armitage)
PENK	rs8181029	C\T	.58	.60	.557
PENK	rs6474063	T\C	.68	.69	.926
PENK	rs1975285	C\G	.76	.76	.916
PENK	rs2576576	G\A	.67	.61	.088
PENK	rs2576581	G\A	.55	.55	.991
POMC	rs1866146	C\G	.68	.65	.349
POMC	rs874401	G\A	.80	.84	.313
PDYN	rs910080	A\G	.73	.69	.267
PDYN	rs2235754	C\T	.65	.62	.422
PDYN	rs6045920	C\A	.85	.84	.639

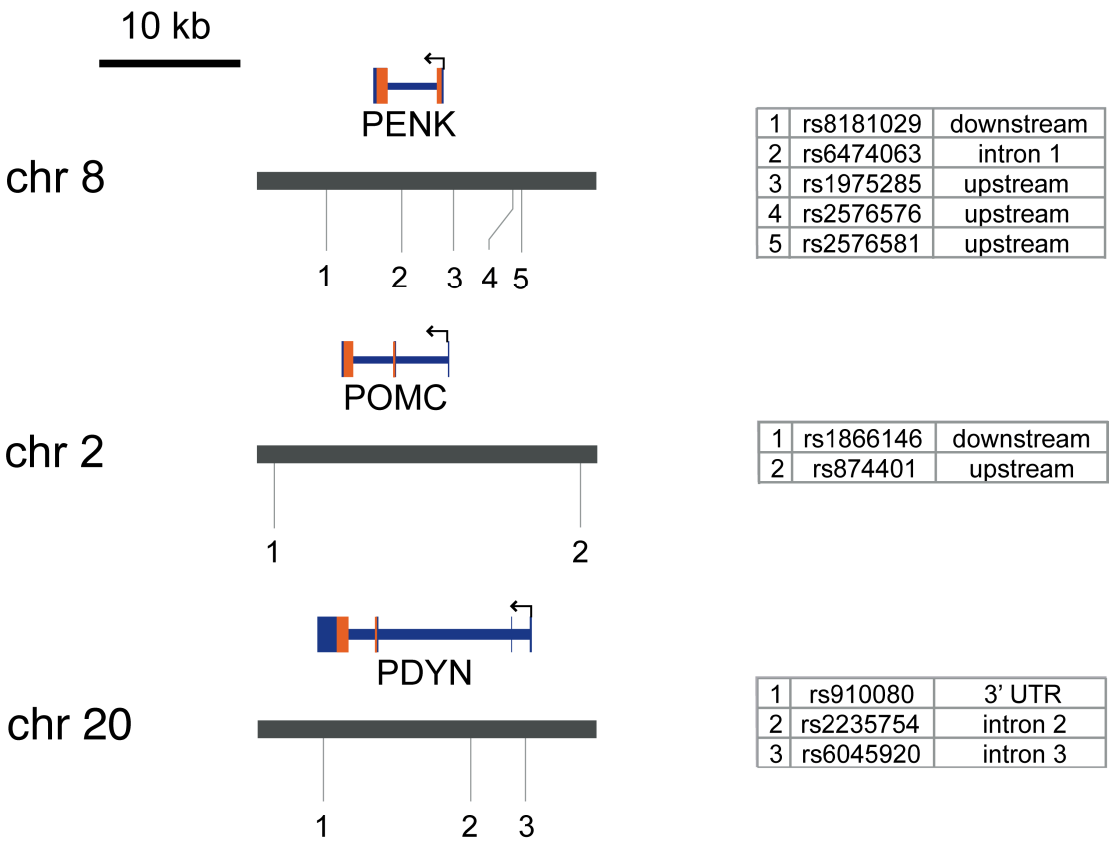

Fig. 4.17: Location of markers used for initial genotyping of the human opioid peptide genes. The gene coding for preproenkephalin (*PENK*) has a size of ∼ 5 kb and is located on chromosome 8. The gene for proopiomelanocortin (*POMC*) is mapped to chromosome 2 with a size of approximately 8 kb. On chromosome 20, the *PDYN* gene (coding for preprodynorphin) is sited, which spans around 15 kb. The direction of transcription is indicated by an arrow.

Figure 4.17 also illustrates the allocation of markers throughout the human preprodynorphin gene locus on chromosome 20pter-p12.2.

Genotype distributions for all polymorphisms were consistent with HWE expectations in both groups. However, no significant frequency difference among cases and controls was observed. For marker rs2576581, located upstream of *PENK*, the A allele was more frequent in cases but this failed to reach significance (p = 0.088). All allele frequencies and p-values are listed in table 4.1.

4.2.2 Detailed genotyping

An in depth genotyping was performed based on results obtained from in-house behavioral analysis of knockout mouse strains for *Penk1*, ß-endorphin (encoded by *Pomc*) and *Pdyn* and based on the frequency difference observed for marker rs2576581. In addition to an increased

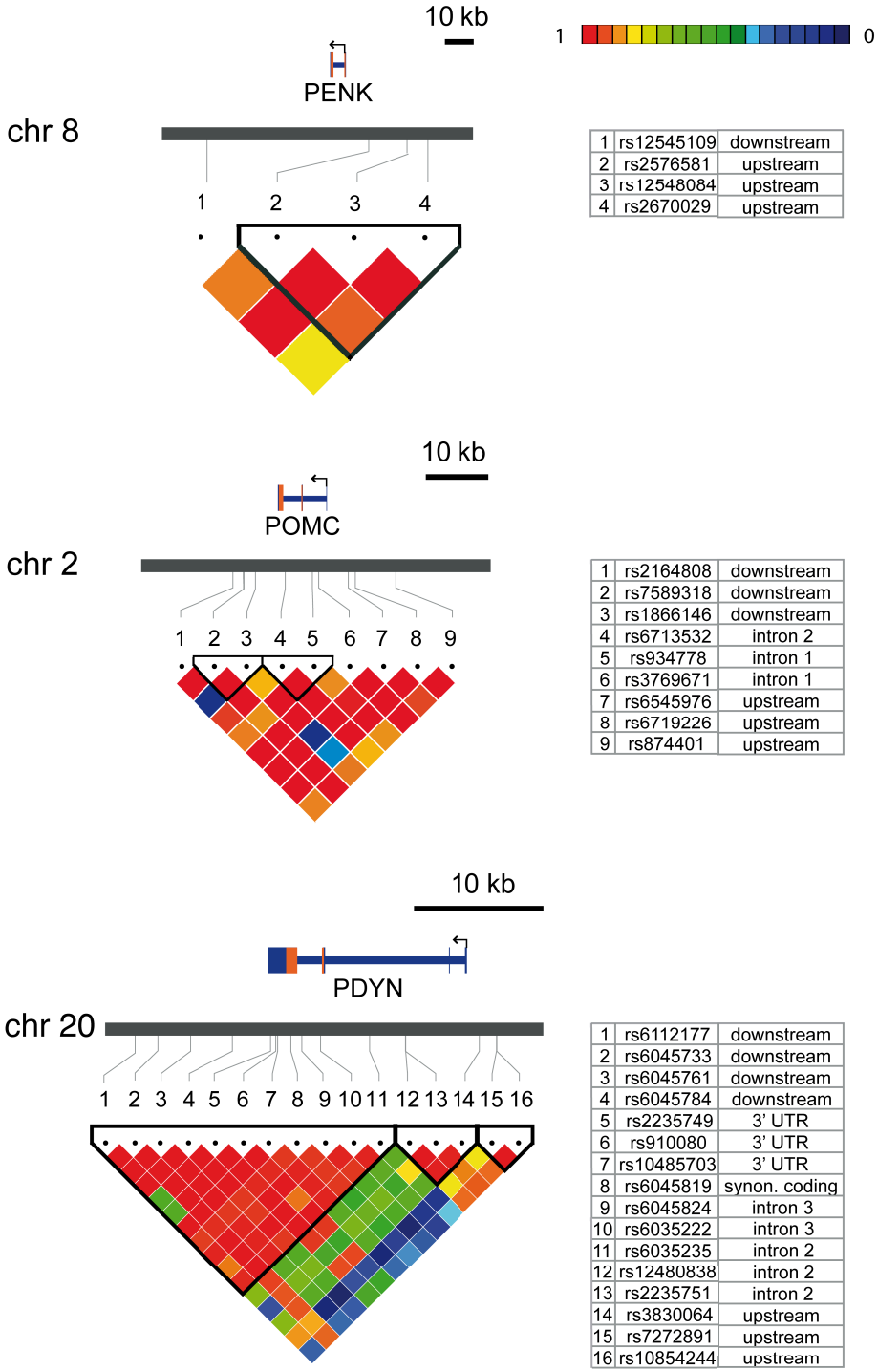

Fig. 4.18: Location and linkage disequilibrium of single nucleotide polymorphisms (SNPs). The gene structure of the human *PENK*, *POMC* and *PDYN* locus and the location of the analyzed SNPs are indicated. The coding regions are plotted in orange. For all three genes the negative strand is the transcription template. Linkage disequilibrium (LD) was calculated on the basis of the German control group. LD-plots were generated with Haploview using the Gold-heatmap color scheme, whereas 1 reflects strong linkage. Black triangles represent predicted haplotype blocks. The direction of transcription is indicated by an arrow.

number of cases, a different control sample that was designed to serve as an epidemiological control sample in the context of the NGFN was provided. The use of this cohort allowed the precise match of cases and controls in terms of gender and age. Linkage disequilibrium and haplotype data for all three genes (based on a reference population) was available at the International HapMap project (Phase II). The data were extracted and appropriate tagging SNPs were chosen to cover the genomic regions of interest.

For *PENK*, all five predicted tagging SNPs that covered a haplotype block of 92 kb (HapMap) encompassing the two exons were genotyped. For marker rs10108778 genotyping signals were not grouped into well-defined clusters. Therefore genotyping data for this marker were excluded from the analysis. The SNP rs12545109 is located 40 kb downstream of exon 2, and rs2670029 is 26 kb upstream of the transcription initiation site (Fig. 4.18). Based on the LD structure of German control subjects, one haplotype block for markers rs2576581, rs12548084, and rs2670029 was indicated (Fig. 4.18). This block corresponds to the haplotype block predicted by HapMap data for Caucasian populations. In contrast, markers at the *POMC* gene locus showed weak LD in Caucasians. Therefore, nine SNPs with an even spacing of approximately 3 kb extending from 11 kb upstream of exon 1 up to 6 kb downstream from exon 3 were selected (Fig. 4.18). In accordance with HapMap data, only weak LD between markers was detected in the German control group. Regarding the *PDYN* gene locus, markers which captured all common haplotypes at a frequency > 5 % in a Caucasian reference population were genotyped. At the same time Xuei and coworkers reported evidence for significant association of *PDYN* SNPs with alcoholism in the COGA cohort (XUEI ET AL., 2006). Therefore markers presented in their study were included for genotyping. This led to an overall number of 16 *PDYN* polymorphisms analyzed in the present study. Based on results of all *PDYN* markers in the German control group, calculation of the haplotype structure revealed three haplotype blocks that resemble those predicted by HapMap. The strongest LD was observed in the 3' part of *PDYN* leading to an haplotype block of around 18 kb (Fig. 4.18).

The analysis of *Penk1*, ß-endorphin and *Pdyn* knockout mice revealed gender-specific differences in alcohol-related behaviors, therefore the allele and genotype distributions were evaluated in the entire sample as well as separately in the female and male subgroups. In the German sample, all markers showed expected allele frequencies (Tab. 4.2 and Tab. 4.4) and revealed no significant deviation from HWE ($p > 0.05$) (Appendix). The unadjusted p-values for allele frequencies of rs12545109 (p = 0.031, OR = 0.73) and rs2576581 in females (p = 0.007, OR = 1.84) (Tab. 4.2) suggested association with the disease phenotype. Both SNPs also showed low p-values for genotype distributions between alcoholic patients and control subjects in the whole sample or the female subgroup, respectively (Tab. 4.3).

After Bonferroni correction for multiple testing, only marker rs2576581 remained below the 5% significance threshold (allele distribution corrected p = 0.028; Armitage's Trend Test corrected p = 0.036). For rs2164808 and rs934778, which are located upstream of the *POMC*

Tab. 4.2: *PENK* and *POMC* SNPs major allele frequency and association with alcohol dependence in the German sample.

Gene	Marker	SNP	Genomic Location[a]	Sample	Control Subjects[b]	Cases	p[c]	OR	CI[d]
PENK	**rs12545109**	C\A	57476460	**All**	.773	.712	**.031**	.729	[.546–.972]
		C\A		Female[e]	.769	.708	.260	.729	[.420–1.265]
		C\A		Male	.774	.712	.060	.723	[.515–1.014]
PENK	**rs2576581**	G\A	57528504	All	.515	.568	.094	1.242	[.963–1.602]
		G\A		**Female**	.418	.586	**.007**	1.971	[1.206–3.222]
		G\A		Male	.552	.565	.729	1.054	[.782–1.422]
PENK	rs12548084	C\T	57540810	All	.946	.945	.924	.973	[.560–1.694]
		C\T		Female	.948	.954	.820	1.139	[.372–3.485]
		C\T		Male	.946	.941	.800	.921	[.486–1.745]
PENK	rs2670029	C\A	57547550	All	.569	.606	.239	1.165	[.903–1.503]
		C\A		Female	.478	.585	.082	1.539	[.946–2.504]
		C\A		Male	.603	.615	.759	1.048	[.776–1.415]
POMC	**rs2164808**	G\A	25230680	All	.557	.496	.056	1.276	[.993–1.640]
		G\A		**Female**	.612	.462	**.014**	1.840	[1.128–3.002]
		G\A		Male	.536	.506	.413	1.130	[.843–1.515]
POMC	rs7589318	G\A	25231876	All	.678	.651	.368	.885	[.679–1.154]
		G\A		Female	.701	.600	.084	.638	[.383–1.063]
		G\A		Male	.669	.668	.956	.992	[.727–1.353]
POMC	rs1866146	T\C	25234077	All	.670	.669	1.000	.997	[.764–1.301]
		T\C		Female	.664	.636	.635	.885	[.534–1.465]
		T\C		Male	.672	.683	.767	1.049	[.766–1.435]
POMC	rs6713532	T\C	25238337	All	.771	.798	.308	1.171	[.864–1.588]
		T\C		Female	.739	.754	.779	1.083	[.622–1.886]
		T\C		Male	.783	.816	.280	1.224	[.848–1.765]
POMC	**rs934778**[e]	T\C	25242728	All	.692	.732	.088	1.217	[.971–1.525]
		T\C		**Female**	.693	.786	**.004**	1.629	[1.165–2.279]
		T\C		Male	.690	.685	.896	.979	[.713–1.344]
POMC	rs3769671[e]	A\C	25243657	All	.967	.974	.404	1.298	[.701–2.403]
		A\C		Female	.974	.970	.725	3.969	[.487–2.812]
		A\C		Male	.957	.978	.124	1.959	[.820–4.681]
POMC	rs6545976	G\T	25248154	All	.967	.959	.489	.790	[.404–1.543]
		G\T		Female	.970	.961	.683	.757	[.199–2.884]
		G\T		Male	.966	.958	.566	.798	[.368–1.729]
POMC	rs6719226	C\G	25249516	All	.966	.959	.571	.827	[.428–1.598]
		C\G		Female	.970	.961	.683	.757	[.199–2.884]
		C\G		Male	.964	.958	.666	.847	[.397–1.806]
POMC	rs874401	C\T	25255784	All	.812	.837	.303	1.189	[.855–1.652]
		C\T		Female	.791	.800	.856	1.057	[.581–1.922]
		C\T		Male	.819	.852	.240	1.268	[.853–1.885]

[a] Genomic location is based on National Center for Biotechnology Information (NCBI) human genome assembly B36.
[b] The allele frequency is shown for the left allele.
[c] Uncorrected p-value of FAMHAP statistic for allele frequency differences between cases and controls.
[d] 95% confidence interval.
[e] Note that the sample size is different for these markers.
Significance is indicated in bold.

gene and inside intron 1, respectively, differences for allele and genotype distributions between German female alcoholic patients and control subjects were detected (Tab. 4.2 and Tab. 4.3). However, after Bonferroni correction, only marker rs934778 showed a significant association in German females (allele distribution corrected p = 0.036; Armitage's Trend Test corrected p = 0.027).

In the case of *PDYN*, a few markers revealed significant frequency differences on an allelic level between female cases and controls only. Here, unadjusted p-values for SNP rs2235749,

Tab. 4.3: *PENK* and *POMC* SNPs genotype distribution and association with alcohol dependence in the German sample.

Gene	Marker	Sample	Control Subjects			Cases			p (Global)[a]	p (Armitage)[b]
PENK	**rs12545109**	All	CC=148	AC=81	AA=15	CC=126	AC=97	AA=22	.104	**.035**
		Female	CC=39	AC=25	AA=3	CC=33	AC=26	AA=6	.475	.260
		Male	CC=109	AC=56	AA=12	CC=92	AC=71	AA=16	.152	.068
PENK	**rs2576581**	All	GG=63	AG=120	AA=56	GG=85	AG=104	AA=52	.103	.104
		Female	GG=11	AG=34	AA=22	GG=24	AG=27	AA=13	**.019**	**.009**
		Male	GG=52	AG=86	AA=34	GG=61	AG=77	AA=38	.499	.736
PENK	rs12548084	All	CC=218	CT=22	TT=2	CC=218	CT=27	TT=0	.288	.924
		Female	CC=60	CT=7	TT=0	CC=59	CT=6	TT=0	.815	.815
		Male	CC=158	CT=15	TT=2	CC=158	CT=21	TT=0	.228	.805
PENK	rs2670029	All	CC=78	AC=124	AA=44	CC=93	AC=111	AA=41	.343	.242
		Female	CC=13	AC=38	AA=16	CC=22	AC=32	AA=11	.155	.072
		Male	CC=65	AC=86	AA=28	CC=71	AC=78	AA=30	.696	.764
POMC	**rs2164808**	All	GG=75	AG=125	AA=47	GG=65	AG=113	AA=67	.090	.060
		Female	GG=24	AG=34	AA=9	GG=15	AG=30	AA=20	**.039**	**.015**
		Male	GG=51	AG=91	AA=38	GG=49	AG=83	AA=47	.507	.420
POMC	rs7589318	All	GG=117	AG=101	AA=29	GG=110	AG=99	AA=36	.612	.388
		Female	GG=33	AG=28	AA=6	GG=26	AG=26	AA=13	.178	.099
		Male	GG=84	AG=73	AA=23	GG=83	AG=73	AA=23	.999	.956
POMC	rs1866146	All	TT=117	CT=97	CC=33	TT=113	CT=102	CC=30	.848	1.000
		Female	TT=28	CT=33	CC=6	TT=26	CT=32	CC=8	.832	.621
		Male	TT=89	CT=64	CC=27	TT=87	CT=69	CC=22	.701	.783
POMC	rs6713532	All	TT=151	CT=79	CC=17	TT=158	CT=75	CC=12	.572	.326
		Female	TT=37	CT=25	CC=5	TT=36	CT=26	CC=3	.778	.777
		Male	TT=114	CT=54	CC=12	TT=122	CT=48	CC=9	.592	.306
POMC	**rs934778**	All	TT=195	CT=191	CC=34	TT=178	CT=130	CC=24	.146	.082
		Female	TT=107	CT=120	CC=14	TT=97	CT=45	CC=10	**.0004**	**.003**
		Male	TT=88	CT=71	CC=20	TT=80	CT=84	CC=14	.283	.896
POMC	rs3769671	All	AA=382	AC=25	CC=1	AA=314	AC=17	CC=0	.561	.409
		Female	AA=221	AC=12	CC=0	AA=141	AC=9	CC=0	.722	.722
		Male	AA=161	AC=13	CC=1	AA=171	AC=8	CC=0	.294	.134
POMC	rs6545976	All	GG=228	GT=16	TT=0	GG=223	GT=20	TT=0	.480	.480
		Female	GG=63	GT=4	TT=0	GG=59	GT=5	TT=0	.677	.677
		Male	GG=165	GT=12	TT=0	GG=163	GT=15	TT=0	.558	.558
POMC	rs6719226	All	CC=231	CG=15	GG=1	CC=222	CG=20	GG=0	.398	.575
		Female	CC=63	CG=4	GG=0	CC=59	CG=5	GG=0	.677	.677
		Male	CC=168	CG=11	GG=1	CC=162	CG=15	GG=0	.428	.671
POMC	rs874401	All	CC=165	CT=71	TT=11	CC=173	CT=64	TT=8	.601	.316
		Female	CC=41	CT=24	TT=2	CC=43	CT=18	TT=4	.463	.858
		Male	CC=124	CT=47	TT=9	CC=130	CT=45	TT=4	.349	.255

[a] Uncorrected p-value of FAMHAP statistic for association between SNPs and alcohol dependence.
[b] p-value of the Armitage's trend test.
Significance is indicated in bold.

rs910080, rs6045819, rs6045824, rs6035253 were below the 5% significance threshold. An odds ratio < 1 indicated a protective effect of the major allele for these markers (Tab.4.4).

Four of these markers (rs2235749, rs910080, rs6045824, rs6035253) also differed significantly in their genotype frequency among female cases and controls (Tab. 4.5). However, only for rs910080 and rs6035235, the global genotype as well as the *Armitage* p-value was significant. Another two markers showed association in the female subgroup only on a genotype level (rs6045733 and rs6035222). For rs6035222 a significant difference in genotype distributions was detected in the whole sample as well (Tab. 4.5). However, subsequent to Bonferroni correction none of the markers remained significant.

Tab. 4.4: *PDYN* SNPs major allele frequency and association with alcohol dependence in the German sample.

Gene	Marker	SNP	Genomic Location[a]	Sample	Control Subjects[b]	Cases	p[c]	OR	CI[d]
PDYN	rs6112177	C\A	1897127	All	.814	.798	.531	.904	[.659-1.240]
		C\A		Female	.843	.795	.310	.723	[.385-1.356]
		C\A		Male	.803	.798	.867	.969	[.672-1.398]
PDYN	rs6045733	G\A	1898858	All	.642	.61	.300	.872	[.674-1.129]
		G\A		Female	.687	.59	.098	.656	[.397-1.083]
		G\A		Male	.625	.615	.787	.959	[.709-1.297]
PDYN	rs6045761	A\G	1901363	All	.883	.88	.888	.972	[.660-1.433]
		A\G		Female	.933	.875	.111	.504	[.214-1.186]
		A\G		Male	.864	.881	.502	1.163	[.748-1.808]
PDYN	rs6045784	T\C	1904663	All	.885	.875	.643	.913	[.621-1.342]
		T\C		Female	.933	.864	.062	.456	[.197-1.056]
		T\C		Male	.867	.879	.635	1.113	[.716-1.729]
PDYN	**rs2235749**	G\A	1907939	All	.719	.697	.459	.901	[.685-1.186]
		G\A		**Female**	.799	.679	**.026**	.534	[.306-.932]
		G\A		Male	.689	.702	.698	1.065	[.775-1.465]
PDYN	**rs910080**	A\G	1908226	All	.727	.711	.592	.927	[.702-1.224]
		A\G		**Female**	.806	.679	**.018**	.509	[.291-.893]
		A\G		Male	.697	.722	.467	1.127	[.816-1.557]
PDYN	rs10485703	A\G	1908313	All	.883	.876	.752	.940	[.641-1.379]
		A\G		Female	.933	.866	.068	.464	[.200-1.074]
		A\G		Male	.864	.879	.540	1.147	[.740-1.778]
PDYN	**rs6045819**	A\G	1909134	All	.885	.869	.467	.868	[.593-1.271]
		A\G		**Female**	.933	.856	**.041**	.428	[.186-.985]
		A\G		Male	.867	.874	.783	1.063	[.688-1.644]
PDYN	**rs6045824**	T\C	1909977	All	.886	.871	.479	.871	[.593-1.278]
		T\C		**Female**	.94	.862	**.032**	.395	[.165-.944]
		T\C		Male	.866	.874	.739	1.077	[.696-1.666]
PDYN	rs6035222	G\A	1911413	All	.877	.852	.254	.809	[.561-1.165]
		G\A		Female	.925	.851	.053	.46	[.206-1.024]
		G\A		Male	.858	.851	.784	.944	[.623-1.43]
PDYN	**rs6035235**	T\G	1915155	All	.889	.871	.405	.849	[.578-1.248]
		T\G		**Female**	.94	.854	**.020**	.371	[.156-.881]
		T\G		Male	.869	.877	.758	1.072	[.690-1.664]
PDYN	rs12480838	C\T	1917824	All	.844	.837	.753	.947	[.672-1.333]
		C\T		Female	.881	.815	.140	.599	[.302-1.188]
		C\T		Male	.831	.844	.634	1.102	[.740-1.641]
PDYN	rs2235751	A\G	1917934	All	.753	.730	.420	.888	[.666-1.184]
		A\G		Female	.766	.731	.523	.833	[.476-1.458]
		A\G		Male	.749	.729	.544	.901	[.645-1.261]
PDYN	rs3830064	A\C	1923679	All	.844	.830	.546	.901	[.643-1.264]
		A\C		Female	.873	.806	.134	.604	[.310-1.174]
		A\C		Male	.833	.838	.867	1.034	[.697-1.535]
PDYN	rs7272891	C\G	1924818	All	.915	.912	.862	.962	[.616-1.5]
		C\G		Female	.896	.909	.709	1.167	[.518-2.627]
		C\G		Male	.922	.912	.635	.879	[.515-1.498]
PDYN	rs10854244	A\T	1925045	All	.755	.728	.326	.867	[.651-1.153]
		A\T		Female	.761	.709	.333	.764	[.443-1.318]
		A\T		Male	.753	.733	.548	.902	[.645-1.262]

[a] Genomic location is based on National Center for Biotechnology Information (NCBI) human genome assembly B36.
[b] The allele frequency is shown for the left allele.
[c] Uncorrected p-value of FAMHAP statistic for allele frequency differences between cases and controls.
[d] 95% confidence interval.
Significance is indicated in bold.

Tab. 4.5: *PDYN* SNPs genotype distribution and association with alcohol dependence in the German sample.

Gene	Marker	Sample	Control Subjects			Cases			p (Global)[a]	p (Armitage)[b]
PDYN	rs6112177	All	CC=163	AC=76	AA=8	CC=155	AC=81	AA=9	.814	.527
		Female	CC=48	AC=17	AA=2	CC=40	AC=25	AA=1	.276	.294
		Male	CC=115	AC=59	AA=6	CC=114	AC=56	AA=8	.837	.867
PDYN	**rs6045733**	All	GG=106	AG=105	AA=36	GG=85	AG=130	AA=31	.069	.296
		Female	GG=34	AG=24	AA=9	GG=19	AG=41	AA=7	**.011**	.09
		Male	GG=72	AG=81	AA=27	GG=65	AG=89	AA=24	.638	.786
PDYN	rs6045761	All	AA=195	AG=46	GG=6	AA=185	AG=54	GG=2	.243	.89
		Female	AA=59	AG=7	GG=1	AA=49	AG=14	GG=1	.203	.124
		Male	AA=136	AG=39	GG=5	AA=135	AG=40	GG=1	.267	.503
PDYN	rs6045784	All	TT=196	CT=45	CC=6	TT=185	CT=57	CC=2	.156	.646
		Female	TT=59	CT=7	CC=1	TT=49	CT=16	CC=1	.109	.068
		Male	TT=137	CT=38	CC=5	TT=135	CT=41	CC=1	.25	.635
PDYN	**rs2235749**	All	GG=133	AG=89	AA=25	GG=117	AG=109	AA=20	.165	.465
		Female	GG=45	AG=17	AA=5	GG=29	AG=33	AA=5	**.014**	**.029**
		Male	GG=88	AG=72	AA=20	GG=87	AG=76	AA=15	.665	.701
PDYN	**rs910080**	All	AA=136	AG=87	GG=24	AA=123	AG=104	GG=19	.253	.599
		Female	AA=45	AG=18	GG=4	AA=29	AG=33	GG=5	**.018**	**.018**
		Male	AA=91	AG=69	GG=20	AA=93	AG=71	GG=14	.578	.478
PDYN	rs10485703	All	AA=195	AG=46	GG=6	AA=187	AG=57	GG=2	.188	.753
		Female	AA=59	AG=7	GG=1	AA=50	AG=16	GG=1	.119	.075
		Male	AA=136	AG=39	GG=5	AA=136	AG=41	GG=1	.258	.54
PDYN	**rs6045819**	All	AA=195	AG=47	GG=5	AA=183	AG=60	GG=2	.198	.465
		Female	AA=59	AG=7	GG=1	AA=48	AG=17	GG=1	.071	**.046**
		Male	AA=136	AG=40	GG=4	AA=134	AG=43	GG=1	.384	.78
PDYN	**rs6045824**	All	TT=196	CT=44	CC=6	TT=184	CT=59	CC=2	.102	.482
		Female	TT=60	CT=6	CC=1	TT=48	CT=16	CC=1	.054	**.037**
		Male	TT=136	CT=38	CC=5	TT=135	CT=43	CC=1	.225	.739
PDYN	**rs6035222**	**All**	GG=193	AG=47	AA=7	GG=175	AG=69	AA=2	**.02**	.254
		Female	GG=59	AG=6	AA=2	GG=48	AG=18	AA=1	**.024**	.065
		Male	GG=134	AG=41	AA=5	GG=126	AG=51	AA=1	.136	.78
PDYN	**rs6035235**	All	TT=197	GT=45	GG=5	TT=184	GT=59	GG=2	.165	.405
		Female	TT=60	GT=6	GG=1	TT=47	GT=17	GG=1	**.033**	**.024**
		Male	TT=137	GT=39	GG=4	TT=136	GT=42	GG=1	.384	.755
PDYN	rs12480838	All	CC=176	CT=65	TT=6	CC=168	CT=69	TT=5	.842	.751
		Female	CC=51	CT=16	TT=0	CC=42	CT=22	TT=1	.248	.115
		Male	CC=125	CT=49	TT=6	CC=125	CT=47	TT=4	.82	.635
PDYN	rs2235751	All	AA=137	AG=92	GG=14	AA=126	AG=103	GG=14	.582	.409
		Female	AA=35	AG=28	GG=1	AA=34	AG=30	GG=3	.602	.482
		Male	AA=102	AG=64	GG=13	AA=91	AG=73	GG=11	.512	.544
PDYN	rs3830064	All	AA=175	AC=67	CC=5	AA=170	AC=70	CC=7	.79	.543
		Female	AA=50	AC=17	CC=0	AA=43	AC=22	CC=2	.205	.118
		Male	AA=125	AC=50	CC=5	AA=126	AC=48	CC=5	.979	.867
PDYN	rs7272891	All	CC=207	CG=38	GG=2	CC=205	CG=35	GG=4	.677	.867
		Female	CC=53	CG=14	GG=0	CC=54	CG=12	GG=0	.693	.693
		Male	CC=154	CG=24	GG=2	CC=150	CG=23	GG=4	.699	.655
PDYN	rs10854244	All	AA=143	AT=87	TT=17	AA=127	AT=104	TT=15	.275	.323
		Female	AA=38	AT=26	TT=3	AA=32	AT=31	TT=4	.578	.309
		Male	AA=105	AT=61	TT=14	AA=94	AT=73	TT=11	.362	.552

[a]Uncorrected p-value of FAMHAP statistic for association between SNPs and alcohol dependence.
[b]p-value of the Armitage's trend test.
Significance is indicated in bold.

Tab. 4.6: *PENK* and *POMC* SNPs major allele frequency and association with alcohol dependence in the Swedish sample.

Gene	Marker	SNP	Genomic Location[a]	Sample	Control Subjects[b]	Cases	p[c]	OR	CI[d]
PENK	rs12545109	C\A	57476460	All	.763	.768	.811	1.03	[.807-1.315]
		C\A		Female	.795	.795	1.000	1.004	[.636-1.584]
		C\A		Male	.748	.756	.765	1.045	[.782-1.397]
PENK	rs2576581	G\A	57528504	All	.536	.522	.599	.946	[.769-1.164]
		G\A		Female	.496	.518	.638	1.092	[.757-1.575]
		G\A		Male	.556	.523	.303	.876	[.680-1.127]
PENK	rs12548084	C\T	57540810	All	.953	.943	.407	.822	[.517-1.306]
		C\T		Female	.957	.939	.366	.682	[.297-1.569]
		C\T		Male	.950	.944	.675	.887	[.507-1.552]
PENK	rs2670029	C\A	57547550	All	.580	.571	.735	.964	[.782-1.189]
		C\A		Female	.547	.577	.516	1.131	[.780-1.639]
		C\A		Male	.595	.566	.358	.888	[.688-1.144]
POMC	rs2164808	G\A	25230680	All	.546	.544	.950	.993	[.806-1.224]
		G\A		Female	.534	.527	.877	.971	[.671-1.406]
		G\A		Male	.551	.550	.975	.995	[.773-1.281]
POMC	rs7589318	G\A	25231876	All	.708	.674	.168	.854	[.682-1.069]
		G\A		Female	.709	.664	.293	.808	[.543-1.202]
		G\A		Male	.707	.678	.319	.871	[.663-1.144]
POMC	rs1866146	T\C	25234077	All	.610	.622	.656	1.049	[.849-1.297]
		T\C		Female	.641	.600	.363	.840	[.577-1.223]
		T\C		Male	.596	.632	.251	1.163	[.899-1.505]
POMC	rs6713532	T\C	25238337	All	.776	.741	.118	.824	[.646-1.051]
		T\C		Female	.795	.732	.114	.704	[.456-1.089]
		T\C		Male	.767	.748	.483	.900	[.671-1.208]
POMC	**rs934778**	T\C	25242728	**All**	.698	.745	**.048**	1.264	[1.002-1.593]
		T\C		Female	.698	.750	.219	1.296	[.857-1.962]
		T\C		Male	.698	.742	.128	1.242	[.939-1.643]
POMC	rs3769671	A\C	25243657	All	.963	.963	.944	.980	[.569-1.688]
		A\C		Female	.966	.926	.055	.440	[.186-1.040]
		A\C		Male	.962	.980	.103	1.888	[.869-4.103]
POMC	rs6545976	G\T	25248154	All	.969	.962	.462	.809	[.459-1.425]
		G\T		Female	.962	.973	.505	1.427	[.499-4.076]
		G\T		Male	.972	.961	.310	.696	[.345-1.405]
POMC	rs6719226	C\G	25249516	All	.969	.962	.451	.805	[.457-1.417]
		C\G		Female	.962	.973	.516	1.414	[.495-4.040]
		C\G		Male	.972	.961	.304	.694	[.344-1.399]
POMC	**rs874401**	C\T	25255784	All	.825	.794	.135	.817	[.627-1.065]
		C\T		**Female**	.872	.791	**.021**	.556	[.337-.919]
		C\T		Male	.803	.795	.748	.950	[.694-1.300]

[a] Genomic location is based on National Center for Biotechnology Information (NCBI) human genome assembly B36.
[b] The allele frequency is shown for the left allele.
[c] Uncorrected p-value of FAMHAP statistic for allele frequency differences between cases and controls.
[d] 95% confidence interval.
Significance is indicated in bold.

To confirm the association results obtained in the German sample, genotyping of a replication cohort was performed. For this purpose, a Swedish cohort of alcoholics and controls was studied. Genotype distributions were in HWE, with the exception of two markers (rs2164808 p = 0.044, rs6045733 p = 0.013), which significantly deviated from HWE in the controls group (Appendix). These two SNPs did not show significant frequency differences between cases and controls (Tab. 4.6).

None of the SNPs typed for *PENK* showed significant frequency differences comparing Swedish cases and controls. Allele and genotype distribution of the *POMC* marker

Tab. 4.7: *PENK* and *POMC* SNPs genotype distribution and association with alcohol dependence in the Swedish sample.

Gene	Marker	Sample	Control Subjects			Cases			p (Global)[a]	p (Armitage)[b]
PENK	rs12545109	All	CC=211	AC=135	AA=19	CC=205	AC=134	AA=15	.822	.806
		Female	CC=75	AC=36	AA=6	CC=70	AC=35	AA=5	.969	1.000
		Male	CC=136	AC=99	AA=13	CC=134	AC=98	AA=10	.845	.757
PENK	rs2576581	All	GG=104	AG=177	AA=78	GG=91	AG=192	AA=75	.464	.593
		Female	GG=28	AG=59	AA=29	GG=28	AG=61	AA=24	.792	.629
		Male	GG=76	AG=118	AA=49	GG=62	AG=130	AA=51	.360	.297
PENK	rs12548084	All	CC=335	CT=33	TT=1	CC=319	CT=39	TT=1	.686	.407
		Female	CC=108	CT=8	TT=1	CC=101	CT=12	TT=1	.608	.393
		Male	CC=227	CT=25	TT=0	CC=216	CT=27	TT=0	.666	.666
PENK	rs2670029	All	CC=121	AC=180	AA=63	CC=107	AC=189	AA=57	.546	.728
		Female	CC=36	AC=56	AA=25	CC=34	AC=59	AA=17	.486	.510
		Male	CC=85	AC=124	AA=38	CC=72	AC=130	AA=40	.544	.341
POMC	rs2164808	All	GG=98	AG=198	AA=65	GG=101	AG=181	AA=70	.644	.95
		Female	GG=34	AG=56	AA=26	GG=34	AG=48	AA=28	.767	.882
		Male	GG=64	AG=142	AA=39	GG=66	AG=133	AA=42	.817	.964
POMC	rs7589318	All	GG=178	AG=158	AA=27	GG=163	AG=146	AA=41	.151	.169
		Female	GG=59	AG=48	AA=10	GG=51	AG=44	AA=15	.463	.307
		Male	GG=119	AG=110	AA=17	GG=111	AG=102	AA=26	.307	.313
POMC	rs1866146	All	TT=129	CT=184	CC=49	TT=143	CT=163	CC=55	.311	.654
		Female	TT=49	CT=52	CC=16	TT=42	CT=54	CC=19	.665	.370
		Male	TT=80	CT=132	CC=33	TT=100	CT=107	CC=36	.084	.243
POMC	rs6713532	All	TT=220	CT=125	CC=19	TT=190	CT=137	CC=22	.266	.118
		Female	TT=74	CT=38	CC=5	TT=58	CT=45	CC=7	.266	.111
		Male	TT=146	CT=87	CC=14	TT=132	CT=92	CC=14	.712	.482
POMC	**rs934778**	All	TT=171	CT=169	CC=26	TT=189	CT=145	CC=17	.116	**.038**
		Female	TT=57	CT=48	CC=11	TT=61	CT=43	CC=6	.423	.218
		Male	TT=114	CT=121	CC=15	TT=127	CT=102	CC=11	.255	.104
POMC	rs3769671	All	AA=341	AC=27	CC=0	AA=335	AC=25	CC=1	.588	.944
		Female	AA=110	AC=8	CC=0	AA=99	AC=15	CC=1	.160	.059
		Male	AA=231	AC=19	CC=0	AA=234	AC=10	CC=0	.098	.098
POMC	rs6545976	All	GG=348	GT=21	TT=1	GG=328	GT=25	TT=1	.746	.473
		Female	GG=109	GT=7	TT=1	GG=104	GT=6	TT=0	.613	.526
		Male	GG=239	GT=14	TT=0	GG=223	GT=19	TT=0	.301	.301
POMC	rs6719226	All	CC=350	CG=21	GG=1	CC=328	CG=25	GG=1	.735	.461
		Female	CC=110	CG=7	GG=1	CC=104	CG=6	GG=0	.617	.536
		Male	CC=240	CG=14	GG=0	CC=223	CG=19	GG=0	.296	.296
POMC	**rs874401**	All	CC=249	CT=101	TT=13	CC=220	CT=119	TT=13	.213	.135
		Female	CC=89	CT=26	TT=2	CC=69	CT=36	TT=5	.074	**.022**
		Male	CC=160	CT=75	TT=11	CC=150	CT=83	TT=8	.563	.747

[a]Uncorrected p-value of FAMHAP statistic for association between SNPs and alcohol dependence.
[b]p-value of the Armitage's trend test.
Significance is indicated in bold.

rs934778 differed significantly among alcoholics and healthy controls in the whole sample. For rs3769671 and rs874401 the association was only detected in the female subgroup (Tab .4.6 and Tab. 4.7).

In case of the *PDYN* locus, rs6112177, rs2235749, rs2235751 and rs10854244 unadjusted p values for allele as well as genotype distributions were identified as significant examining the whole sample. For three of them (rs2235749, rs2235751, rs10854244) the same was seen in the male subgroup.

Tab. 4.8: *PDYN* SNPs major allele frequency and association with alcohol dependence in the Swedish sample.

Gene	Marker	SNP	Genomic Location[a]	Sample	Control Subjects[b]	Cases	p[c]	OR	CI[d]
PDYN	**rs6112177**	C\A	1897127	**All**	.795	.848	**.009**	1.432	[1.092-1.878]
		C\A		Female	.791	.86	.051	1.622	[.995-2.645]
		C\A		Male	.798	.842	.069	1.355	[.976-1.880]
PDYN	rs6045733	G\A	1898858	All	.887	.911	.127	1.307	[.926-1.847]
		G\A		Female	.876	.904	.347	1.325	[.737-2.382]
		G\A		Male	.892	.916	.214	1.313	[.854-2.018]
PDYN	rs6045761	A\G	1901363	All	.895	.908	.406	1.158	[.819-1.638]
		A\G		Female	.876	.905	.333	1.341	[.740-2.429]
		A\G		Male	.903	.909	.765	1.067	[.696-1.637]
PDYN	rs6045784	T\C	1904663	All	.889	.905	.338	1.181	[.840-1.661]
		T\C		Female	.869	.891	.466	1.235	[.700-2.179]
		T\C		Male	.899	.910	.536	1.144	[.747-1.753]
PDYN	**rs2235749**	G\A	1907939	**All**	.616	.700	**.001**	1.454	[1.169-1.81]
		G\A		Female	.616	.696	.073	1.423	[.967-2.093]
		G\A		Male	.616	.701	.005	1.458	[1.118-1.902]
PDYN	rs910080	A\G	1908226	All	.891	.910	.226	1.238	[.876-1.749]
		A\G		Female	.874	.904	.298	1.364	[.758-2.454]
		A\G		Male	.900	.914	.427	1.190	[.774-1.831]
PDYN	rs10485703	A\G	1908313	All	.723	.762	.091	1.227	[.968-1.554]
		A\G		Female	.712	.743	.456	1.170	[.774-1.768]
		A\G		Male	.727	.769	.131	1.250	[.936-1.670]
PDYN	rs6045819	A\G	1909134	All	.729	.771	.068	1.249	[.984-1.587]
		A\G		Female	.728	.752	.556	1.134	[.746-1.722]
		A\G		Male	.730	.779	.074	1.305	[.974-1.748]
PDYN	rs6045824	T\C	1909977	All	.891	.910	.226	1.238	[.876-1.749]
		T\C		Female	.874	.904	.298	1.364	[.758-2.454]
		T\C		Male	.900	.914	.427	1.190	[.774-1.831]
PDYN	rs6035222	G\A	1911413	All	.886	.903	.274	1.206	[.862-1.688]
		G\A		Female	.870	.900	.307	1.350	[.758-2.404]
		G\A		Male	.893	.906	.511	1.150	[.758-1.743]
PDYN	rs6035235	T\G	1915155	All	.883	.893	.533	1.110	[.799-1.541]
		T\G		Female	.868	.883	.645	1.139	[.654-1.986]
		T\G		Male	.890	.899	.634	1.105	[.734-1.662]
PDYN	rs12480838	C\T	1917824	All	.851	.880	.108	1.281	[.947-1.734]
		C\T		Female	.855	.874	.56	1.173	[.686-2.005]
		C\T		Male	.849	.881	.141	1.316	[.912-1.900]
PDYN	**rs2235751**	A\G	1917934	**All**	.726	.780	**.02**	1.333	[1.045-1.701]
		A\G		Female	.745	.784	.342	1.238	[.797-1.923]
		A\G		**Male**	.718	.775	**.041**	1.356	[1.012-1.816]
PDYN	rs3830064	A\C	1923679	All	.827	.855	.151	1.231	[.927-1.634]
		A\C		Female	.827	.835	.834	1.054	[.646-1.720]
		A\C		Male	.827	.863	.125	1.312	[.927-1.858]
PDYN	rs7272891	C\G	1924818	All	.907	.924	.239	1.249	[.862-1.811]
		C\G		Female	.905	.948	.079	1.903	[.919-3.943]
		C\G		Male	.908	.914	.728	1.081	[.697-1.675]
PDYN	rs10854244	A\T	1925045	All	.713	.759	.05	1.266	[1.00-1.602]
		A\T		Female	.726	.743	.666	1.096	[.723-1.661]
		A\T		Male	.707	.765	.041	1.348	[1.012-1.796]

[a] Genomic location is based on National Center for Biotechnology Information (NCBI) human genome assembly B36.
[b] The allele frequency is shown for the left allele.
[c] Uncorrected p-value of FAMHAP statistic for allele frequency differences between cases and controls.
[d] 95% confidence interval.
Significance is indicated in bold.

Tab. 4.9: *PDYN* SNPs genotype distribution and association with alcohol dependence in the Swedish sample.

Gene	Marker	Sample	Control Subjects			Cases			p (Global)[a]	p (Armitage)[b]
PDYN	**rs6112177**	**All**	CC=232	AC=115	AA=17	CC=260	AC=92	AA=9	**.037**	**.01**
		Female	CC=75	AC=35	AA=7	CC=85	AC=26	AA=3	.172	.061
		Male	CC=157	AC=80	AA=10	CC=173	AC=65	AA=6	.191	.069
PDYN	rs6045733	All	AA=281	AG=73	GG=4	AA=299	AG=58	GG=3	.299	.126
		Female	AA=91	AG=23	GG=3	AA=93	AG=20	GG=1	.551	.359
		Male	AA=190	AG=50	GG=1	AA=204	AG=37	GG=2	.251	.206
PDYN	rs6045761	All	TT=296	CT=70	CC=4	TT=290	CT=59	CC=3	.707	.405
		Female	TT=91	CT=23	CC=3	TT=90	CT=19	CC=1	.557	.346
		Male	TT=205	CT=47	CC=1	TT=199	CT=40	CC=2	.706	.762
PDYN	rs6045784	All	TT=292	GT=74	GG=4	TT=289	GT=57	GG=5	.4	.343
		Female	TT=90	GT=25	GG=3	TT=88	GT=20	GG=2	.78	.48
		Male	TT=202	GT=49	GG=1	TT=200	GT=37	GG=3	.302	.533
PDYN	**rs2235749**	**All**	GG=126	AG=193	AA=42	GG=176	AG=155	AA=31	**.001**	**.0005**
		Female	GG=45	AG=53	AA=18	GG=54	AG=52	AA=9	.148	.072
		Male	GG=81	AG=140	AA=24	GG=120	AG=102	AA=22	**.001**	**.003**
PDYN	rs910080	All	TT=289	CT=71	CC=4	TT=301	CT=59	CC=3	.474	.225
		Female	TT=89	CT=23	CC=3	TT=94	CT=20	CC=1	.51	.311
		Male	TT=200	CT=48	CC=1	TT=205	CT=38	CC=2	.466	.42
PDYN	rs10485703	All	GG=182	AG=152	AA=23	GG=208	AG=137	AA=18	.215	.083
		Female	GG=61	AG=39	AA=13	GG=62	AG=47	AA=6	.191	.466
		Male	GG=121	AG=113	AA=10	GG=144	AG=89	AA=12	.081	.112
PDYN	rs6045819	All	AA=187	AG=148	GG=23	AA=213	AG=132	GG=17	.175	.063
		Female	AA=65	AG=36	GG=13	AA=64	AG=45	GG=6	.167	.572
		Male	AA=122	AG=112	GG=10	AA=147	AG=86	GG=11	.055	.061
PDYN	rs6045824	All	AA=289	AG=71	GG=4	AA=301	AG=59	GG=3	.474	.225
		Female	AA=89	AG=23	GG=3	AA=94	AG=20	GG=1	.51	.311
		Male	AA=200	AG=48	GG=1	AA=205	AG=38	GG=2	.466	.42
PDYN	rs6035222	All	AA=284	AG=75	GG=4	AA=295	AG=64	GG=3	.543	.271
		Female	AA=88	AG=24	GG=3	AA=93	AG=21	GG=1	.512	.316
		Male	AA=196	AG=51	GG=1	AA=200	AG=42	GG=2	.546	.502
PDYN	rs6035235	All	GG=277	AG=80	AA=2	GG=287	AG=71	AA=3	.635	.521
		Female	GG=86	AG=26	AA=2	GG=90	AG=23	AA=2	.874	.648
		Male	GG=191	AG=54	AA=0	GG=195	AG=47	AA=1	.468	.616
PDYN	rs12480838	All	CC=263	CT=95	TT=7	CC=278	CT=79	TT=4	.261	.103
		Female	CC=85	CT=25	TT=4	CC=88	CT=25	TT=2	.7	.572
		Male	CC=178	CT=70	TT=3	CC=187	CT=54	TT=2	.308	.127
PDYN	**rs2235751**	**All**	AA=180	AG=150	GG=21	AA=214	AG=121	GG=17	**.04**	**.018**
		Female	AA=61	AG=42	GG=7	AA=67	AG=40	GG=4	.564	.335
		Male	AA=119	AG=108	GG=14	AA=144	AG=81	GG=13	**.044**	**.037**
PDYN	rs3830064	All	AA=244	AC=106	CC=9	AA=267	AC=85	CC=10	.184	.152
		Female	AA=79	AC=29	CC=5	AA=83	AC=26	CC=6	.845	.845
		Male	AA=165	AC=77	CC=4	AA=181	AC=59	CC=4	.211	.114
PDYN	rs7272891	All	CC=303	CG=58	GG=5	CC=309	CG=53	GG=1	.23	.243
		Female	CC=95	CG=20	GG=1	CC=104	CG=10	GG=1	.154	.086
		Male	CC=208	CG=38	GG=4	CC=203	CG=42	GG=0	.122	.729
PDYN	**rs10854244**	**All**	AA=176	AT=153	TT=25	AA=211	AT=129	TT=23	.075	**.048**
		Female	AA=59	AT=46	TT=8	AA=66	AT=39	TT=10	.556	.673
		Male	AA=117	AT=107	TT=17	AA=143	AT=89	TT=13	.093	**.037**

[a]Uncorrected p-value of FAMHAP statistic for association between SNPs and alcohol dependence.
[b]p-value of the Armitage's trend test.
Significance is indicated in bold.

4.2.3 Haplotype analysis

Haplotype analysis provides a valuable tool to access information about marker combinations. A haplotype also provides information of all not-analyzed SNPs in LD with markers of this haplotype. Combinations of neighboring markers were successively analyzed. No *PENK* or *PDYN* haplotypes with significant frequency differences between cases and controls in both cohorts were found. Interestingly, for *POMC* an association with the T-A haplotype of markers rs934778 and rs3769671 in the group of alcohol-dependent women in the German and the Swedish sample was detected. To further substantiate these results the number of German females was increased by genotyping additional cases and controls for the two markers. Including them into haplotype analysis additionally confirmed the association (German females p = 0.012, Swedish females p = 0.016) (Tab. 4.10).

Tab. 4.10: Haplotype frequencies and association of *POMC* with alcohol dependence in the German and Swedish sample.

Sample	Marker		German			Swedish		
	rs934778	rs3769671	Control Subjects	Cases	p	Control Subjects	Cases	p
All	C	A	.28	.25	.27	.26	.21	.077
	T	A	.69	.73		.70	.74	
	C	C	.03	.02		.04	.04	
Females	C	A	.28	.19	**.012**	.27	.18	**.016**
	T	A	.69	.78		.69	.75	
	C	C	.02	.02		.03	.07	
Male	C	A	.27	.29	.26	.27	.24	.12
	T	A	.69	.68		.69	.74	
	C	C	.04	.02		.04	.02	

Significance is indicated in bold.

Chapter 5

Discussion

Within the scope of this thesis the generation of a new mouse model was reported. In this mouse strain, *Penk1*$^{loxe/loxe}$, the coding region of the murine preproenkephalin gene is flanked by two *loxP* sites and *lacZ* is introduced as a reporter gene. The introduction of *loxP* sites allows the region- or cell-specific knockout of enkephalin and the *lacZ* gene is expressed after cre recombinase-mediated deletion of the enkephalin coding exons. In this context, a second constitutive enkephalin knockout strain denoted as *Penk1*$^{loxd/loxd}$ expressing *lacZ* under the control of the endogenous enkephalin promoter was established. Additionally, the virus-mediated knockout in *Penk1*$^{loxe/loxe}$ mice was analyzed and initial experiments were conducted. These data will be discussed in the first two sections of this chapter, whereas the results obtained for *PENK*, *POMC* and *PDYN* in the human case-control samples will be discussed in the last section.

5.1 Generation and validation of the new mouse model

One aim of this thesis was the generation of a mouse line with a floxed preproenkephalin locus. The cre/lox technology enables the analysis of gene functions in specific tissues or cell types, or during specific stages of a disease process.

A targeting construct was electroporated into ES cells to mutate the murine *Penk1* locus. Homologous recombination of the target construct was observed in the expected frequencies in relation to the generation of constitutive Penk$^{-/-}$ mice (Prof. A. Zimmer, personal communication). The frequency was also in line with commonly reported homologous recombination frequencies of 1–3% (CAPECCHI, 1989). An exponential relationship between the length of the homologous region and the targeting frequency was described, with an increase in homology leading to higher recombination frequencies (CAPECCHI, 1989). Since the 5' *loxP* site introduced in the *Penk1* gene locus was located at the margin of the homology region, its integration into the mutant gene locus was uncertain. This *loxP* site was located around 5 kb

upstream of the *neo* gene that was introduced as selectable marker. The frequency of coconversion decreases with the distance of two genetic regions (AHN AND LIVINGSTON, 1986). A study on mitotic gene conversion length in yeast indicated coconversion rates of less than 25% between two markers more than 1 kb apart (AHN AND LIVINGSTON, 1986). Nevertheless, one clone showed full length recombination. This ES cell clone containig the mutated *Penk1* gene was successfully injected in blastocysts, leading to chimeric animals that transmitted the mutation to progeny.

The mutated gene locus consisted of a *neo* gene, a *lacZ* gene as reporter and three *loxP* sites flanking the *Penk1* coding exons and the *neo* gene. In these mice multiple recombination events can take place. To remove one 3' *loxP* site and to excise the *neo* gene, these mice were mated to mouse strains expressing cre recombinase during embryonic development. The frequency and extent of the excision depends on the length of the floxed sequence. Shorter sequences led to higher excision frequencies (LAKSO ET AL., 1996). Because of their proximity, recombination between the *loxP* sites flanking the *neo* gene should be favored. Mating of mice, expressing cre recombinase under the Pgk-1 promoter, to the floxed *Penk1* mice always led to deletion of the entire floxed region. The successful deletion of the coding exons and the *neo* gene proved the functionality of the *loxP* sites. Recombination either occurred directly between the 1st and the 3rd *loxP* site or sequential recombination events took place due to ongoing or strong cre recombinase activity. It is likely that sequential recombination events took place because recombination between adjacent *loxP* sites is favored (LAKSO ET AL., 1996). In the *Pgk-cre* strain, cre recombinase expression is under dominant maternal control, as cre recombinase was already active in the early diploid phase of oogenesis (LALLEMAND ET AL., 1998). Therefore, recombination can take place as early as the pronuclear fusion. This possibly explains the high efficiency of excision observed in matings with floxed *Penk1* mice.

In *EIIa-cre* animals, cre expression is under the control of the adenoviral EIIa promoter. This promoter confines the expression of cre recombinase to a very early state of embryogenesis, most likely to the one-cell embryo (DOOLEY ET AL., 1989). However, it was reported that in these mice cre recombinase expression could occur past the zygote stage, resulting in mosaicism due to incomplete recombination (LAKSO ET AL., 1996). Indeed, in contrast to matings of *Penk1*$^{lox/wt}$ x *Pgk-cre* animals, offspring of *Penk1*$^{lox/wt}$ mice and *EIIa-cre* mice showed to some extend a mosaic pattern of different alleles. Hence the loxe allele was present as well, demonstrating successful excision of the *neo* gene. Further mating of these offspring fixed the genetic alterations and enabled the generation of the two new mouse lines, *Penk1*$^{loxe/loxe}$ and *Penk1*$^{loxd/loxd}$.

Mutation of the *Penk1* gene locus led to decreased preproenkephalin expression. In *Penk1*$^{lox/lox}$ mice mRNA levels were reduced compared to wild type littermates. It was reported that genetic modification, for example introduction of a *neo* gene, can influence gene expression of nearby

genes (VALERA ET AL., 1994; HLAVATY ET AL., 2004). *In vitro*, introduction of a *neo* gene altered expression of endogenous genes (VALERA ET AL., 1994) and led to decreased expression of a eGFP transgene (HLAVATY ET AL., 2004). However, reduced mRNA levels were also observed in mice homozygous for the loxe allele in which the *neo* gene is not present. As this reduction had the same magnitude in *Penk1*$^{lox/lox}$ and *Penk1*$^{loxe/loxe}$ a specific influence of the *neo* gene on preproenkephalin expression could be excluded.

Another possible reason for reduced mRNA levels might be the location of the first *loxP* site. In transcript ENSMUST00000070375, this *loxP* site is located in the 5'UTR. It is known that the UTRs can influence mRNA stability or expression (WANG ET AL., 2006; KEYSER ET AL., 2009). The analysis of transcript-specific expression levels revealed that both transcripts had lower expression levels in *Penk1*$^{loxe/loxe}$ mice and the magnitude of reduction was similar for ENSMUST00000070375 and ENSMUST00000108381. This indicates that the location of the first *loxP* site in the 5' UTR of ENSMUST00000070375 did not lead to a selective down-regulation of this transcript. The detected difference in expression levels was more pronounced using TaqMan® assays. That was probably due to their higher specificity compared to SYBR green detection (TIAN ET AL., 2004; HEIN ET AL., 2001). The analysis of *Penk1*$^{loxd/loxd}$ mice proved the absence of both *Penk1* transcripts after cre recombinase-mediated knockout.

A further reason for the reduced mRNA level could be an influence of the *lacZ* reporter gene on mRNA expression. In the targeted gene locus, the splice acceptor site and the ATG of the *Penk1* gene were fused in frame to the *lacZ* coding sequence. This enables *lacZ* expression under the control of the enkephalin promoter after excision of the *Penk1* coding exons 2 and 3. It is very unlikely that the *lacZ* sequence was already transcribed in homozygous lox or loxe mice as the stop codon of *Penk1* should terminate transcription before. Indeed, ß-galactosidase activity was only detected in *Penk1*$^{loxd/loxd}$ mice, suggesting that the *lacZ* gene is only transcribed subsequent to the knockout of enkephalin. Therefore, the presence of the *lacZ* transcript and alternative splicing of *Penk1* exon 2 and 3 did not account for the reduced mRNA level in *Penk1*$^{lox/lox}$ and *Penk1*$^{loxe/loxe}$ mice.

However the introduction of the *lacZ* and the *neo* gene modified the 3' flanking of the *Penk1* gene locus. Different studies reported that 3' flanking regions of genes are involved in transcriptional regulation (ZARUDNAYA ET AL., 2003; CHEN ET AL., 2006). Modification of the 3' region can for example influence the binding of *cis*-acting transcriptional enhancers or repressors (CHEN ET AL., 2006). Therefore the most probable explanation for the reduced preproenkephalin mRNA level is that the 3' introduction of the *lacZ* and the *neo* sequence impair the binding of a transcriptional enhancer.

It was proposed that Leu-enkephalin can be generated from preprodynorphin via a different cleavage pathway (DAY ET AL., 1998; ZAMIR ET AL., 1984). The results reported herein revealed that reduction or absence of preproenkephalin mRNA did not lead to a compensatory up-regulation of preprodynorphin expression, though the presence of Leu-enkephalin

in *Penk1*$^{loxd/loxd}$ mice supports the suggestion made by Day and co-workers. However, Leu-enkephalin anti-sera often show a cross-reactivity to Dynorphin A (GARZÓN AND PICKEL, 2004; MILNER ET AL., 1989). The anti-serum used in this work exhibits a cross-reactivity of 29 % to dynorphin A (according to specification provided by the supplier). Therefore, arround one third of the detected peptide might be dynorphin A and not Leu-enkephalin. Since the peptide level detected in *Penk1*$^{loxd/loxd}$ mice represent 37 % of the amount observed in *Penk1*$^{wt/wt}$ mice, one could speculate that most of it was dynorphin A.

Altogether the peptide levels are in line with the expression data. A reduction of Met- and Leu-enkephalin was observed in mice homozygous for the loxe allele, Met-enkephalin was absent in null allele mice, whereas Leu-enkephalin was further reduced in these knockout mice. The higher amount of Met-enkephalin compared to Leu-enkephalin is attributed to the fact that preproenkephalin can give rise to four copies of Met- and only one copy of Leu-enkephalin (WEISINGER, 1995; ZAMIR ET AL., 1984).

Penk1$^{-/-}$ mice are commonly used as a genetic model for enhanced anxiety responses. In this mouse strain, an anxiogenic phenotype was observed in the zero maze test, independent of genetic background and animal facility (BILKEI-GORZÓ ET AL., 2008; BILKEI-GORZO ET AL., 2004; KÖNIG ET AL., 1996). The zero maze paradigm was therefore chosen to evaluate whether the reduced enkephalin level in *Penk1*$^{loxe/loxe}$ mice resulted in phenotypic differences. For two of the parameters measured, namely distance traveled and time spent in the open area, no significant genotype effect was observed. Reduced values for these two parameters are the most obvious signs of an anxiogenic phenotype. Additionally, a significant higher latency to enter the closed area of the maze was connected to an increased anxiety (BILKEI-GORZÓ ET AL., 2008; BILKEI-GORZO ET AL., 2004). Hence, the reduced expression levels of *Penk1*$^{loxe/loxe}$ compared to *Penk1*$^{wt/wt}$ mice resulted in slightly higher anxiety levels in *Penk1*$^{loxe/loxe}$ mice, indicated by the higher latency to enter the closed area of the maze. However, compared to constitutive *Penk1* knockout mice (BILKEI-GORZÓ ET AL., 2008; BILKEI-GORZO ET AL., 2004; KÖNIG ET AL., 1996), the anxiety phenotype observed in *Penk1*$^{loxe/loxe}$ is very weak.

The *Penk1*$^{loxe/loxe}$ mice provide a valuable tool to further clarify the role of enkephalin in addiction related-behaviors. Enkephalins are widely expressed in brain structures involved in reward and reinforcement. Additionally, constitutive *Penk1* knockout mice were shown to influence drug-related behaviors for many drugs of abuse (VALVERDE ET AL., 2000; BERRENDERO ET AL., 2005; RACZ ET AL., 2008). For example nicotine was not rewarding in *Penk1*$^{-/-}$ mice (BERRENDERO ET AL., 2005). Another example is the reduction of Δ9-tetrahydrocannabinol (THC) withdrawal symptoms in enkephalin-deficient mice (VALVERDE ET AL., 2000). The subpopulations of enkephalinergic neurons modulating these processes are so far unclear (BERRENDERO ET AL., 2005).

The analysis of conditional knockout mice can provide new insights. One example is the CB1 receptor that mediates most of the cannabinoid effects in the CNS (VALVERDE ET AL.,

2000; MONORY ET AL., 2007). These receptors are highly expressed on GABAergic neurons and this CB1 subpopulation was believed to mediate most of the effects of exogenously administered and endogenously released cannabinoids (FREUND ET AL., 2003). Instead, the analysis of conditional CB1 knockout mice revealed that CB1 signaling on glutamatergic, but not on GABAergic neurons, accounts primarily for the behavioral effects induced by high doses of THC (MONORY ET AL., 2007). Analysis of the newly generated *Penk1*$^{loxe/loxe}$ mice will help to elucidate the role of enkephalin in processes relevant for drug addiction.

5.2 Region-specific knockout of enkephalin

The newly generated *Penk1*$^{loxe/loxe}$ mouse line provides the possibility to study the effect of region- or cell-specific knockout of preproenkephalin. Thus, it was crucial to establish techniques that allow the detection of such knockouts. The tissue distribution of enkephalin was assessed by *in situ* hybridization. The pronounced expression of preproenkephalin in the striatum was completely in line with expression data published before (YOSHIKAWA ET AL., 1984; WEISINGER ET AL., 1992; BRANCH ET AL., 1992; WEISINGER, 1995). After knockout of the *Penk1* coding exons the signal was absent. This results confirmed that *in situ* hybridization can be used for the analysis of virus-mediated knockout.

Alternatively, the *lacZ* gene was introduced into the *Penk1* gene locus to function as a reporter gene. By this, X-gal staining on brain sections demonstrating ß-galactosidase activity provides another tool to prove the absence of enkephalin. In *Penk1*$^{loxd/loxd}$ mice, ß-galactosidase activity was detected in regions of former enkephalin expression (ZAMIR ET AL., 1984; GARZÓN AND PICKEL, 2004), demonstrating the functionality of the *lacZ* reporter gene. One limitation of this method was the lower sensitivity compared to *in situ* hybridization, as it is based on protein activity instead of directly assessing gene expression.

Since the applied viruses express either eGFP or cre recombinase tagged with eGFP, another analysis method was the detection of GFP by immunohistochemistry. In the present study, this technique was used to detect the tissue distribution of the virus.

Injection of cre recombinase expressing viruses resulted in a knockout of enkephalin at the injection site, whereas injection of the eGFP expressing virus did not effect preproenkephalin mRNA expression. This demonstrated the efficacy of the virus-mediated knockout in floxed *Penk1* animals. Targeting of the NAc led to GFP expression in the accumbens core and shell region. However, the viral expression was not detected in all parts of the NAc. Instead, the observed transduction for AAV2 was always essentially restricted to the injection site. The distribution of AAV2 observed in these experiments was in line with previous studies, also showing restricted transduction of AAV2 viruses (BURGER ET AL., 2005; TAYMANS ET AL., 2007; HOWARD ET AL., 2008). If AAV2-Cre viruses do not transduce all parts of the NAc, preproenkephalin expression is not completely abolished in the NAc. One option to reach virus

transduction in the whole NAc would be the use of other AAV serotypes. It is known that other serotype capsids could increase the virus distribution. For two other human derived AAV serotype capsids (1 and 5) and for the non-human primate derived AAV7 capsid efficient transduction in the adult mouse brain was described (TAYMANS ET AL., 2007). On the other hand, they often exhibited retrograde transduction in untargeted areas of the CNS and showed a different tropism including increased transduction of glia cells and oligodendrocytes (TAYMANS ET AL., 2007; BURGER ET AL., 2005). The advantage of AAV2 viruses is that retrograde transduction was not observed and its expression was primarily neuronal (TAYMANS ET AL., 2007; BURGER ET AL., 2005; HOWARD ET AL., 2008). This has to be considered when using alternative serotypes. Another study using higher concentrated AAV2 solutions achieved virus-mediated knockout in the hippocampus, a much larger brain region (MONORY ET AL., 2006). Therefore the best option would be to use higher concentrated AAV2s to increase the virus distribution.

Dopamine release in the NAc is essential for the rewarding properties of most addictive drugs (FELTENSTEIN AND SEE, 2008; NESTLER, 2004A; SPANAGEL ET AL., 1992). To evaluate the influence of enkephalin on nicotine-induced dopamine release, nicotine-related behaviors were studied in the newly generated floxed preproenkephalin mice. In a preliminary experiment, injections of either cre recombinase (CRE) or GFP (GFP) expressing viruses into the NAc were performed. Subsequently, these mice underwent the nicotine self-administration paradigm. This experiment revealed no difference between either CRE- or GFP-injected animals. Both experimental groups learned to self-administer nicotine solution indicating that nicotine was rewarding for both groups. In contrast, it was shown before that *Penk1*$^{-/-}$ mice did not exhibit nicotine-induced place preference, and the nicotine-dependent increase in extracellular dopamine levels in the NAc was attenuated (BERRENDERO ET AL., 2005). These results in *Penk1*$^{-/-}$ demonstrated that enkephalin contributes to the rewarding properties of nicotine. It is unclear, which neurons meditate the enkephalinergic modulation of dopamine release. Enkephalinergic interneurons as well as enkephalinergic inputs from the NAc are present in the VTA (GARZÓN AND PICKEL, 2004). In a different study, nicotine administration induced the release of enkephalin in the NAc (HOUDI ET AL., 1991). Thus, the role of enkephalin in the NAc and the VTA needed further investigation. The results observed in the *Penk1*$^{loxe/loxe}$ animals do not suggest a role for preproenkephalin expressed in NAc cell bodies in mediating nicotine reinforcement. In this experiment only 15 of 37 virus-injected mice underwent the self-administration procedure. Interpretation of these results remains speculative, as the brains of these mice need to be checked for the virus transduction. In addition, the final number of animals was small for such behavioral experiments. The small animal number is due to a high mortality of the mice during catheter implantation for the self-administration experiments. However, the moderate variance observed during the experiments indicate that results would not substantially change with increased animal numbers. Still, a role of enkephalin from NAc

afferents in dopamine release cannot be excluded. Furthermore, as discussed above the virus probably did not spread in the NAc, indicating that to some extent enkephalin was still present in some NAc neurons. Further experiments, including viral-mediated knockout of enkephalin in the VTA, are needed to clarify the role of enkephalinergic neurons on dopamine release in the NAc.

5.3 Association of opioid peptide genes with alcoholism

The contribution of variants in genes coding for the three endogenous opioid precursor proteins to alcohol addiction in humans was systematically evaluated in a case-control study. Data obtained in an exploratory genotyping did not point towards an association of these genes with alcoholism. This is probably due to the small number of cases in this initial group, as the power to detect genetic association for complex phenotypes largely depends on sample size (COLHOUN ET AL., 2003; HOEFGEN ET AL., 2005; MAXWELL ET AL., 2008). The integration of linkage data into marker selection and data analysis augments the coverage of genetic variation for a gene locus of interest (SCHAID, 2004; MENG ET AL., 2003). The extended sample size and the marker selection based on LD data for the detailed genotyping therefore increased the power to detect genetic association.

5.3.1 Contribution of *PENK* variants

The LD observed for the German control group and the predicted haplotype structure for the *PENK* SNPs indicated high linkage and the presence of one haplotype block. This is in line with the data provided by the HapMap project for a Caucasian reference population, as high LD and location in one Haplotype block was predicted for SNPs in the *PENK* gene locus. The allele frequencies detected in the present study were similar in the German and the Swedish sample and in line with the minor allele frequencies (MAFs) indicated in the HapMap database.

The analysis of *PENK* SNPs did not provide evidence for an association with alcoholism. For rs12545109, the elevated MAF in German cases was not significant after correction for multiple testing and was also not replicated in the Swedish sample. The significant frequency difference detected for rs2576581 in females was due to a reduced allele frequency in the female control group. Thus, this seemed to be a false positive signal, since for the general population no frequency differences between males and females were reported for this marker and these findings were not replicated in Swedish females. In line with the results presented here, an analysis of a different set of *PENK* variants in the COGA cohort did not indicate an association with alcohol dependence. Another study analyzed a CA repeat in the *PENK* promoter region and reported negative findings (CHAN ET AL., 1994). Different studies on *Penk1* knockout animals also support the results, as no significant differences in ethanol consumption or ethanol induced place-preference was observed (RACZ ET AL., 2008; KOENIG AND OLIVE, 2002).

Together with the observed increase in ethanol consumption in *Penk1*$^{-/-}$ mice after stress exposure (RACZ ET AL., 2008), these data and the results reported herein implicate a role of enkephalin in mediating stress relief rather than a general contribution to alcohol consumption.

5.3.2 Contribution of *PDYN* variants

LD and the haplotype block structure were calculated on the basis of the German control population. Both, LD and haplotype structure, resembled those predicted for the *PDYN* locus according to HapMap project data for a Caucasian reference population. However, the contribution of variants in the *PDYN* locus remained somewhat unclear.

In the German subgroup, allele frequencies differed significantly among female cases and controls for several SNPs (rs910080, rs6045819, rs6045824, rs6035235). These markers also showed significant differences in genotype distributions. Another three markers (rs6045733, rs2235749, rs6035222) differed significantly in their genotype distributions between female cases and controls. For these SNPs the significant p-values resulted from an increase in the number of heterozygous cases. This is reflected by a non significant *Armitage* p-value for rs6045733 and rs6035222, indicating the absence of a linear trend among the three genotypes. Therefore these results seemed to be false positive. Additionally, none of the p-values remained significant after correction for multiple testing.

The association results observed in the Swedish sample did not overlap with results observed in the German sample. In the Swedish sample four markers differed significantly between cases and controls (rs6112177, rs2235749, rs2235751, rs10854244). Only for rs2235749 significant differences for allele and genotype distribution were also observed in the German cohort. In contrast to the results of the German sample, the G allele instead of the A allele was more frequent in Swedish cases, and the effect was seen in the males subgroup. In general the association results in the Swedish sample were observed in the whole sample as well as in the male subgroup, which also contradicts the effects mostly seen in the German female group. Thus, the absence of overlapping results in these two cohorts indicates that the *PDYN* variants analyzed did not contribute to an increased risk to become alcohol-dependent.

In contrast, Xuei and co-workers reported association of *PDYN* variants with alcoholism (XUEI ET AL., 2006). In this study nine markers showed significant association with alcoholism. The SNPs analyzed overlap with the markers studied in the scope of this thesis (rs6045784, rs2235749, rs910080, rs10485703, rs6045819, rs6035222, rs2235751, rs3830064). Comparing the association results with the findings of Xuei et al., the role of PDYN variants remained ambiguous. They found significant p-values for marker rs2235749 that differed significantly in Swedish males, the whole Swedish sample and in German females. For another three of their associated SNPs significant p-values were observed in the German cohort (rs910080, rs6045819, rs6035222). In contrast, rs2235751 associated in the Swedish cohort were not associated in the Xuei cohort. However, the family-sample used by Xuei et al. showed high

comorbidity with other drug dependencies and gender influences were not considered (XUEI ET AL., 2006; REICH ET AL., 1998). Therefore, the studies cannot be compared one-to-one, which might be the reason for the inconsistent results. A recent study reports association of one *PDYN* 3' UTR haplotype comprised of three SNPs (rs910080, rs910079, rs2235749) in a Caucasian sample for cocaine dependence and a combined sample for cocaine and alcohol dependence. The risk haplotype was also associated with lower *PDYN* mRNA levels in post-mortem brains in the same study (YUFEROV ET AL., 2009). This indicates that variants in the 3' UTR of the *PDYN* locus could influence preprodynorphin expression. As the association results of Yuferov *et al.* and Xuei *et al.* were obtained in samples showing high comorbidity with other substance dependencies, *PDYN* seems to contribute to addiction in general rather than to alcohol dependence alone. The results of a quantitative trait loci (QTL) analysis using recombinant QTL ingression strains indicated linkage close to the gene coding for the κ-opioid receptor, and different studies showed evidence for association of the OPRK gene in humans (VADASZ ET AL., 2000; XUEI ET AL., 2006; ZHANG ET AL., 2008), indicating a κ-opioid receptor mediated contribution to an increased risk to develop alcohol dependence. The role of the gene coding for its endogenous ligand dynorphin remains to be clarified in further studies.

5.3.3 Contribution of *POMC* variants

For the analyzed genomic region located around the *POMC* locus the overall LD in the German control group was weak, resembling the data provided by the HapMap Project for a Caucasian reference population. The allele frequencies detected in the present study were similar in the German and the Swedish sample and were also in line with the MAFs indicated in the HapMap database.

Allele frequencies and genotype distributions of *POMC* variants differed significantly between German alcoholics and controls in the female subgroup for rs2164808 and rs934778. The latter SNP remained significant after Bonferroni correction for multiple testing and significant association was also observed in the Swedish sample. In the Swedish group the higher frequency of the T allele in cases was not gender-specific. Marker rs874401 significantly associated in Swedish females did not show association in the German cohort. Generally, throughout both samples, significant p-values were mostly observed in the female subgroup, indicating a gender-specific contribution of this gene locus. Subsequent haplotype analysis then revealed that the T-A haplotype of rs934778 and rs3769671 was significantly more frequent among female cases in both cohorts, verifying the association of the *POMC* gene locus with alcoholism in females.

Two other groups studied the contribution of *POMC* variants to alcoholism, although the SNPs partly differed between the studies and also from SNPs reported herein (XUEI ET AL., 2007; ZHANG ET AL., 2009). Only rs6713532 was analyzed throughout all studies and constantly showed no association. Xuei and co-workers also analyzed rs934778, but did not find

a significant association with alcohol dependence. Nevertheless, this SNP showed a significant association with a more severe subtype of alcoholism, which was characterized by comorbidity with opioid dependence (XUEI ET AL., 2007), supporting the association observed in the German females and the Swedish sample. The five polymorphisms studied in four different samples by Zhang and co-workers overlap with SNPs analyzed in this work (rs1866146, rs6713532, rs3769671, rs6545976, rs6719226), however, rs934778 was not included. In their samples, alcoholics also showed comorbidity with either cocaine or opioid dependence. For none of the markers, association with alcohol dependence in an African-American family sample, in an European-American family sample, or in an African-American case-control sample was found. However, in an European-American case-control sample significant association of rs6713532 and rs1866146 with alcohol dependence was observed. Unfortunately none of the other studies performed a gender specific analysis. In the latter study age and gender of cases and controls were not matched. To correct their results for potential gender and age effects, they performed a logistic regression analysis. This revealed one haplotype (C-G-A-T-T) significantly contributing to the risk to develop alcoholism (ZHANG ET AL., 2009). The haplotype consisted of the rs3769671 A allele that was also part of the haplotype detected more frequently in cases in the present work.

Additionally, data from animal experiments underline the implication of the *POMC* gene in alcohol dependence. Studies using ß-endorphin or μ-receptor knockout mice revealed differences in ethanol consumption and preference, whereas knockout mice either did not self-administer ethanol or the consumption was decreased compared to wild type mice. These effects were more pronounced in female animals (RACZ ET AL., 2008; HALL ET AL., 2001; GHOZLAND ET AL., 2005), pointing towards a gender-specific contribution. In addition, the stress-induced increase in plasma adrenal-corticothrophin (ACTH), another product of *POMC*, was prevented by a single consumption of alcohol (DAI ET AL., 2007).

The consistent results for the two marker haplotype reported here were in line with findings from two other groups reporting association of overlapping markers or haplotypes. In addition, different mouse studies reported alterations in ethanol consumption in ß-endorphin knockout mice and the ACTH level was influenced by alcohol consumption. Altogether these data substantiate a role for *POMC* in alcoholism. As *POMC*-derived peptides (especially ß-endorphin and ACTH) modulate the stress response (BILKEI-GORZO ET AL., 2008; ZHOU ET AL., 2009), variations in these gene locus might account for individual differences in stress-reactivity, which might in turn influence ethanol consumption and the vulnerability for relapse.

Summary and conclusion

One outcome was the successful generation of conditional *Penk1* knockout mice which enable the cell- or tissue-specific deletion of enkephalin. The mutated Penk allele contains two *loxP* sites allowing cre recombinase-mediated deletion of the coding exons. A *lacZ* reporter gene resulting in ß-galactosidase expression under the enkephalin promoter after cre-mediated recombination was also inserted. In these mice, knockout of preproenkephalin results in ß-galactosidase activity in brain regions where enkephalin is known to be expressed. Thus, the functionality of both genetic modifications was demonstrated.

Molecular analysis of the floxed *Penk1* mice revealed a reduction in mRNA and peptide levels. As a consequence, these mice have a slightly anxiogenic phenotype. Although this weak phenotype has to be considered it does not impair the applicability of these mice. Because the floxed *Penk1* allele always represent the control group, phenotypes measured after tissue- or cell-specific deletion can be attributed to the absence of enkephalin.

Successful deletion of enkephalin in the targeted brain region was demonstrated by stereotactic injection of cre recombinase expressing viruses. Ongoing experiments targeting the nucleus accumbens and the VTA will provide new insights into the role of enkephalinergic neurons in mediating nicotine reward and reinforcement. Using this new mouse model, the contribution of enkephalin to a wide range of opioid-mediated phenotypes can be studied. Now brain regions involved in addiction-related behaviors or neuronal structures involved in pain relief can be assessed in detail.

The second outcome was the identification of *POMC* variants contributing to human alcohol addiction. A systematic analysis of the genomic regions coding for all three endogenous opioid peptides was performed in two independent case-control samples. Association analysis illustrated the importance to replicate findings in an independent cohort to exclude potential false positive signals. The assumed role for *POMC*-encoded peptides in alcohol dependence was further substantiated by this work. Interestingly, this contribution was gender-specific. A POMC two-marker haplotype was significantly more frequent in female cases across both samples studied. Subsequently, the role of *POMC* was confirmed by independent studies. The correlation of distinct alcohol-related behaviors with proopiomelanocortion and ß-endorphin particularly demands further studies in humans.

Appendix

Supplier information

Applichem	http://www.applichem.de/
Applied Biosystems	http://www.appliedbiosystems.com/
Bachem	http://www.bachem.com/
Invitrogen	http://www.invitrogen.com/
Metabion	http://www.metabion.com/
Merck	http://www.merck.de/
New England Biolabs	http://www.neb.com/
Peqlab	http://www.peqlab.de/
Qiagen	http://www.qiagen.com/
Roche	http://www.roche.de/
Roth	http://www.carl-roth.de/
Sequenom	http://www.sequenom.com/
Sigma	http://www.sigmaaldrich.com/

Primer

Tab. A.1: Sequence information about the primers used in the scope of thesis.

Method	Primer	Sequence 5'-3'
realtime PCR	beta-actin for	CAC ACT GTG CCC ATC TAC GA
	beta-actin rev	AGT GGC CAT CTC CTG CTC GA
	Penk1 for	GAG AGC ACC AAC AAT GAC GAA
	Penk1 rev	TCT TCT GGT AGT CCA TCC ACC
Southern probe	enk2-E46h	GTC CGT TTG AGG GTG TTG TT
	enk2-5'S-1r	TGA CGC AGG CCC TAC GCC AA

Databases

Ensembl genome browser	http://www.ensembl.org/index.html
HapMap project	http://hapmap.ncbi.nlm.nih.gov/
PubMed	http://www.ncbi.nlm.nih.gov/sites/entrez
UCSC genome browser	http://genome.ucsc.edu/

Hardy-Weinberg equilibrium

Tab. A.2: P-values for Hardy-Weinberg equilibrium in the German Sample.

Gene	Marker	P HWE (controls)	P HWE (cases)
PENK	rs12545109	0.387	0.593
PENK	rs2576581	0.938	0.062
PENK	rs12548084	0.1	0.361
PENK	rs2670029	0.663	0.423
POMC	rs2164808	0.691	0.225
POMC	rs7589318	0.32	0.083
POMC	rs1866146	0.079	0.353
POMC	rs6713532	0.142	0.428
POMC	rs934778	0.703	0.46
POMC	rs3769671	0.285	0.769
POMC	rs6545976	0.597	0.503
POMC	rs6719226	0.176	0.502
POMC	rs874401	0.35	0.492
PDYN	rs6112177	0.811	0.692
PDYN	rs6045761	0.235	0.083
PDYN	rs6045824	0.111	0.365
PDYN	rs6035235	0.091	0.289
PDYN	rs6045733	0.087	0.439
PDYN	rs6045784	0.075	0.643
PDYN	rs2235749	0.111	0.296
PDYN	rs910080	0.286	0.22
PDYN	rs10485703	0.075	0.243
PDYN	rs6045819	0.057	0.085
PDYN	rs6035222	0.213	0.243
PDYN	rs12480838	1.000	0.496
PDYN	rs2235751	0.779	0.234
PDYN	rs3830064	0.629	0.95
PDYN	rs7272891	0.86	0.093
PDYN	rs10854244	0.453	0.296

Tab. A.3: P-values for Hardy-Weinberg equilibrium in the Swedish Sample.

Gene	marker	P HWE (controls)	P HWE (cases)
PENK	rs12545109	0.665	0.233
PENK	rs2576581	0.869	0.157
PENK	rs12548084	0.845	0.867
PENK	rs2670029	0.779	0.081
POMC	rs2164808	0.044	0.494
POMC	rs7589318	0.316	0.347
POMC	rs1866146	0.191	0.448
POMC	rs6713532	0.821	0.683
POMC	rs934778	0.068	0.102
POMC	rs3769671	0.465	0.469
POMC	rs6545976	0.267	0.482
POMC	rs6719226	0.265	0.482
POMC	rs874401	0.492	0.529
PDYN	rs6112177	0.573	0.8
PDYN	rs6045761	0.759	0.92
PDYN	rs6045824	0.95	1
PDYN	rs6035235	0.775	0.265
PDYN	rs6045733	0.013	0.703
PDYN	rs6045784	0.877	0.956
PDYN	rs2235749	0.24	0.45
PDYN	rs910080	0.38	0.546
PDYN	rs10485703	0.877	0.956
PDYN	rs6045819	0.699	0.818
PDYN	rs6035222	0.137	0.541
PDYN	rs12480838	0.639	0.538
PDYN	rs2235751	0.158	1
PDYN	rs3830064	0.528	0.312
PDYN	rs7272891	0.253	0.417
PDYN	rs10854244	0.286	0.584

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Statutory declaration

I hereby affirm that I prepared this thesis entitled: "Endogenous opiod peptides in drug addiction" entirely by myself except where otherwise stated. All text passages that are literally or correspondingly taken from published or unpublished papers/writings are indicated as such. All materials or services provided by other persons are equally indicated. Parts of this thesis are published as listed:

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* **equal contribution**

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