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The endogenous opioid system in alcoholism: Translational studies in humans and rodents

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Für meine Familie

ABSTRACT

In most parts of the world, alcohol is consumed for social and recreational reasons. However, the initially controllable use can become compulsive and alcohol dependence develops. Late dependence is characterized by persistent neuroadaptations in various brain neurotransmitter systems, including the endogenous opioid and dopamine system, which are thought to underlie relapse. Many hypotheses on the state of brain neurotransmitter systems are based on positron emission tomography (PET) studies. However, the interpretation of those data is challenging as PET signals are sensitive not only to receptor but also ligand levels. For instance, increased μ-opioid receptor (MOR) PET binding potentials are interpreted as elevated receptor levels. Those are thought to be the target of the anti-relapse medication naltrexone, an opioid antagonist. However, naltrexone's effect size is relatively small and only a subset of alcohol-dependent patients appears to benefit. Furthermore, only few studies on the opioid and dopamine system during protracted abstinence are available. This is surprising as this phase is characterized by high relapse propensity and, thus, is clinically highly relevant.
Therefore, this thesis aims to demonstrate the state of the opioid and dopamine system during alcohol abstinence. A translational approach was applied by analyzing these systems in four separate studies (Study I-IV) in post-mortem brain tissue of human alcoholics and an animal model of alcohol dependence.

In Study I, transcriptional and protein levels (receptor binding sites) of the MOR are found to be strongly reduced in the striatum of alcoholics. Additionally, a PET study associates decreased striatal MOR binding potential with higher relapse risk. Decreased MOR expression is mirrored by data from alcohol-dependent rats in Study II. Furthermore, the precursor of the MOR-ligand β-endorphin *Pomc* is significantly reduced. Signaling at the δ-opioid receptor (DOR) appears to be decreased in alcohol dependence while the κ-opioid receptor (KOR) system is upregulated. In Study III, chronic naltrexone treatment counteracts the changes in MOR/*Pomc* levels by significantly increasing expression and further enhanced KOR density. The DOR, in contrast, seems not to be a target of naltrexone under the applied experimental conditions. Because the endogenous opioid system is known to modulate dopamine release, the dopamine system was investigated in Study IV. Here, human post-mortem tissue reveals strongly decreased dopamine transporter and D1 receptor levels in alcoholics while D2 is unchanged. These findings are further supported by the alcohol-dependent animals where an oscillatory-like regulation of the dopamine system is observed during acute withdrawal and protracted abstinence. While previous studies report on a hypodopaminergic state during acute withdrawal, here, a hyperdopaminergic state is demonstrated during protracted abstinence by measurements of striatal dopamine release and a meta-analysis followed by functional validations. Based on these data, both hypo- and hyperdopaminergic states are suggested as phases with increased vulnerability for alcohol relapse.

In summary, the results presented in this thesis provide consistent evidence for a severe dysregulation of the endogenous opioid and dopamine system during alcohol abstinence that demands reinterpretation of existing PET data. It is proposed for future studies to combine the analysis of human post-mortem tissue and established animal models with PET studies to achieve a more precise picture of the state of brain neurotransmitter systems in alcoholic patients. Moreover, decreased MOR and dopamine receptor/transporter levels may represent molecular markers of the disease course that can be used to develop personalized treatment approaches.

ZUSAMMENFASSUNG

In den meisten Teilen der Welt wird Alkohol zu gesellschaftlichen Anlässen konsumiert. Allerdings kann sich das anfänglich kontrollierbare Alkoholtrinken zum Zwang und zur Alkoholabhängigkeit entwickeln. Diese ist durch lang anhaltende Neuroadaptionen in verschiedenen Neurotransmittersystemen, einschließlich des endogenen Opiat- und Dopaminsystems, im Gehirn geprägt. Zahlreiche Hypothesen zum Status dieser Neurotransmittersysteme basieren auf Positronen-Emissions-Tomographie (PET) Studien. Allerdings ist die Interpretation dieser Daten schwierig, da PET Signale sowohl von Rezeptor- als auch Ligandenkonzentrationen abhängen. Beispielsweise wurden erhöhte μ-Opiatrezeptor (MOR) Bindungspotential in PET Studien als erhöhte Rezeptorendichte interpretiert. Es wird angenommen, dass dies der Angriffspunkt von Naltrexon ist, einem Opiat-Antagonisten zur Rückfallprävention. Die Effektstärke von Naltrexon ist jedoch relativ gering und nur eine Untergruppe von Patienten profitiert von der Behandlung. Außerdem gibt es nur relativ wenige Studien, die sich mit dem Opiat- und Dopaminsystem in der Langzeitabstinenz beschäftigen. Dies ist überraschend, da diese Phase durch eine hohe Rückfallrate gekennzeichnet und dadurch klinisch hoch relevant ist.
Aus diesen Gründen setzt sich diese Dissertation das Ziel, den Status des Opiat- und Dopaminsystems in der Alkoholabhängigkeit zu charakterisieren. In einem translationalen Ansatz werden in vier Studien (Studien I-IV) post-mortem Gehirngewebe von Alkoholikern sowie ein Tiermodell für Alkoholabhängigkeit untersucht.

In Studie I wird eine starke Verminderung von MOR Transkripten und Proteinen im Striatum von Alkoholikern berichtet. Eine PET-Studie assoziiert die reduzierten striatalen MORs mit einem erhöhten Rückfallrisiko. In Studie II spiegeln sich diese Effekte in alkoholabhängigen Ratten wider. Zudem ist hier der Vorläufer des MOR-Liganden β-endorphin *Pomc* stark reduziert. Die Signalweiterleitung am δ-Opiatrezeptor (DOR) ist vermindert während das κ-Opiatrezeptor (KOR) System hochreguliert ist. Chronische Behandlung mit Naltrexon steuert den Veränderungen in der MOR/*Pomc* Expression in Studie III entgegen indem es sowohl MOR als auch *Pomc* signifikant erhöht. Die Dichte von KOR wird ebenfalls verstärkt. Unter den verwendeten experimentellen Bedingungen scheint DOR jedoch kein Angriffspunkt von Naltrexon zu sein. Da bekannt ist, dass das Opiatsystem das Dopaminsystem moduliert, wurde letzteres in Studie IV untersucht. Die Analyse der humanen Gehirnproben zeigt stark verminderte Dopaminrezeptor D1 und –transporter Level in Alkoholikern, während der D2 Rezeptor unverändert ist. Diese Beobachtung wird zudem von Ergebnissen in den alkoholabhängigen Ratten unterstützt, die eine dynamische Regulation des Dopaminsystems im akuten Alkoholentzug und in der fortgeschrittenen Abstinenz aufweisen. Während bisherige Studien von einer hypodopaminergen Phase im akuten Entzug berichten, wird hier eine hyperdopaminerge Phase in der Langzeitabstinenz gezeigt. Beides, also sowohl die hypo- als auch die hyperdopaminerge Phase, werden als Zustände mit erhöhtem Rückfallrisiko interpretiert.

Zusammenfassend liefern die Ergebnisse dieser Dissertation Beweise für die Dysregulation des endogenen Opiat- sowie des Dopaminsystems in der Alkoholabhängigkeit und Abstinenz, die eine Neuinterpretation der vorliegenden PET-Daten fordern. Zukünftige PET-Studien sollten mit der Analyse von humanem post-mortem Material und etablierten Tiermodellen kombiniert werden, um ein präziseres Bild der Neurotransmittersysteme in Alkoholikern zu erlangen. Zudem wird vermutet, dass verminderte MORs und Dopaminrezeptoren/-transporter als molekulare Marker für die Krankheit gesehen und zur Entwicklung personalisierter Behandlungsmöglichkeiten genutzt werden können.

CONTENT

ABBREVIATIONS

AcbC	nucleus accumbens core region
AcbS	nucleus accumbens shell region
BLA	basolateral nucleus of the amygdala
BP_{ND}	binding potential
CeA	central nucleus of the amygdala
CIE	chronic intermittent exposure to alcohol vapor
CPM	counts per minute
CPu	caudate putamen
DA	dopamine
DALYs	disability adjusted life years
DAT	dopamine transporter
DOR	δ-opioid receptor
DSM-IV/DSM-5	Diagnostic and Statistical Manual for Mental Disorders, $4^{th}/5^{th}$ edition
D1	dopamine receptor D1
D2	dopamine receptor D2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
HPLC	high pressure liquid chromatography
ICD-10	International Classification of Disease, 10^{th} edition
KOR	κ-opioid receptor
MOR	μ-opioid receptor
MSN	medium spiny neuron
NC	nucleus caudatus
NTX	naltrexone
Pdyn	preprodynorphin
Penk	preproenkephalin
Pomc	proopiomelanotropin
qRT-PCR	quantitative real-time polymerase chain reaction
RIN	RNA integrity number
ROI	region of interest
SNP	single nucleotide polymorphism
VS	ventral striatum (including nucleus accumbens)
VTA	ventral tegmental area

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K. Björk, A. Thorsell, G. Tanda, **N. Hirth**, M. Heilig, A.C. Hansson, W.H. Sommer. *Beta-arrestin 2 knockout mice exhibit sensitized dopamine release and increased reward in response to alcohol*, Psychopharmacology, 2013 Dec;230(3):439-49

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N. Hirth*, M. W. Meinhardt*, H. R. Noori*, H. Salgado, O. Torres-Ramirez, S. Uhrig, L. Broccoli, S. Perreau-Lenz, G. Köhr, R. Spanagel, W. H. Sommer, A. C. Hansson. *Convergent evidence from alcohol dependent humans and rats for a hyperdopaminergic state during abstinence,* 2015, *Proceedings of the National Academy of Science,* under revision (Study IV in this thesis)

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R. E. Bernardi, K. Zohsel, **N. Hirth**, M. Rietschel, M. Laucht, R. Spanagel, W.H. Sommer. *A gene-by-sex interaction for nicotine reward: Evidence from epidemiology and humanized mice*, 2015, in preparation

1 INTRODUCTION

1.1 ALCOHOL DEPENDENCE

As a psychoactive substance, alcohol has addiction- and dependence-inducing properties and its harmful use is a major risk factor for death, disease and disability. Worldwide, it accounts for 5.9 % of all deaths and 5.1 % of disability-adjusted life years (DALYs, years of life lost due to premature mortality or lost due to time lived in less than full health) (1). It not only affects the consumer but has serious impact on society and economy, and on every person connected to the consumer. The European Union (EU) is one of the heaviest drinking regions in the world, with alcohol consumption almost double of the global average. In 2010, every person older than 15 years consumed an average of 10.2 liters of pure alcohol. As a result, alcohol is a major factor for premature deaths in the EU that can be attributed to cancers, liver cirrhosis and injuries caused by alcohol (2).

In the EU, 7.5% of the population older than 15 years is thought to suffer from alcohol use disorders (AUD) with 4 % actually being alcohol dependent (1). The term "dependence" refers to physical adaptations that result in withdrawal symptoms whereas "addiction" describes behavioral changes that cause the loss of control over drug consumption despite its consequences. It is a major challenge to understand why some individuals become alcohol dependent whereas others do not. Genetic (3, 4), developmental, as well as environmental factors (5) have an impact on the risk to develop dependence. Alcohol dependence has been described as chronically relapsing disorder. Initially controllable drug consumption relies on the subjective drug-induced effects, i.e. the increase of positive subjective feelings or relieve of negative states (Figure 1A). In some individuals, this limited drug consumption shifts to compulsive drug seeking and taking which no longer can be controlled or limited. Withdrawal symptoms and negative emotional states emerge when alcohol use is discontinued. This stage is characterized by craving for positive and negative reinforcing effects of alcohol that were previously experienced. Craving can be induced by drug-associated (conditioned) cues (6), drug priming, or stress, and is contributing to relapse (7, 8). Living through this cycle of intoxication, withdrawal, craving, and relapse repeatedly results in neuroadaptive changes in various brain neurotransmitter systems, including the dopamine (DA) and endogenous opioid system (9)(Figure 1A).

Various brain regions are involved in mediating the rewarding effects of alcohol (Figure 1B). As early as 1954, Olds and Milner (10) started to identify those regions by implanting

electrodes into the brain of rats. By pressing a lever the animals could voluntarily self-stimulate themselves by directly activating brain reward pathways while bypassing normal physiological inputs. The medial forebrain bundle, a complex axon bundle including serotonergic and noradrenergic projections as well as dopaminergic projections from the ventral tegmental area, was identified as critical for self-stimulation (11). Alcohol dependence and withdrawal induce severe dysregulations in this brain reward system (Figure 1B). Simultaneously, stress and anti-reward systems are increasingly activated (12-15).

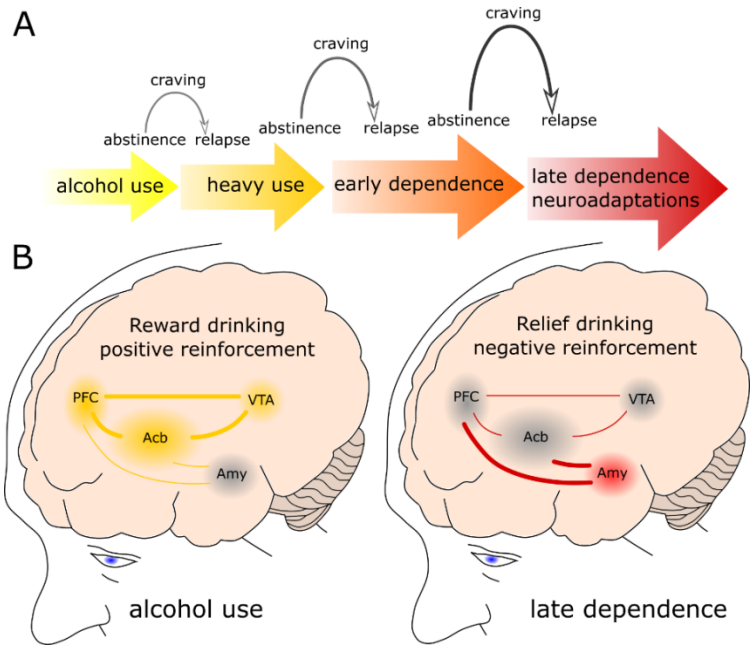

Figure 1: Development of alcohol dependence over time. (A) Initial alcohol use that is linked to positive reinforcing and pleasurable effects of alcohol is followed by the loss of control and compulsive alcohol intake. This progression is accompanied by the shift of positive to negative reinforcement where alcohol is consumed to achieve relief from negative emotional states. The state of late dependence is characterized by long-lasting neuroadaptations that also persist into protracted abstinence. (B) In a non-dependent individual alcohol is consumed for its positive reinforcing and rewarding effects. These are mediated by neurocircuitries involving the nucleus accumbens (Acb), ventral tegmental area (VTA) and prefrontal cortex (PFC). During alcohol dependence, the reward processes within these systems are dysregulated and the amygdala (Amy) is increasingly active. Thereby, negative emotional states are emerging and alcohol is consumed for its relieving effects (negative reinforcement). Adapted from (12, 14).

In summary, alcohol dependence is a chronically relapsing disorder that is characterized by reduced reward functions and increased dysphoric states. A clear diagnosis of the disease is needed to successfully help patients to remain abstinent.

1.1.1 DIAGNOSIS OF ALCOHOL DEPENDENCE

Diagnosis of alcohol use disorders and dependence often is challenging and physicians use psychiatric manuals as a guideline. In 2013, the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders was published (DSM-5, (16)). It differs from the previous edition DSM-IV which distinguished between alcohol abuse and dependence, now integrating these two disorders into a single one (alcohol use disorder, AUD). It is defined by the occurrence of at least two specified symptoms (see below) and its severity is indicated by the number of symptoms present (mild 2-3, moderate 4-5, severe 6 or more symptoms) as shown in Table 1.

Another widely used diagnostic manual is the "International Statistical Classification of Disease and Related Health Problems" (ICD-10) which was introduced by the World Health Organization. Diagnostic criteria of ICD-10 and DSM coincide.

Table 1: Diagnostic criteria for alcohol use disorder according to DSM-5 (from (16))

1)	"Alcohol is often taken in larger amounts or over a longer period than was intended."
2)	"Persistent desire or unsuccessful efforts to cut down or control alcohol use."
3)	"A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects."
4)	"Craving, or a strong desire or urge to use alcohol."
5)	"Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home."
6)	"Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol."
7)	"Important social, occupational, or recreational activities are given up/reduced because of alcohol use."
8)	"Recurrent alcohol use in situations in which it is physically hazardous."
9)	"Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol."
10)	"Tolerance, as defined by either of the following: a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect b. A markedly diminished effect with continued use of the same amount of alcohol"
11)	"Withdrawal, as manifested by either of the following: a. The characteristic withdrawal syndrome for alcohol (refer to criteria A and B of the criteria set for alcohol withdrawal) b. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms"

1.1.2 PHARMACOLOGICAL INTERVENTIONS FOR RELAPSE PREVENTIONS

A major challenge in the treatment of alcohol dependence is the reduction of relapse to drinking behavior. The risk for relapse can be reduced by psychological interventions such as cognitive-behavioral therapies or motivational enhancement interviewing but also pharmacotherapy or the combination of both. To date, only few pharmacological anti-relapse medications are approved.

Disulfiram (Antabus®)

Disulfiram has been used to treat alcohol dependence since the 1940's and interacts with the alcohol metabolism by inhibition of the enzyme acetaldehyde dehydrogenase. Thereby it leads to elevated acetaldehyde levels causing hangover-like symptoms such as sweating, headache, nausea, and vomiting. Patients associate these aversive states with drinking and can be discouraged to further ingest alcohol. However, the efficacy is weak with showing low effects on alcohol craving and those patients that want to drink can easily stop taking disulfiram. Thus, it should be taken by patients that are motivated to stop drinking or receive their medication under supervision (17, 18).

Acamprosate (Campral®)

Acamprosate (calcium-bis(*N*-acetylhomotaurinate)) is a well-tolerated and safe pharmacological treatment and it has been shown to reduce the risk for relapse (19). However, it does not affect craving (20). Acamprosate is thought to modulate glutamatergic systems by interacting with N-methyl-D-aspartate (NMDA) and metabotropic-5 glutamate (mGlur5) receptors (21-23). A common theory in the alcohol research field is that chronic alcohol leads to a hyperglutamatergic state in alcohol withdrawal which may drive relapse (24, 25). Acamprosate therapy has been shown to reduce glutamate concentrations in alcohol-dependent patients (26) and to reduce consumption in alcohol drinking mice by dampening this hyperglutamatergic state (27, 28). However, the exact molecular mode of action of acamprosate is not clear and it has been suggested that acamprosate's effects are caused by calcium as the active moiety of the drug (29).

Naltrexone (ReVia®, Vivitrol®)

The unselective opioid receptor antagonist naltrexone (NTX) displays the highest affinity for the MOR followed by KOR and DOR was approved in the US by the Food and Drug Administration (FDA) in 1994 after two studies reported reduced drinking in dependent subjects after NTX therapy (30, 31). In Germany, NTX is available since 2010. As MOR antagonist, NTX is thought to block the rewarding effects of alcohol by reducing MOR-mediated dopamine release in the striatum. The recommended dose of 50 mg of NTX has been shown to almost completely block the MOR (95 %) and to a lower percentage (21%) the DOR in human subjects (32). A dose of 150mg was sufficient to block about 90% of the KOR (33).

The efficacy of NTX has been shown by a meta-analysis (34). However, its effect size is relatively small and only a subset of patients appears to benefit from NTX therapy. Thus, many scientists and physicians demand for personalized treatment approaches (35).

Nalmefene (Selincro®)

Nalmefene received authorization for the European Union in 2013 and is the first pharmacotherapy approved for reduction of alcohol consumption and for "as-needed" use. Patients are asked to take their medications if they feel at risk to return to heavy drinking (36). The efficacy of nalmefene treatment to reduce alcohol consumption in dependent patients was demonstrated in "as-needed" clinical trials (37, 38).

In contrast to NTX which is an opioid receptor antagonist, nalmefene shows antagonistic activity at the MOR and DOR but also partial agonistic activity at the KOR (39). Its affinity for KOR and DOR is higher than that of NTX. It is assumed that nalmefene could be more helpful than NTX by acting at the KOR and thereby antagonizing the rewarding and reinforcing effects of alcohol. Administration of nalmefene into the nucleus accumbens (Acb) of alcohol-dependent rats reduced self-administration to a higher degree than in non-dependent rats and this effect was attributed to KOR mediated mechanisms (40, 41). However, the superiority of nalmefene over NTX in humans remains to be under debate and additional studies are warranted (42).

Off-label use

In addition to the medications specifically approved for the reduction of alcohol intake, different pharmaceutics approved for other indications are studied and used for the therapy of alcohol dependence. A brief overview is given in Table 2.

Table 2: Approved (light grey) and off-label (white) pharmacotherapies for the treatment of alcohol dependence. References show reviews on the use of the medication in alcohol dependence.

Compound	Mode of action	Approved in US	Approved in EU	Reviewed in/ Reference
Naltrexone	Opioid receptor antagonist	1994	2010	(34)
Nalmefene	Opioid receptor antagonist	1995	2013	(43, 44)
Disulfiram	Acetaldehyde dehydrogenase inhibitor	1948	1950's	(17, 18)
Acamprosate	Modulator of glutamate system	2004	1989	(19, 45)
Gabapentin	Enhancement of GABA activity	1993 (adjuvant)	1995 (antiepileptic, to treat neuropathic pain)	(46)
Baclofen	$GABA_B$ receptor agonist	1988 (to treat multiple sclerosis, spinal cord injury)	1970 (myotonolyticum)	(47)
Ondansetron	Serotonin receptor antagonist	2006 (to treat chemotherapy and postsurgical nausea)	1990 (to nausea and vomiting)	(48)
Topiramate	Glutamate receptor antagonist, facilitates GABA currents	1996 (anticonvulsant/ antiepileptic)	1998 (antiepileptic)	(48, 49)

Gabapentin is an approved medication for the treatment of epilepsy and neuropathic pain but has also been successfully used for the therapy of mild withdrawal and alcohol dependence. It appears to increase the time to first heavy drinking, reduces the number of heavy drinking days, and has positive effects on mood and sleep (46).

Baclofen's effects have been studied preclinically where it reduced alcohol self-administration. Although some clinical trials report promising results (50-52) the overall picture is inconsistent and more clinical trials are required (47).

Ondansetrone which is approved for chemotherapy and the reduction of postsurgical nausea has positive effects on abstinence days and drinking intensity (48, 53, 54). However, more studies are needed to establish its beneficial effects in the treatment of alcohol dependence.

Topiramate has been shown to reduce heavy drinking but side effects are strong and reduce clinical utility (48).

As only few approved pharmacotherapies are available for alcohol relapse prevention, further studies on potential treatment targets are warranted.

1.2 THE ENDOGENOUS OPIOID SYSTEM

Opium has been used for recreational and medical reasons for thousands of years as it induces euphoria, analgesia, and sleep. However, the most prevalent and active alkaloid in opium, morphine, has only been isolated at the beginning of the 19th century by the German pharmacist Sertüner (Figure 2) (55). While the term "opiates" summarizes natural alkaloids like morphine contained in opium, "opioids" refers to all substances inducing morphine-like effects which can be blocked by opioid receptor antagonists such as NTX. This includes opiates, synthetic substances, and endogenous opioid peptides.

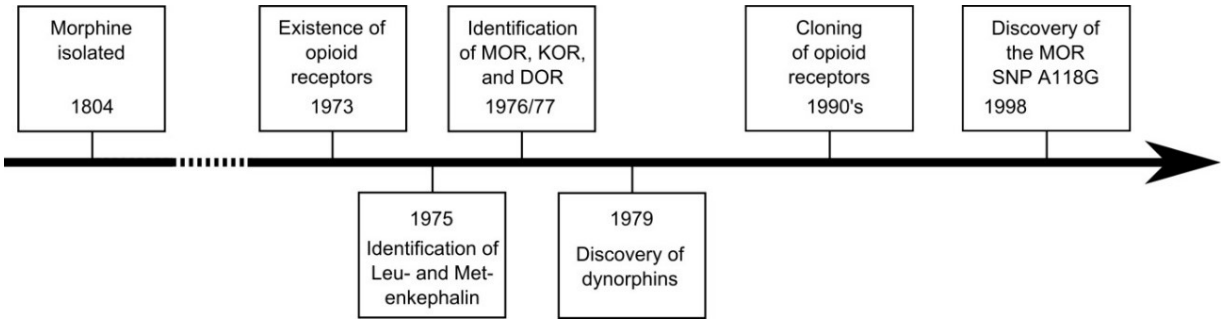

Figure 2: Timeline of the discovery of the endogenous opioid system. Even though morphine, a natural alkaloid of opium, was known since the early 19th century, it took until the 1970's to identify the opioid receptors and their endogenous ligands (56-60).

Although it was believed that opioid-like compounds have endogenous binding sites in the nervous tissue, it took several years to identify and classify the opioid receptors (56-60). Eventually, three different receptors were characterized: μ- (MOR), δ- (DOR), and κ- (KOR)

opioid receptors. Also in the 1970's, the endogenous opioid peptides, Leu-enkephalin and Met-enkephalin (61), β-endorphin (62), and dynorphins were discovered (63).

1.2.1 THE ENDOGENOUS OPIOID LIGANDS

All endogenous opioid peptides are produced by proteolytic cleavage of the three precursor proteins proopiomelanocortin (Pomc), preproenkephalin (Penk), and preprodynorphin (PDYN) (64-67). In the mammalian brain, *Penk* and *Pdyn* mRNA expression is widely distributed while *Pomc* expressing cell bodies are restricted to only few regions: the median eminence/arcuate nucleus of the hypothalamus, the pituitary, and nucleus tractus solitarius (68, 69)(Figure 3). However, Leriche et al. (70) detected *Pomc* mRNA also in the prefrontal cortex, the Acb and ventral tegmental area (VTA). *Pomc* is the precursor of several biologically active neuropeptides, such as β-endorphin, β-lipotropin, adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (68, 71). *Penk* gives rise to Leu-enkephalin, Met-enkephalin, Met-enkephalin-Arg6-Phe7, and Met-enkephalin-Arg6-Gly7-Leu8 (72). Dynorphin A and B as well as neoendorphin and leu-enkephalin are derived from *Pdyn* (65, 73).

All endogenous opioids possess a common NH-terminal Tyr-Gly-Gly-Phe-[Met/Leu] sequence which is referred to as the opioid motif. This sequence is responsible for the interaction with the opioid receptors. However, the opioid peptides show varying affinities for the different receptors. While endorphins bind equally strong to the MOR and DOR (74, 75), enkephalins show higher affinity for DOR than MOR and almost negligible affinity for KOR (60). Dynorphins exert their effects primarily through KOR (76).

Table 3: Endogenous opioid peptides and their receptors (for references see text and (77))

Precursor	Endogenous peptide	Affinity for opioid receptors
Preproenkephalin	Met-enkephalin Leu-enkephalin	DOR, MOR (DOR >> MOR)
Proopiomelanocortin	β-endorphin	MOR, DOR (MOR = DOR)
Prodynorphin	Dynorphin A and B Leu-enkephalin α- and β-neoendorphin	KOR, MOR, DOR (KOR >> MOR and DOR)

1.2.2 THE OPIOID RECEPTORS

Cloning of the MOR, DOR, and KOR revealed the characteristic topology of G-protein coupled seven-transmembrane receptors (GPCRs) and a high sequence homology among the receptors. Intracellularly, they interact with G_i/G_o proteins. Upon activation of the receptor by endogenous or exogenous ligands the membrane potential, neuronal excitability and neurotransmitter release decreases by opening of G-protein gated inwardly rectifying potassium ion channels (GIRK), inhibiting voltage gated calcium ion channels and decreasing intracellular adenylyl-cyclase-mediated cAMP production. Moreover, second-messenger systems and gene expression are affected.

1.2.2.1 THE NEUROANATOMICAL DISTRIBUTION OF OPIOID RECEPTORS

The opioid receptors and endogenous opioids are broadly distributed throughout the peripheral and central nervous system. Their distribution and expression level can be analyzed by receptor autoradiography and *in situ* hybridization and is well studied in the rodent brain. Each opioid receptor has a defined expression pattern in the rodent brain, with MOR being the most represented opioid receptor in many subregions of the amygdala, thalamus, mesencephalon, and in the striatum. KOR is most expressed in brain structures such as the striatum, basal anterior forebrain, hypothalamus, and pituitary. DOR distribution is more restricted with expression in the striatum, olfactory tract, cortices and some subregions of the amygdala. In few brain structures, there is only one opioid receptor present, e.g. the MOR in specific thalamic nuclei while in many other regions the MOR and KOR are co-distributed (for review see (69)).

The pattern of the distribution of opioid receptors and their ligands in the rodent brain is shown in Figure 3.

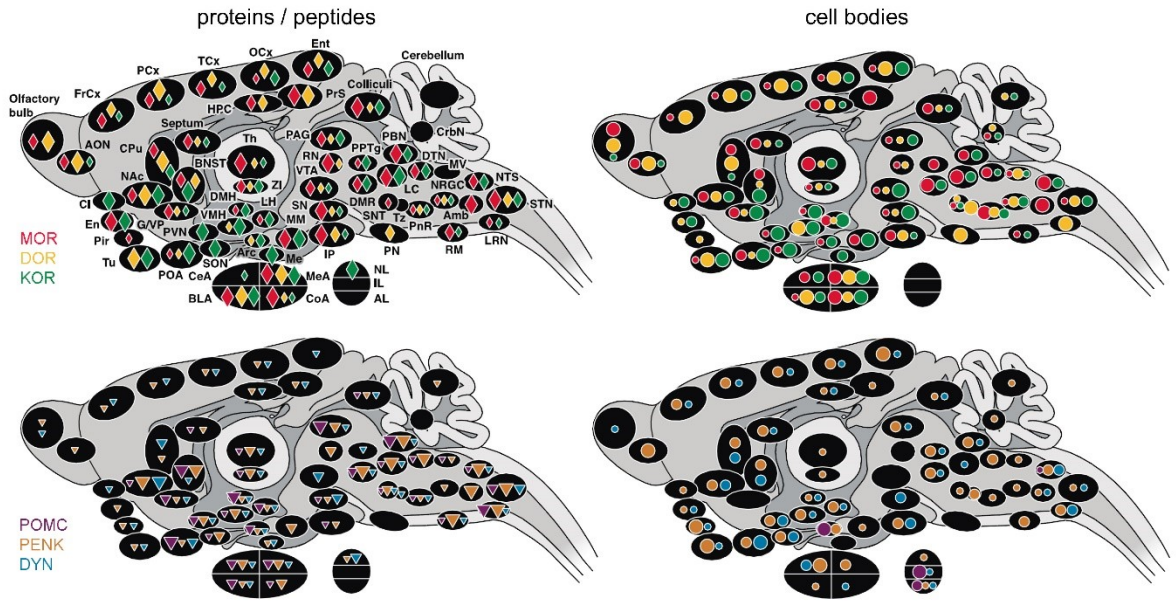

Figure 3: Anatomical distribution of opioid receptors and their endogenous ligands. (**A**) Receptor protein distribution in the rodent brain (red – MOR, yellow – DOR, green – KOR). The size of the icon representing the specific receptor indicated the amount of expression in the shown brain area. (**B**) Distribution of opioid receptor mRNA expressing cell bodies. (**C**, **D**) Expression of proopiomelanocortin (POMC – purple), preproenkephalin (PENK – orange), and dynorphin (DYN – blue) peptides (**C**) and mRNA (**D**). Adapted from (69).

In the human brain, expression of opioid receptors has been studied since the early 1980s and the expression pattern of MOR (78-93), DOR (79, 82, 84, 86, 89, 90, 93, 94), and KOR (79, 86, 89, 90, 92, 93, 95) are well known. Overall, the distribution is similar to the rodent brain. However, human brain structures do not always match the rodent brain, e.g. the caudate putamen (one combined structure) in the rodent brain corresponds to two regions in the human brain (nucleus caudatus and putamen). Thus, the expression pattern of receptors can be different. For instance, Voorn et al. (78) demonstrated MOR expression in a dorso-ventral gradient with higher expression in the ventral part of the nucleus caudatus in human post-mortem brain slices. In the rodent brain, however, the MOR is enriched in striatal patches (striosomes).

1.2.2.2 REGULATION OF OPIOID RECEPTORS

Opioid receptors can be regulated at different levels, including transcription and translation, de-/resensitization and internalization processes, or receptor affinity (Figure 4). Additionally, intracellular signaling can be affected by disturbances in signaling cascades or altered

coupling of the receptor to G-proteins. The adapter protein β-arrestin (βArr) is an important modulator of opioid receptor signaling. After ligand-induced activation, G-protein receptor kinases (GRK, mainly GIRK2 and GRK3) phosphorylate the receptor which increases the affinity for β-arrestin2 binding and, thus, triggers endocytosis. The receptor can afterwards be degraded or recycled back to the cell surface membrane (96). In various animal models, a role of β-arrestin2 in alcohol reward processes has been proven. For instance, alcohol-preferring AA rats show elevated levels of β-arrestin2 expression (97). Furthermore, knockout mice lacking β-arrestin2 show maximum alcohol-induced DA release at lower doses as compared to wildtype mice and display increased conditioned place preference indicating an important role of β-arrestin2 in alcohol reward.

Interestingly, the β-arrestin2 protein has been shown to be associated with MOR availability and function (98-102). For instance, enhanced MOR G-protein coupling was observed in the β-arrestin2 knockout mice after a low dose of alcohol (98). However, the β-arrestin2-dependent receptor regulation is not specific for opioid receptors but occurs in various GPCRs including DA receptors (103). Additionally, arrestins can mediate G-protein-independent signaling processes.

In the presented thesis, various levels of opioid receptor regulation, i.e. transcription, cell surface density, G-protein coupling and β-arrestin2 expression in association with MOR, are analyzed.

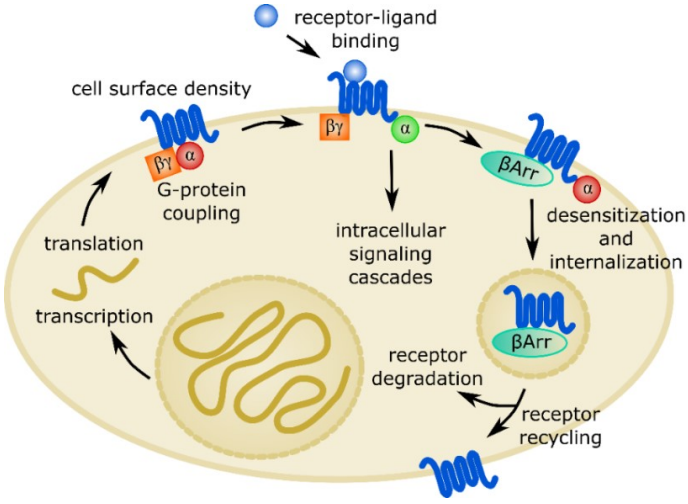

Figure 4: Possible levels of regulation of opioid receptors. Expression of receptors can be affected by changes on the transcriptional or translational level, thereby affecting cell surface densities. Post-translational or conformational changes may have an impact on receptor-ligand affinity. Intracellularly, the opioid receptors are coupled to G-proteins. A decrease in coupling can influence receptor signaling. Upon ligand binding, G-protein subunits (α and βγ) are activated and signaling cascades are initiated. The receptor is phosphorylated and β-arrestin binding induces receptor internalization.

1.2.3 THE ENDOGENOUS OPIOID SYSTEM IN ALCOHOL DEPENDENCE

The endogenous opioid system is – together with other neurotransmitter systems, mainly the dopamine system – involved in modulating reward and is critical in addictive behaviors and has been the subject of numerous preclinical and clinical studies.

1.2.3.1 THE μ-OPIOID RECEPTOR (MOR)

Human studies

In human subjects, positron emission tomography (PET) brain scans are the method of choice to investigate alterations of neurotransmitters or receptors. Mitchell et al. (104) measured the displacement of the radiolabelled MOR agonist [^{11}C]-carfentanil to study alcohol-induced changes in the level of endogenous opioids before and immediately after alcohol consumption in heavily drinking and control subjects. In both groups, carfentanil binding was significantly reduced in the Acb following alcohol consumption indicating alcohol-induced accumbal β-endorphin release (104). Furthermore, it is proposed that this activation of the endogenous opioid system also leads to increased dopamine release. Indeed, remifentanil activation of MOR causes dopamine release as measured by displacement of the radiotracer [^{18}F]-fallypride (105).

Various studies investigated the opioid receptor status in human alcoholics using [^{11}C]-carfentanil or [^{11}C]-diprenorphin PET studies reporting increased or unchanged MOR availability (106-108). Furthermore, Heinz et al. correlated the increased MOR availability with craving (107). This and the ability of opioid antagonists to block [^{11}C]-carfentanil binding in abstinent alcoholics (32) gives the rational to treat alcohol dependence with opioid antagonists such as naltrexone and nalmefene.

The interpretation of PET studies, however, is challenging. When using receptor agonists such as [^{11}C]-carfentanil as tracers, the measured binding potential BP_{ND} cannot directly be translated into the amount of surface receptors because endogenous opioids compete with the tracer for receptor binding sites (Figure 5). Hence, increased MOR availability as measured in the studies mentioned above could represent a decrease in endogenous ligands leaving more receptors available for the PET tracer. It is therefore highly important to determine the actual state of the opioid receptors in alcohol dependence and abstinence as they are the target of the anti-relapse pharmacotherapies naltrexone and nalmefene. One method to achieve this, is the autoradiographic analysis of human post-mortem brain tissue.

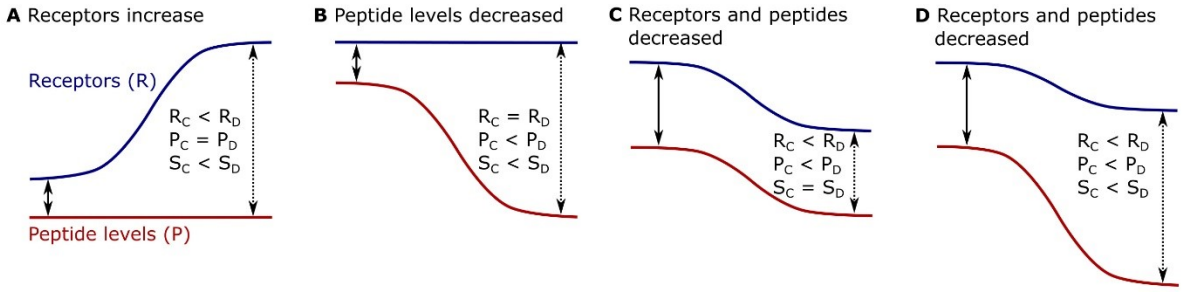

Figure 5: An increase in PET signal can be caused by altered receptor density or peptide levels or both. PET signals measured in healthy controls and diseased individuals are indicated by solid and dashed arrows, respectively. (**A**) Elevated receptor densities with stable ligand levels result in an increase in PET signal. (**B**) Similar results can be observed when receptor levels stay constant but ligand levels are decreased since the radiotracer does not have to compete with the endogenous ligand. (**C**) When receptors and ligands both are decreased to a similar extent, no alterations in PET signal intensity can be found. (**D**) With a stronger decrease in peptide levels, however, an increased in PET signal is seen. R_C/R_D – receptor density in controls/diseased individuals, P_C/P_D – peptide levels in controls/diseased individuals, S_C/S_D – signal intensity measured by PET in controls/diseased individuals.

Animal studies

The MOR is the most studied opioid receptor in the context of alcohol addiction. Mice deficient of the MOR gene have been generated in various labs. Those animals do not self-administer alcohol and show reduced anxiolytic effects of low doses of alcohol (109). This indicates an important role of the MOR in alcohol dependence. However, the state of the MOR after chronic alcohol administration is unclear as results on MOR expression appear to be inconsistent (Table 4). This is most likely caused by the differences in study designs as various animal strains, alcohol administration paradigms, and methods for MOR detection were used. Furthermore, animals used in these studies are most likely not severely alcohol dependent. The focus of most studies was to analyze the expression of MOR immediately after chronic alcohol administration or during acute withdrawal.

Table 4: Overview of studies investigating the state of MOR after chronic alcohol. Results are inconsistent. ↑ increase, ↓ decrease, ↔ unchanged MOR expression or G-protein coupling, 2BFC – voluntary alcohol consumption in a two bottle free choice paradigm.

Animals	Alcohol paradigm	MOR detection	Finding (Striatum)	Reference
C57BL6 and DBA/2 mice	2BFC (10% ethanol)	PCR	↔ also during acute withdrawal	(110)
Fawn-Hooded rats	2BFC (5% ethanol)	[^{125}I]FK-3382 binding	↑	(111)
Wistar rats	Ethanol as only liquid source	[^{3}H]-Damgo binding	↓ Also during acute withdrawal	(112)
Fawn-Hooded rats	2BFC (5% ethanol)	Damgo stimulated [^{35}S]-GTPγS binding	↓ slight recovery after 48h withdrawal	(113)
Fawn-Hooded rats	2BFC (5% ethanol)	[^{125}I]FK-3382 binding	↑ at different time points during withdrawal	(114)
Long Evans rats	Ethanol self-administration	Damgo stimulated [^{35}S]-GTPγS binding	↔	(115)
Sprague-Dawley rats	Liquid diet (6.7% ethanol)	Damgo stimulated [^{35}S]-GTPγS binding	↔	(116)
Sprague-Dawley rats	Liquid diet (6.7% ethanol)	Immunohistochemistry	↓	(117)
Wistar rats	2BFC (10% ethanol)	Damgo stimulated [^{35}S]-GTPγS binding	↓	(118)
Wistar rats	Ethanol as only liquid source	Membrane binding [^{3}H]-Damgo	↔	(119)
Wistar rats	2BFC (6% ethanol)	PCR	↑ after 2 and 4 months of 2BFC, but not after 10 months	(120)

The influence of the MOR single nucleotide polymorphism A118G

Many polymorphisms have been found in the opioid receptor genes. For the *OPRM1* gene encoding the MOR, the single nucleotide polymorphism (SNP) A118G (rs1799971) is the most studied in the context of drug dependence (121). It was first described in 1998 (122) as

an exchange of adenine by guanine at nucleotide 118 (A118G) resulting in an amino acid exchange in the amino-terminal extracellular domain of the MOR, i.e. asparagine (N) is replaced by aspartate (D) (N40D). It affects MOR glycosylation, stability (123) and has been proposed to increase the receptors affinity for the endogenous ligand β-endorphin (122). However, several studies failed to replicate the latter (124-127). The frequency of the less common (minor) G-allele varies among populations with about 40-50% in Asians but only 15-30% in Europeans and is very rare (1-3%) in individuals of African or Hispanic ancestry (128-130). It might cause decreased *OPRM1* expression in G-allele carriers (125, 131, 132). However, these studies did not include striatal brain tissue but rather analysed the global brain than specific areas, post-mortem pons tissue or cell cultures.

Various studies indicate a role of this genetic variant in alcohol dependence. Results on the association between the SNP and the risk for alcohol dependence vary (133-137) but a meta-analysis shows no increased risk for substance dependence (138). A number of studies indicate a role of this genetic variant on the reinforcing and rewarding effects of alcohol (139-142).

To extend the knowledge on the impact of the *OPRM1* SNP A118G (rs1799971) different transgenic animal models were generated, including rhesus macaques with a functionally equivalent SNP (C77G resulting in P26R)(143), a mouse model carrying the equivalent substitution in the mouse *Oprm1* gene (A112G) (144), and two mouse lines possessing the human MOR sequence carrying either the A- or G-allele (127). Studies in these animals support the assumption of a role of this SNP in alcohol-related behavior and suggest better treatment outcome of G-allele carriers after NTX therapy (145).

1.2.3.2 THE δ-OPIOID RECEPTOR (DOR)

Human studies

The DOR is far less well studied than MOR. Human PET imaging studies in alcoholic subjects are very limited and revealed unchanged DOR availability measured with the radiotracer [^{11}C]-methyl-naltrindole (106, 146). Plasma levels of the DOR ligand enkephalin appears to be unchanged during alcohol withdrawal and abstinence (147). This is in contrast to the decline in plasma β-endorphin levels that have been observed during withdrawal (147).

Animal studies

Mice deficient of DOR consume more alcohol as compared to wildtype mice indicating a role of DOR in alcohol intake behavior. This may be linked to increased anxiety in these animals and, thus, represent a self-medication approach to reduce anxiety levels (148, 149). After chronic alcohol intake, however, DORs are increased or unchanged (110, 112, 117).

Application of DOR antagonists in the VTA induce alcohol consumption while activation of the DOR by the specific agonist DPDPE ([D-Pen^{2,5}]Enkephalin, [D-Pen^{2},D-Pen^{5}]enkephalin) decreases alcohol intake (150). This most likely is mediated by inhibiting GABAergic terminals by DOR agonists in alcohol consuming rats and this is linked to anxiety (150, 151).

However, the state of DOR in alcohol-dependent animals during protracted abstinence was, so far, not subject to investigations and remains unclear.

1.2.3.3 THE *K*-OPIOID RECEPTOR (KOR)

The development and evaluation of KOR-selective ligands suitable for PET studies is in progress (33, 152) but no data of KOR availability in alcoholic subjects are available. Therefore, studies of the state of the KOR/dynorphin system are restricted to post-mortem tissue analysis of control and alcoholic subjects. Previous post-mortem studies found increased dynorphin A and B in prefrontal brain regions and the hippocampus (153, 154) while dynorphin A was decreased in the putamen (155). In the nucleus caudatus, dynorphin A and B were unchanged (155). Unfortunately, neither *OPRK1* mRNA nor KOR surface density were analyzed in the same sample set of human post-mortem tissue and, thereby, no complete overview can be given on the state of KOR/dynorphin system in the striatum of those alcoholics.

Animal studies

Kissler et al. found increased G-protein coupling and dynorphin immunoreactivity in alcohol-dependent rats during withdrawal and these increases in KOR activity have been linked to aversion as measured by augmented 22 kHz ultrasonic vocalizations (156). Interestingly, blockade of KOR by intra-amygdala infusions of nor-BNI resulted in decreased alcohol self-administration in dependent but not non-dependent rats in the same study (156). Also intra-accumbal and intra-cerebroventricular infusion of nor-BNI attenuated operant responding for

alcohol selectively in dependent animals while not affecting non-dependent self-administration (40, 41). Moreover, primates voluntarily consuming alcohol display supersensitivity of the KOR/dynorphin system in the ventral and dorsal striatum that correlates with drinking behavior (157).

These observations indicate an upregulation of the KOR/dynorphin system in alcohol dependence which increases the anhedonic effects associated with alcohol consumption and withdrawal. Indeed, increased dynorphin and KOR mRNA expression in the Acb has been reported (158-160).

1.2.3.4 COMBINED ACTION OF OPIOID RECEPTORS AND THEIR LIGANDS

Acute alcohol administration releases β-endorphin, enkephalin, and dynorphin in a time specific profile: β-endorphin increases within 30 min after acute alcohol administration which is followed by an increase in dynorphin 1.5 to 2 hours later (161, 162). While β-endorphin and enkephalin are known to mediate the positive reinforcing effects by activating MOR and DOR, dynorphins are implicated in mediating the aversive effects of alcohol by stimulating KOR. It has been proposed that after chronic alcohol consumption positive effects are attenuated and negative effects are increased (163, 164)(Figure 1).

1.2.3.5 THE OPIOID SYSTEM AS TARGET FOR PHARMACOLOGICAL RELAPSE PREVENTION

As briefly mentioned above, the pharmacotherapies naltrexone (NTX) and nalmefene are targeting the endogenous opioid system to reduce alcohol relapse in dependent individuals.

In human alcoholics, the efficacy of NTX has been supported by meta-analysis (34). However, its effect size is small (165). This might reflect heterogeneity among patients as some individuals seem to improve dramatically while others show no response to NTX pharmacotherapy demanding for personalized treatment approaches (165). Indeed, it has been reported that a family history of alcoholism, which can reflect both environmental and/or genetic factors, positively influences therapeutic effects (166-168), while NTX can even increase alcohol drinking in individuals without a family history of alcohol dependence (167).

In animals with high alcohol consumption NTX has been proven to reduce alcohol intake (169) as well as preference (170) and to block alcohol-seeking in rats (171-173). On the cellular level, NTX reduces dopamine release in the striatum of rats (174, 175). This is

somehow surprising considering the PET studies stating a hypodopaminergic state during abstinence.

NTX has a relatively low plasma half-life of about four hours in humans and is metabolized to β6-naltrexol by the enzyme dihydrodiol dehydrogenase. This active metabolite has a half-life of about eleven hours but its role in NTX therapy is, so far, unresolved (176). In Wistar rats, NTX's half-life is only about one hour after intravenous bolus injection but β6-naltrexol is below the detection limit indicating that it is not a major metabolite in rats (177).

The majority of studies, however, focus on the influence of the SNP A118G of the *OPRM1* gene as a determinant of treatment outcome. The first meta-analysis reviewing publications on the association between the SNP A118G and response to NTX treatment in alcohol dependent patients found that G-allele carriers had lower relapse rates than AA-allele carriers (178). However, some studies do not support the hypothesis that the G-allele moderates the response to NTX (179-181) and a more recent meta-analysis states that more studies are needed to prove the impact of this genetic variant on NTX treatment outcome (182). On the molecular level, a PET study demonstrated slightly higher NTX occupancy in G-allele carriers as compared to controls although the results did not reach significance (132).

Animal models for the A118G SNP in mice and monkeys support the assumption of a better treatment outcome in G-allele carriers. NTX attenuated alcohol preference (143) and intake in monkeys carrying the minor allele that were trained to self-administer alcohol (183). In a mouse model carrying the A118G SNP, naltrexone moderated alcohol reward processes and reduced alcohol consumption in 118GG but not 118AA mice (145).

Another factor influencing NTX efficacy might be the severity of alcohol dependence. Various authors suggest that more severely diseased patients benefit less from NTX pharmacotherapy (37, 184).

Furthermore, several studies report that NTX modulates cortisol release by affecting the hypothalamic-pituitary-adrenal (HPA) axis (Figure 6), a major stress response pathway, which is dysregulated in alcohol-dependent patients (185, 186). Alcohol-dependent patients display a dysregulated HPA axis response, i.e. blunted cortisol response. By antagonizing MOR function, NTX has been shown to enhance cortisol levels in healthy controls and alcohol-dependent subjects.

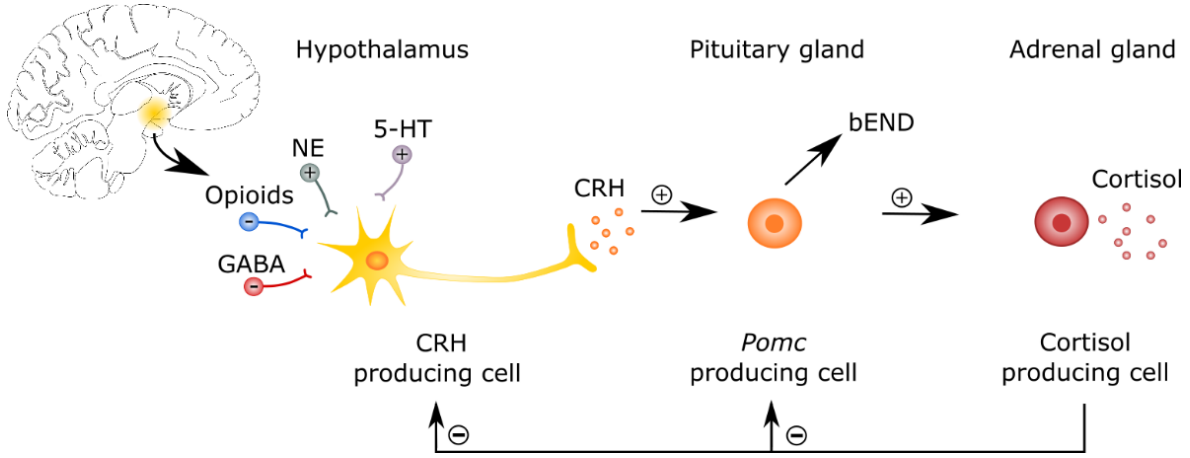

Figure 6: Stress and alcohol induce the production of corticotropin-releasing hormone (CRH) in the hypothalamus. The release of CRH is regulated by opioids, GABA, norepinephrine (NE), and serotonin (5-HAT). CRH causes β-endorphin release in the same brain region. Additionally, CRH is transported to the pituitary gland where it stimulates the production of *Pomc*. *Pomc* is then transformed to ACTH, β-endorphin (bEND) and others. ACTH induces cortisol synthesis and release from the adrenal gland. When cortisol reaches a certain level, CRH and ACTH production are reduced via feedback mechanisms. Adapted from (185).

Taken together, previous research on the endogenous opioid system proves its importance in the development and maintenance of alcohol dependence. However, the state of the opioid system in alcohol abstinence as target of the anti-relapse therapies naltrexone and nalmefene remains unclear as human PET studies are ambiguous to interpret.

1.3 THE DOPAMINE SYSTEM

The description of dopamine (DA) as an independent neurotransmitter of the central nervous system (187, 188) was followed by the identification and classification of the dopamine receptors (189, 190) which are now divided into two classes: D1-like (including D1 and D5 receptors) and D2-like (including D2, D3, and D4 receptors) dopamine receptors. All dopamine receptors are members of the GPCR family but differ in their affinity for dopamine, pharmacological profiles, mechanisms of action, and have distinct expression patterns throughout the nervous system (191). D1 receptors are highly expressed in nigrostriatal, mesolimbic, and mesocortical areas, including the dorsal (caudate-putamen) and ventral (Acb) striatum, amygdala and frontal cortex. D2 receptors are also enriched in the striatum but additionally expressed in other regions such as the substantia nigra and VTA. D3, D4, and D5 receptors appear to have lower and/or more restricted patterns of distribution in the brain

(191). The postsynaptically expressed D1-like receptors enhance the formation of cyclic adenosine monophosphate (cAMP) and protein kinase A activity (192). D2-like receptors, which are pre- and postsynaptically located (193), inhibit cAMP synthesis. Presynaptically expressed receptors act as autoreceptors that are usually activated at lower dopamine concentrations and provide an important negative feedback mechanism by regulating neuronal firing rate and dopamine synthesis and release (194-197).

Dopamine itself is synthesized in neurons and stored in vesicles until the dopaminergic neurons are activated. The dopamine containing vesicles fuse with the cell membrane by calcium-dependent mechanisms and dopamine is released into the synaptic cleft. The dopamine transporter (DAT) on presynaptic cells clears the dopamine from the synaptic cleft into the cytosol from which it is transported back into vesicles. Dopaminergic neurons are tonically active, meaning low amounts of neurotransmitter in the nanomolar scale are continuously released by single spike action potentials and achieve a stable extracellular dopamine level. In response to behaviorally relevant stimuli the neurons respond with phasic burst firing releasing dopamine in micro- to millimolar concentrations (198).

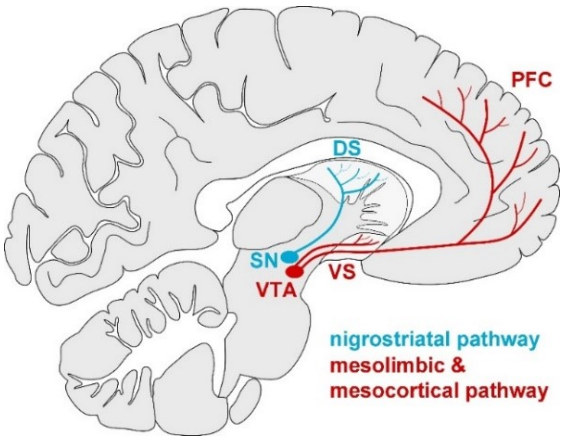

Figure 7: Dopaminergic pathways in the human brain. Dopaminergic cell bodies within the substantia nigra (SN) project via the nigrostriatal pathway to the dorsal striatum (DS). Dopaminergic neurons within the ventral tegmental area (VTA) innervate the ventral striatum (VS) and the prefrontal cortex (PFC) via the mesolimbic and mesocortical pathway, respectively.

Dopaminergic neurons in the brain are relatively few in number and their somata are restricted to defined areas. Fuxe and Dahlström (199) were the first to map these cell groups in 1964 which are organized in several pathways that originate in the midbrain. In the rodent

nigrostriatal pathway (Figure 7, blue), neurons with their cell bodies located in the substantia nigra project mainly to the dorsal striatum, i.e. the caudate (200) and are particularly important for locomotor regulation. Limbic and cortical areas are innervated by dopaminergic neurons from the VTA (201). Based on the projection fields the mesocortical and mesolimbic dopamine pathways are distinguished (Figure 7, red). The mesocortical projections innervate prefrontal cortical areas and are implicated in higher motor execution of behavior, motivation and cognition. The dopaminergic neurons of the mesolimbic pathway project to the ventral striatum, including the Acb, olfactory tubercle, septal area, amygdala, and the bed nucleus of the stria terminalis (202) which is crucial for the mediation of emotion and reward.

1.3.1 THE DOPAMINE SYSTEM IN ALCOHOL DEPENDENCE

Human studies

The method of choice to investigate the brain dopamine system in humans is brain imaging by positron emission tomography (PET). While there are no PET data available on the state of the dopamine D1 receptor in human alcoholics, studies focusing on D2-like receptors mostly used the radiotracer [^{11}C]-raclopride. Displacement of this PET tracer has been used as indirect measure of alcohol-induced dopamine release in healthy social drinkers (127, 203). In alcohol dependent patients, a blunted dopamine response to drug administration and reduced availability of D2-like receptors has been reported (204-210). This decrease in [^{11}C]-raclopride binding has collectively been interpreted as a decrease in D2-like receptors and low mesolimbic extracellular dopamine levels. However, the interpretation of *in vivo* PET data is ambiguous as decreases in PET signal can either be caused be decreased receptor availability or increased endogenous ligands (Figure 5). Endogenous ligands compete with the radiotracer for binding the receptor and, thus, can change the signal intensity. However, some studies report unchanged or even increased D2 receptor densities (211, 212). Interestingly, naltrexone reduces alcohol-induced striatal dopamine release (174, 175) which seems to be at odds with the importance of a hypodopaminergic state for relapse propensity.

Animal studies

Various studies established that alcohol consumption results in dopamine release in animals and human subjects that is associated with reward (13), while acute withdrawal from chronic

alcohol decreases dopamine neurotransmission (213-215). This is accompanied by reduced levels of tyrosine hydroxylase, the enzyme catalyzing the rate limiting step in the production of dopamine (213), an increase in reuptake of dopamine by the DAT and D2 autoreceptor supersensitivity (215).

Decreased activity of dopaminergic neurons originating within the VTA and the reduced accumbal dopamine release during withdrawal have led to the assumption that alcohol dependence is a reward deficit disorder (216-218). These findings are supported by human positron emission tomography studies (see above "*Human studies*").

In summary, the state of the dopamine system during acute withdrawal is well established showing decreased dopamine release. Although human PET studies also investigated changes in this system during abstinence, the interpretation of the available data is challenging. Additionally, the state of the dopamine system during (protracted) alcohol abstinence remains unclear.

1.4 THE DOPAMINE AND OPIOID SYSTEM INTERACT TO MEDIATE REWARD

The mesolimbic dopamine and endogenous opioid system interact to mediate rewarding effects. Alcohol enhances dopamine release in the Acb via a GABAergic feedback mechanism to the VTA. Alcohol increases the firing rates of accumbal GABAergic neurons innervating the VTA by activating MORs. This results in inhibition of tonically activated GABAergic neurons within the VTA which normally hold dopaminergic neurons of the mesolimbic pathway under inhibitory control. Dopaminergic neurons projecting to the Acb are thereby disinhibited resulting in increased dopamine release in the Acb after alcohol administration (219, 220). Additionally, DORs in the Acb and VTA increase striatal dopamine release and KORs located in the Acb inhibit dopamine release (220-222). This feedback mechanism is described in Figure 8.

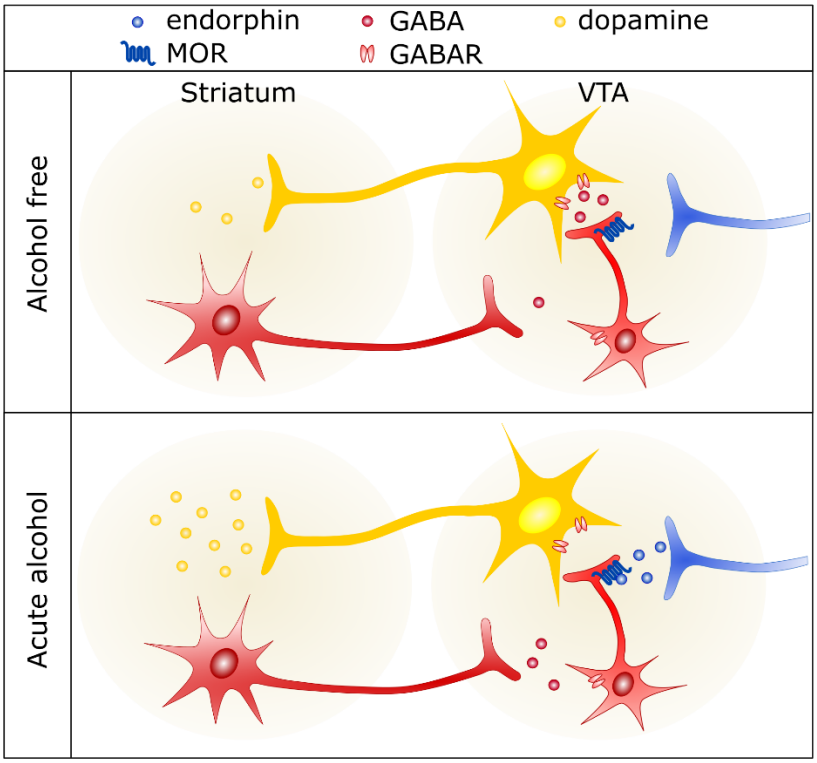

Figure 8: Feedback loop between the nucleus accumbens (Acb) and ventral tegmental area (VTA). In an alcohol-free state, small GABAergic interneurons (red) keep the dopaminergic neurons (yellow) of the mesolimbic pathway under inhibitory control. With alcohol present, the firing rate of accumbal GABAergic neurons projecting innervating the VTA is increased, thereby inhibiting the small interneurons within the VTA. Additionally, endogenous opioids, i.e. β-endorphin, are released which are further decreasing neuronal excitability of the small GABAergic neurons by activating the MOR. This causes disinhibition of dopaminergic projection neurons and increased accumbal dopamine release. Adapted from (223).

In the late 1970's and early 1980's, also endorphins and enkephalins have been proposed to possess intrinsic rewarding properties (224-226) as endogenous opioids as well as other MOR and DOR agonists such as Damgo and DPDPE are self-administered into the Acb and VTA by laboratory animals (227, 228). KOR agonists, however, produce aversive effects (229). The endogenous opioid system is thought to exert its rewarding effects, at least in part, by modulating the mesolimbic dopamine system (220, 222, 230). However, only in 2001 Olive et al. (231) finally demonstrated that drugs of abuse, including alcohol, trigger rewarding effects by releasing endogenous opioids.

Additionally, several studies suggest the existence of a reverse regulation of the endogenous opioid system by dopamine. Stimulation of dopamine D1 receptors appears to activate the dynorphin system whereas D2 receptors hold the enkephalin system under inhibitory control

(232). Furthermore, dopamine-induced release of β-endorphin has been observed in the Acb by *in vivo* microdialysis experiments and was blocked by dopamine antagonist pre-treatment (233).

Even though many studies investigated alcohol dependence-induced alterations in the brain, the focus was set on time points during acute or chronic alcohol administration or alcohol withdrawal. Studies on neuroadaptive changes during protracted alcohol abstinence are less frequent. One aim of this thesis is to fill these gaps.

1.5 THE POST-DEPENDENT ANIMAL MODEL

Animal models of psychiatric disorders, including alcohol dependence, are usually evaluated by their face, predictive and construct validity (234). *Face validity* describes the similarity of the model to the actual disease symptoms in humans. *Predictive validity*, refers to the model's ability to accurately respond to (pharmacological) treatments, meaning a treatment that has been found to be useful in human alcoholics has a comparable effect in the animal model. *Construct validity* can be described as the similarity between mechanisms underlying the behavior of the model and the actual disease.

To date, various animal models for alcohol dependence have been established which can be divided into two main classes. The first category depends on alcohol-induced neuroadaptations after chronic drug exposure while the second class is based on genetically encoded alcohol preference. In this thesis a model of the first category, the post-dependent animals, has been used to study long-lasting neuroadaptations in protracted abstinence.

Alcohol dependence develops through the repeated exposure to and withdrawal from the drug resulting in increased withdrawal severity. To mimic these processes, the chronic intermittent exposure to alcohol vapor (CIE) is a well-established tool (Figure 9). It is a reliable and easily controllable method to increase brain alcohol to relevant levels. Alcohol vapor is inhaled by the animals for 14 to 16 hours/day over several weeks or months. Consequently, animals show excessive voluntary alcohol intake, compulsive behavior with loss of control, increased tolerance to the drug, and hypersensitivity to stress (235). This phenotype, comprising long-lasting neuroadaptations that remain even during long periods of abstinence, was named the "post-dependent state" (14).

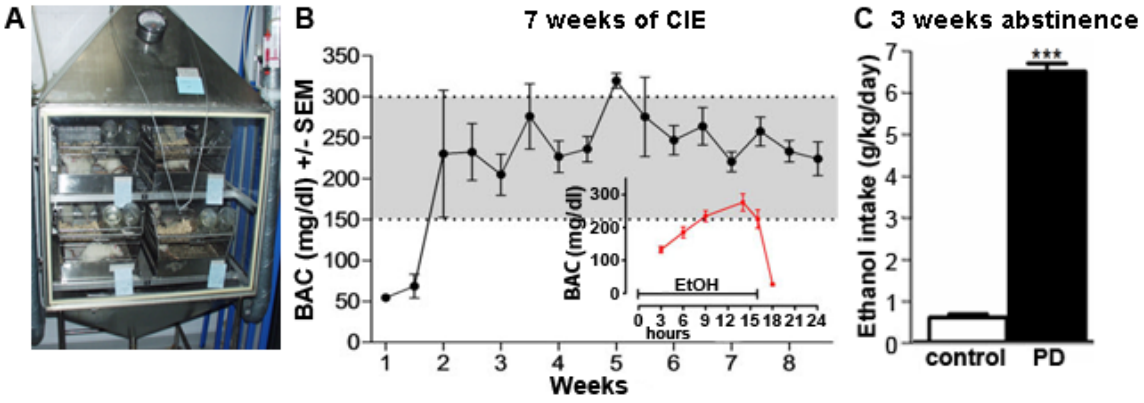

Figure 9: The post-dependent animal model. (**A**) Rats are housed in their home-cages in the exposure chambers and are exposed to chronic intermittent cycles of alcohol vapor (CIE) or air (controls) for 7 weeks. (**B**) Ideally, blood alcohol concentrations (BAC) rise daily to levels of 150-250 mg/dl. The inlet shows the time course of blood alcohol levels over one day of alcohol exposure. During the 16 hours of alcohol vapor exposure levels increase continuously. The remaining hours, animals are exposed to air and blood alcohol concentrations decrease rapidly. This procedure results in the post-dependent phenotype. (**C**) Following three weeks of abstinence, the post-dependent rats consume high amounts of alcohol in a two-bottle fee choice paradigm. Adapted from (235).

The *construct validity* of the alcohol-vapor induced post-dependent state has been shown for different brain neurotransmitter systems and regions. For example, similar dynamic changes in brain glutamate levels during withdrawal and abstinence have been observed in humans and post-dependent rats (236). Additionally, increased activity of the amygdala in response to stress has been shown in post-dependent animals (237, 238) as well as human alcoholics (239). Concerning the *face validity* of this animal model, there has been some criticism as the alcohol administration is forced onto the animals and the mode of intake differs from human alcoholics. However, the consequences of dependence are the major subject of studies and are more important than how dependence develops. The disease symptoms in post-dependent animals correspond well to the human situation and fulfill several criteria of the DSM-IV/-5, such as withdrawal signs, tolerance, loss of control and an increase in voluntary alcohol consumption. Most importantly, the post-dependent animal model shows high *predictive validity* and allows for studies aiming at the development of new medications. For example, the administration of the opioid receptor antagonists naltrexone and nalmefene, both of which are clinically approved anti-relapse medications, produces a decrease in alcohol consumption in post-dependent animals. In addition, acamprosate has been shown to reduce alcohol intake in post-dependent rats but did not affect non-dependent drinking (240). Established medication effects in post-dependent animals were recently reviewed in (235).

In this thesis, the term “post-dependent” will be used to describe animals where alcohol dependence was induced by seven weeks of CIE.

2 AIMS

Lack of knowledge:

- Theories on the state of the endogenous opioid and dopamine system during alcohol abstinence are based on human PET studies, e.g. using [^{11}C]-carfentanil, investigating patients in long-term abstinence. However, the interpretation of PET data is very challenging as results are not only influenced by the density of the receptor itself but also by the availability of the endogenous ligand. Additionally, the impact of the *OPRM1* SNP A118G on MOR density levels has not conclusively been established.

- Many studies focused on alcohol-induced neuroadaptations in MOR system that is thought to be the target of the anti-relapse medications naltrexone and nalmefene. However, the nature of these neuroadaptations during alcohol abstinence and their underlying mechanisms remain unclear as the focus of preclinical alcohol research was set on the acute effects of the drug and withdrawal after induction of alcohol dependence.

- The state of the endogenous opioid system, i.e. the DOR/enkephalin system, in protracted abstinence is so far not clear, even though it may also be a target of NTX and nalmefene treatment.

- The regulation of the dopamine system and its state during protracted abstinence is not completely resolved, although a hypodopaminergic state has been observed during acute withdrawal. However, this appears to contradict the observation that NTX reduces alcohol-induced dopamine release in dependence.

The knowledge on the state of the endogenous opioid and dopamine system during protracted abstinence is crucial for the development of new and the improvement of existing pharmacotherapies for relapse prevention. Furthermore, it may help to establish personalized treatments as many researchers and physicians are demanding for (184, 241).

The following aims were defined for this thesis:

Aim 1:

To demonstrate the state of the endogenous opioid system in alcohol dependence

Aim 2:

To study the effects of chronic naltrexone on the endogenous opioid system in protracted alcohol abstinence

Aim 3:

To demonstrate neuroadaptations in the dopamine system during acute alcohol withdrawal and protracted abstinence

List of studies:

Study I:

Low μ-opioid receptor status in alcohol dependence assessed by combined PET and post-mortem brain analysis (**Aim 1**)

Study II:

Neuroadaptations in the endogenous opioid system in protracted abstinence (**Aim 1**)

Study III:

Impact of chronic naltrexone on the endogenous opioid system in alcohol dependence (**Aim 2**)

Study IV:

Convergent evidence from alcohol dependent humans and rats for a hyperdopaminergic state during abstinence (**Aim 3**)

3 MATERIALS AND METHODS

3.1 HUMAN STUDIES

3.1.1 HUMAN POST-MORTEM TISSUE

Microdissected frozen brain tissue samples of alcoholic and healthy control subjects were obtained from the New South Wales Tissue Resource Centre (NSW TRC) at the University of Sydney, Australia (http://www.neura.edu.au/sydneybrainbank). Brain regions included in the experiments were the nucleus caudatus (NC) and the ventral striatum including the Acb (VS). Control subjects (n=43) consumed less than 20 g of alcohol per day while alcoholics (n=43) were mostly drinking more than 80 g of alcohol. Subjects assigned to the "alcohol abuse/dependence" group were diagnosed using the Diagnostic Instrument for Brain Studies – Revised (DIBS-R) which is consistent with the criteria of the Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV). Out of 43 alcoholic subjects, 16 displayed blood alcohol levels of 0.195 ± 0.132 g / 100 ml ("intoxicated") while the remaining 27 subjects were "not intoxicated". Smokers and ex-smokers were combined in one group while non-smokers and "unknown" were also pooled, as to include only the two variables "smoker" or "non-smoker" in the statistical analysis. Detailed information on subjects such as age, toxicology, tissue pH, post-mortem interval, is shown in Suppl. Table 1.

Additionally, a smaller and more homogenous sample set (here referred to as "core sample set") was selected consisting of age-matched alcoholic and control subjects. All subjects have a post-mortem interval <40 hours, brain tissue pH~6, no positive blood alcohol levels and no significant amounts of substances known to affect the expression of neurotransmitter systems (e.g. opioids, benzodiazepines, concentrations <0.1 mg/L).

3.1.2 POSITRON EMISSION TOMOGRAPHY (PET) STUDY IN ALCOHOL-DEPENDENT PATIENTS

The PET study to assess MOR availability ([^{11}C]-carfentanil binding potential BP_{ND}) in alcohol-dependent patients was conducted by Derik Hermann (Department of Addictive Behavior and Addiction Medicine, Central Institute of Mental Health Mannheim, Medical Faculty Mannheim, Heidelberg University, Germany), Matthias Reimold (Department of Nuclear Medicine, University of Tübingen, Germany) and colleagues and data are kindly

provided for this thesis. Because these data are part of a combined manuscript and helpful for interpretation, they are included in this thesis.

Participants

Forty alcohol-dependent patients from the Central Institute of Mental Health Mannheim, Germany, and the University of Tübingen, Germany, were included in this study. All patients were treated according to a protocol similar to the PREDICT study protocol (37) and the time until first heavy relapse (defined as 5 or more drinks per day for men or 4 or more drinks per day for women) was set as primary outcome. Inclusion criteria for the PET study were age (18-65 years), current DSM-IV/ICD 10 diagnosis of alcohol dependence, minimum of 14 drinks (females) or 21 drinks (males) per week over a consecutive 30-day period prior to detoxification, at least 2 weeks of inpatient detoxification, and an alcohol abstinence of 3-28 days. Exclusion criteria were other psychiatric diagnoses, psychotropic medication, positive drug urine screen test within the last 30 days, severe medical illness, pregnancy or lactating, and 5-fold elevated liver enzymes. Two patients did not meet criteria and were excluded from the statistical analysis due to considerably decreased striatal perfusion or lack of genetic information. Of the remaining 38 patients, 31 were carriers of the *OPRM1* genotype 118AA and seven 118AG. Clinical characteristics of participants are shown in Table 5.

Inpatients were treated for 2-3 weeks for withdrawal symptoms (if necessary) and with a psychosocial program to enhance motivation and abstinence. At the end of the inpatient treatment, the PET scan was performed in medication-free patients. One day afterwards, double-blind randomized naltrexone (50 mg, N=20) or placebo (N=18) treatment started for three months. This was accompanied by a simultaneous outpatient follow-up with biweekly medical management sessions for six months and a final visit after one year.

All participants signed an informed consent statement that had been approved by the Ethics Committee of the Mannheim Medical Faculty of the University of Heidelberg.

PET scan and image reconstruction

The patient's head was placed on an elastic mould and fixed with adhesive tape inside the PET scanner (GE Advance PET scanner, GE-Medical Systems, Milwaukee, USA). Three fiducial markers were attached to the scull to allow for support correction of head movements.

After intravenous bolus injection of a maximum of 19 mCi (700MBq) [^{11}C]-carfentanil, the cerebral distribution of radioactivity was measured over 72 minutes (2-dimension acquisition mode). A transmission scan with 500 000 kilo counts was used for attenuation correction and filtered back-projection (128 x 128 pixels = 30 cm) with a Hanning filter (cutoff, 4.6 mm) was employed to reconstruct attenuation corrected images.

Characteristic	**Alcohol-dependent patients (N = 38)**
Sex (female/male)	11 / 27
Age (years)	46 ± 7
OPRM1 genotype A118G	31 AA / 7 AG
Married	37 %
OCDS	15 ± 6
OCDS thoughts	5 ± 3
OCDS behavior	10 ± 4
Alcohol dependence scale (ADS)	16 ± 6
AUDIT	26 ± 7
Age of onset alcohol dependence (years)	32 ± 10
Genotype A118G	31 AA / 7 AG
Drinking days in the last 90 days (N)	73 ± 23
Drinks (12g) per drinking day (N)	17 ± 10
Gamma-glutamyl transferase (U/l)	128 ± 160
Alanine transaminase (U/l)	44 ± 27
Volume of erythrocytes (MCV in fL)	95 ± 5
Depressiveness (BDI)	8 ± 5
Anxiety (STAI State)	44 ± 8
Current smokers/ex-smoker/never	30 / 2 / 6
ICD-10 criteria alc.-dep. (N; max 6)	5.1 ± 1.1
DSM-IV criteria alc.-dep. (N; max 7)	5.9 ± 1.2

Table 5: Clinical characteristics of alcohol-dependent participants of the PET study. OCDS: Obsessive Compulsive Drinking Scale, ADS: Alcohol dependence scale AUDIT: Alcohol Use Disorders Identification Test, BDI: Beck Depression Inventory, STAI: State trait anxiety inventory

Image Analysis

The availability of the MOR, as defined as [^{11}C]-carfentanil binding potential BP_{ND}, was assessed from time activity curves from three striatal regions of interest (ROI). These three-dimensional ROIs, predefined in MNI space, were placed on PET images after correction for

head movement, stereotactic normalization with SPM5 and spatial smoothing (8 mm full width at half maximum). ROI positions were adjusted with the aid of ROI outlines being projected on early (0-5 min after injection) and late (30-72 min after injection) summation images without changing their size and shape in order to adjust for anatomic variations.

For pharmacokinetic analysis, the multi-linear reference tissue method (242) was used that is algebraically identical to the widely used Logan method (243) but exhibiting a lower noise dependent bias (242). The occipital cortex was chosen as reference tissue as MOR density is negligible in this region. Reference tissue washout of 0.1 min^{-1} and a pre-equilibrium interval of t*=18 min was used as parameters. The resulting BP_{ND} usually is interpreted in terms of $BP_{ND} = f_{ND} \times B_{max}/KD$, with f_{ND} being the free fraction of tracer in the first tissue compartment; B_{max}, the concentration of available binding sites (MOR); and K_D, the equilibrium dissociation constant (244).

3.1.3 GENOTYPING FOR *OPRM1* A118G (RS1799971)

Post-mortem samples

Genomic DNA was isolated from tissue samples using the QIAam DNA micro kit (Qiagen, USA) and the *OPRM1* A118G single nucleotide polymorphism was detected by TaqMan® SNP Genotyping Assay (C_8950074_1; Applied Biosystems, Carlsbad, USA) on an ABI 7900 HT RT-PCR system with SDS 2.2.2 software (10 µl reaction volume containing 10 ng genomic DNA, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min).

PET study participants

Genomic DNA from whole blood was isolated. Genotyping was performed by primer extension reaction, using matrix-assisted, laser-desorption/-ionization, time-of-flight mass-spectrometry (iPLEX Assay, Sequenom, San Diego, USA) and with a 7900HT Fast Real-Time PCR System (C_8950074_1; Applied Biosystems, Carlsbad, USA).

3.2 ANIMALS

3.2.1 CHRONIC INTERMITTENT ALCOHOL VAPOR EXPOSURE (CIE) - POST-DEPENDENT ANIMAL MODEL

Male Wistar rats obtained from Charles River were group-housed (four animals per cage) under a 12 hours light/dark cycle with *ad libitum* access to water and food. Animals were exposed to alcohol vapor for 16 hours per day followed by normal air for 5 days a week to achieve blood alcohol levels of 150-250 mg/dl. This CIE lasted seven weeks and has been shown to induce alcohol dependence including increased voluntary alcohol consumption, long-lasting neuronal and behavioral adaptations (173, 235, 240). Animals of the control group were kept under similar conditions breathing air. Except for the time course experiment, animals were sacrificed three weeks after the last alcohol exposure. All animals for the opioid system studies were sacrificed during their active cycle (3–5 hours after light off, **Study II** and **III**) but during their inactive phase for the time course experiment (2–3 hours after light on, **Study IV**, see 3.2.2). After decapitation, brains were snap frozen in -40 °C isopentane and stored at -80 °C until further processing.

3.2.2 ANALYSIS OF DIFFERENT TIME POINTS DURING ABSTINENCE IN POST-DEPENDENT RATS

For the time course analysis (**Study IV**), animals were subjected to CIE for seven weeks as described above and either sacrificed immediately after the last exposure (day 0), one day (day 1), three (day 3) or seven days (day 7) or three weeks later (day 21). On day 0, the animals still displayed positive blood alcohol values of 273 ± 52 mg/dl. Animals were kept under a 12 hours light /dark cycle and were killed at the same Zeitgeber time (2–3 hours after light on).

3.2.3 NALTREXONE EFFECTS IN POST-DEPENDENT RATS

To investigate the effects of chronic alcohol treatment on receptor and ligand expression, rats were exposed to CIE (“exposed”) or air (“not exposed”) for seven weeks as described above and kept abstinent for three weeks. Afterwards, the alcohol exposed as well as control animals received one daily naltrexone (2.5 mg/kg) or saline injections (i.p., 1.5 hours after light off) for 14 days. Three to four hours after the last injection, animals were sacrificed. The experimental design is visualized in Figure 20.

3.3 TISSUE PREPARATION (CRYOSECTIONS) OF HUMAN AND RAT TISSUE

Transcript levels, receptor/transporter densities, and G-protein coupling of specific receptors were analyzed by *in situ* hybridization, receptor autoradiography, and [^{35}S]-GTPγS assay techniques, respectively, on cryosections of human as well as rat brain tissue. For this, cryosections were prepared using a cryostat (Leica CM1950) and mounted onto glass slides. For receptor autoradiographies and [^{35}S]-GTPγS assays, glass slides were coated with gelatin beforehand.

Cryosections of human microdissected tissue samples of the caudate nucleus (NC) and the ventral striatum including the Acb (VS) were cut at a thickness of 10µm. For quality control each section was stained with a Nissl staining (Cresyl Violet) for morphological analysis after performing the quantitative measurements by receptor autoradiography as described below. For the Nissl staining, the sections were incubated in 4% paraformaldehyde for 15 min, washed in PBS and water for 2 min, followed by incubation in 0.1 % Cresyl Violet for 20 min. Sections were briefly washed in water and dehydrated in increasing ethanol concentrations (70%, 80%, 99% EtOH) and xylene, coverslipped and analyzed under light microscope. Overall, the morphology was intact in most cases, with some minor extent of artifacts. Based on this and reports on remarkable stability of proteins in post-mortem brain tissue (245), no samples were excluded for quantitative analyses.

Coronal cryosections of rat brains (12µm) were collected at Bregma levels (i) 1.2 to 0.7mm (striatum) and (ii) -5.2 to -5.6mm (midbrain) according to the brain atlas "The Rat Brain in Stereotaxic Coordinates" (246).

3.4 EXPRESSION ANALYSES – MRNA QUANTIFICATION IN HUMAN AND RAT TISSUE

3.4.1 QUANTITATIVE REAL-TIME PCR FOR RNA FROM HUMAN POST-MORTEM TISSUE

Quantitative real-time PCR (qRT-PCR) was used to analyze mRNA expression in human post-mortem brain tissue samples. After isolation (RNeasy Micro Kit), 100 ng total RNA were reverse transcribed using the High Capacity RNA-to-cDNA Master Mix. Quantitative RT-PCR was perform on triplicates of each sample using the Power SYBR®Green PCR Master Mix (reaction volume 20 µl, 40 cycles of 95 °C for 15 sec, 60 °C for 1 min). Melting profiles of each sample were recorded to check for aberrant fragment amplifications. Primer details are listed in Table 6.

Gene	RefSeq	Forward	Reverse
OPRM1	NM_000914.4	5‘-AGAGACCACCCCTCCACGGC-3‘	5‘-ACCCTGTTAGGGCAACGGAGCA-3‘
DRD1	NM_000794.3	5‘-ACGACCCCAAGGCAAGGCGT-3‘	5‘-TCGGGGCTGTTGCTTTTCTGGT-3‘
DRD2	NM_016574.3	5‘-CAGACGCCGCAAGCGAGTCA-3‘	5‘-TCCTCTCGGGTGGGCTGGTG-3‘
AluSx	---	5‘-TGGTGAAACCCCGTCTCTACTAA-3‘	5‘-CCTCAGCCTCCCGAGTAGCT-3‘
GAPDH	NM_002046.4	5‘-CATGAGAAGTATGACAACAGCCT-3‘	5‘-AGTCCTTCCACGATACCAAAGT-3‘

Table 6: List of primers used for qRT-PCR in human post-mortem tissue.

SDS 2.2.2 software (ABI) was employed to analyze SYBR®Green fluorescence intensity and calculation of the theoretical cycle number when a defined threshold was reached (Ct-value). Relative quantification was done by the ΔΔCT-method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal normalizer. AluSx (173) was tested as internal normalizer in a smaller sample set (n = 10/group) but was not used for subsequent experiments as results were similar to GAPDH.

To ensure good RNA quality, RNA integrity number (RIN) values were recorded and samples with RIN values <7 and tissue pH<6 were excluded from qRT-PCR experiments in the analysis of the complete sample set (N=43/group).

3.4.2 IN SITU HYBRIDIZATION ON RAT BRAIN SECTIONS

Fixation

For fixation, sections were warmed to room temperature and incubated in 4 % PFA in PBS for 15 min, washed for 10 min in PBS, and twice in sterile water for 5 min. After treatment with 0.1 M HCl for 10 min and two times 5 min with PBS, brain sections were incubated in 0.1 M triethanolamine (pH 8) and 0.25 % acetic anhydride for 20 min in order to acetylate proteins. Subsequently, sections were washed twice in PBS for 5min, once in sterile water for 1 min and dehydrated in a graded series of ethanol (70 %, 80 %, 99 %; 2 min each). After air drying, sections were stored at -80 °C in sealed boxes with silica gel to avoid moisture.

Probe generation

Gene-specific riboprobes are described in Table 7 and were generated by PCR. Product size was checked by 1.5 % agarose gel electrophoresis. Radioactively-labelled riboprobes were generated by *in vitro* transcription. For this, 200 ng unlabeled riboprobe was incubated with

1x transcription buffer, 12.5 nmol ATP, CTP, GTP, 50 pmol UTP, and 125 pmol Uridine 5-(α-thio)triphosphate-[^{35}S] (Perkin Elmer, Massachusetts, USA), 1 U RNase inhibitor and 1 U polymerase for 90 to 120 min at 37 °C. Afterwards, the DNA template was digested by DNAseI (20 min, 37 °C) and riboprobes were purified using IllustraTM MicrospinTM S-200 HR Columns.

Gene	RefSeq	Position (bp) from - to		Reference
Oprm1	NM_013071	1226	1479	(247)
Oprd1	NM_012617	148	569	(247)
Oprk1	NM_017167	1298	1555	(247)
Pomc	NM_139326	11	344	-
Penk	NM_017139	1086	779	(247)
Pdyn	NM_019374	586	991	(247)
Arbb2	NM_012911.1	1238	1679	(97)
Th	NM_012740	1594	1843	(248)

Table 7: Gene-specific RNA riboprobes used for *in situ* hybridizations on rat brain sections.

Probe hybridization and washing

Fixed tissue sections were incubated in prehybridization buffer (100 mM Tris-HCl, pH 7.6, 5 mM EDTA, 5x Denhardt's solution, 1.25mg/ml yeast tRNA, 40 mM NaCl) diluted 1:1 with deionized formamide for 2 to 4 hours at 37 °C followed by incubation with hybridization mix containing 10 000 CPM / µl at 55 °C over night. The hybridization mix consisted of 50 % deionized formamide, 150 mM DTT, 330 mM NaCl, and 10 % dextran sulfate, 1x basic mix (10x basic mix: 200 mM Tris-HCl, pH 7.6, 10 mM EDTA, 10 x Denhardt's solution, 5 mg/ml yeast tRNA, 1 mg/ml polyadenylic acid). Sections were washed once for 40 min followed by two washing steps for 30 min in 1x SSC at 42 °C. If necessary, sections were incubated in formamide (1:1 diluted with 1x SSC) for 1 hour followed by two times 1x SSC. RNase treatment (2mg / 100 ml RNase buffer) was carried out at 37 °C for 1 hour. Enzyme reaction was stopped by washing the sections in 1x SSC at 55 °C twice for 30 min. Sections were dipped in water for 2 min and dehydrated in a graded series of ethanol (70 %, 80 %, 99 %; 2 min each). Fujifilm BAS imaging plates were exposed to the sections for 1 week.

3.5 EXPRESSION ANALYSES - PROTEIN QUANTIFICATION IN HUMAN AND RAT TISSUE

3.5.1 RECEPTOR AUTORADIOGRAPHIES ON HUMAN AND RAT BRAIN TISSUE SECTIONS

All receptor/transporter autoradiographies were performed under saturated conditions. K_d-values, the dissociation equilibrium constant describing the affinity for a specific receptor, as well as B_{max}-values, describing the maximum density of the receptor, are listed in Table 8.

	Rat brain		**Human brain**	
	K_d [nM]	**B_{max} [fmol/mg]**	**K_d [nM]**	**B_{max} [fmol/mg]**
[^{3}H]-Damgo	0.7 ± 0.1 (249)	10.3 ± 1.8 (249)	1.6 ± 0.3 (250)	37 ± 5 (250)
[^{3}H]-DPDPE	1.8 ± 0.6 (251)	2.2 ± 0.2 (251)	2.72 ± 0.21 (251)	20.78 ± 3.13 (251)
[^{3}H]-U-69,593	3.8 ± 0.2 (252)	12.6 ± 0.8 (252)	3.94 (253)	1.5 (253)
[^{3}H]-raclopride	2.08 (254)	20.0 (254)	1.25 (255)	~9.5 (255)
[^{3}H]-SCH23390	0.7 (256)	347 (256)	1.37 (255)	~13 (255)
[^{3}H]-mazindol	18.2 (257)	0.0073 (257)	18.5 (258)	1.6 (258)

Table 8: K_d and B_{max} values of radioligands used for saturated receptor autoradiographies on human and rat brain tissue sections.

MOR autoradiography

Sections were preincubated in 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, and 1 mM EDTA twice for 15 min. Afterwards, incubation buffer containing 1 nM or 8 nM [^{3}H]-Damgo (Damgo, [Tyrosyl-3,5-^{3}H(N)]-, spec. activity 50-51 Ci/mmol, Perkin Elmer, Massachusetts, USA) for rat and human tissue, respectively, was applied onto sections and incubated for 2 hours at 30 °C. Incubation buffer consisted of 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM Bacitracin, and 0.1 % bovine serum albumin. For measuring non-specific binding on adjacent sections, 1µM CTOP (Tocris, Bristol, UK) was added. Sections were washed three times for 2 min at 4 °C in 50mM Tris-HCl, pH 7.4, dipped in ice-cold water and dried in a cold air stream.

DOR autoradiography

Sections were washed in 50 mM Tris-HCl, pH 7.4, for 30 min followed by incubation in 8 nM of [^{3}H]-DPDPE (Enkephalin, [Tyrosyl-2,6-^{3}H(N)]- (2-D-Penicillamine,5-D-Penicillamine, Perkin Elmer, Massachusetts), spec. activity 51.3 Ci/mmol) for 1 hour at 4 °C. Non-specific binding was determined in presence of 1µM naltrindole (Sigma-Aldrich, Missouri, USA).

Sections were then twice washed in washing buffer for 30 sec at 4 °C, dipped in ice cold water and air dried.

KOR autoradiography

For KOR, the same buffers as for MOR receptor autoradiography were used. Sections were preincubated twice for 20 min, followed by incubation in 10 nM [^{3}H]-U69,593 (spec. activity 44.6 Ci/mmol, Perkin Elmer, Massachusetts) for 1 hour at room temperature. Non-specific binding was determined in presence of 1 µM nor-BNI (Tocris, Bristol, UK). Washing was done as for MOR receptor autoradiography.

D1 and D2/D3 receptor autoradiography

Sections were pretreated in 50 mM Tris, pH 7.4, 5 mM MgCl2, and 1 mM EDTA two times for 15 min, followed by incubation with the radioligand in incubation buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% bovine serum albumin). Total binding of the D1 receptor was determined using 3 nM [^{3}H]-SCH23390 (SCH23390, [N-Methyl-3H]-, spec. activity 81.9 Ci/mmol, Perkin Elmer, Massachusetts) and 10 µM Mianserin (Tocris, Bristol, UK) in human tissue. Non-specific binding was achieved by adding 10 µM flupenthixol (Tocris, Bristol, UK). For rat tissue, 1nM [^{3}H]-SCH23390 with (non-specific) or without (total) 10 µM SKF were used. To determine the density of D2/D3 binding sites, 5 nM [^{3}H]-raclopride (total binding, spec. acitivity 74.4 Ci/mmol, Perkin Elmer, Massachusetts) and 30 µM sulpiride (non-specific, Tocris, Bristol, UK) were used. After 2 h incubation at 30 °C, the sections were twice washed in 50 mM Tris-HCl, pH 7.4, dip in cold water and dried in a cold air stream.

DAT autoradiography

Sections were preincubated in 50 mM Tris-HCl, pH 7.9, 300 mM NaCl, and 5 mM KCl for 5 min at 4 °C. Total binding was determined by adding 2 nM or 4 nM [^{3}H]-mazindol (spec. activity 20.7 Ci/mmol, Perkin Elmer, Massachusetts) and 0.3 µM desipramine (Tocris, Bristol, UK) for human and rat tissue, respectively. For non-specific binding, 100 µM nomifensine (Tocris, Bristol, UK) were added. Incubation took place at 4 °C for 40 min, followed by two times 1 min in ice-cold buffer and dipping in ice-cold water.

3.5.2 G-PROTEIN COUPLING OF OPIOID RECEPTORS ASSESSED BY [^{35}S]-GTPγS ASSAYS

MOR [^{35}S]-GTPγS assay

Sections were washed in 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 1 mM EDTA twice for 15 min and then pretreated in incubation buffer containing 1 mM GDP. Incubation buffer contained 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 0.1 % bovine serum albumin. G-protein coupling of MOR was determined by adding 10 mM GDP, 80 pM [^{35}S]-GTPγS (Perkin Elmer, Massachusetts) and the MOR specific agonist Damgo (1µM, Tocris, Bristol, UK). Basal G-protein coupling was measured in absence of Damgo but presence of the vehicle (acetonitrile). Incubation took place at 30 °C for 1 hour. Sections were then washed in 20 mM Tris-HCl and 100 mM NaCl two times for 2 min, rinsed in ice-cold water and air dried.

DOR and KOR [^{35}S]-GTPγS assay

Sections were rinsed in preparation buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl) for 10 min followed by incubation in preparation buffer containing 1 mM GDP for 20 min. The sections were then incubated for 2 hours at room temperature in preparation buffer with 1 mM GDP, 40 pM [^{35}S]-GTPγS and either DOR (10 µM DPDPE, Sigma-Aldrich, Missouri, USA) or KOR (10 µM U50,488H, Tocris, Bristol, UK) specific agonist or vehicle (acetonitrile or water). Afterwards, sections were washed twice in 50 mM Tris-HCl, pH 7.4, at 4 °C, dipped in ice cold water and air dried.

3.5.3 AUTORADIOGRAPHIC IMAGE ANALYSIS

After performing *in situ* hybridizations, receptor bindings or [^{35}S]-GTPγS autoradiograhpies on tissue sections, Fujifilm BAS imaging plates (Fujifilm, Tokyo, Japan) were exposed to the sections. The plates were then scanned with a phosphoimager (Typhoon FLA 700, GE Healthcare, Germany). Mean grey values were measured using the MCID software (MCID Image Analysis Software Solutions for Life Sciences). For *in situ* hybridization experiments, a sense probe was generated to measure unspecific binding that was subtracted from antisense signals. In [^{35}S]-GTPγS assay studies, basal and stimulated (in presence of a specific agonist) was measured on adjacent sections and the percentage of stimulated after agonist application was calculated for every sample. Total and non-specific binding (in presence of a specific

blocker) were determined for receptor binding assays on adjacent sections and the non-specific signal was subtracted from the total signal. Based on the known radioactivity in ^{14}C standards, image values of *in situ* hybridization and [^{35}S]-GTPγS assay measurements were converted to nanocurie per mg (nCi/mg). Values of measurements of the autoradiographies were converted to femtomol per mg (fmol/mg) based on ^{3}H standard values and the specific activity of the tritiated ligand. These values were used for statistical analysis.

3.6 NEUROTRANSMITTER MEASUREMENTS

3.6.1 DOPAMINE *IN VIVO* MICRODIALYSIS

This experiment was performed by Dr. Marcus Meinhardt and Dr. Stéphanie Perreau-Lenz (Institute of Psychopharmacology, Central Institute of Mental Health Mannheim, Medical Faculty Mannheim, Heidelberg University, Germany) and results are part of a combined manuscript. The results are presented here to support the interpretation of my data.

Surgery

Post-dependent and control rats (450-550g) were group housed before surgery and single housed afterwards. Animals were anesthetized with 1.5 – 2 % isofluran and placed in a stereotaxic frame (Kopf Instruments, California, USA). Guide cannulas (CMA11, 20 gauge, 14mm, CMA Microdialysis, Kista, Sweden) were implanted 2.0 mm above the Acb shell region unilaterally at the coordinates anterior/posterior + 1.6 mm, medial/lateral ± 0.8 mm, and dorsal/ventral 5.6 mm based on Bregma, midline, and dura (246). The implant was anchored using stainless steel screws and dental acrylic. After surgery, animals were allowed to recover for one week.

In vivo microdialysis procedure

The microdialysis experiment was conducted in freely moving rats on day 21 after the last alcohol-exposure. The dialysis probes (CMA11 11/2, 2 mm active membrane) were inserted into the implanted guide cannula 12 hours before starting the experiment to minimize damage-induced release of neurotransmitters and metabolites. Samples were then collected every 15 min at a flow rate of 1.5 µl/min. In order to measure baseline dopamine levels in

control and post-dependent animals, six baseline samples were collected. Afterwards, the animals were injected with saline (i.p.) as a control. This was followed by consecutive injections of ethanol (15 % v/v in water): 1 g/kg ethanol 30 min after the saline injection and 2 g/kg ethanol 60 min after the first ethanol injection. Sampling continued for the whole experiment. Placement of the dialysis probes was verified after the experiment and the location of at least 80 % of the active membrane within the AcbS was the inclusion criterion for this study.

High pressure liquid chromatography (HPLC) analysis

HPLC was used to determine the dopamine content in the microdialysis samples. An ALEXIS 100 cooled-micro LC-EC system (Antec Leyden, Zoeterwoude, Netherlands) with a microbore VT-03 flow cell was employed for electrochemical detection. The working potential of the cell was set at 400 mV and the oven temperature of the DECADE II at 35°C. The mobile phase (pH 6, containing 50 mM phosphoric acid, 400 mg/l OSA, 0.1 mM EDTA, 8 mM KCl, 15% methanol) and was perfused with a flow rate of 200 µl/min. Duplicates of each sample (4 µl aliquots) were injected onto a reversed phase column (C18, ALF-205 column, 50x2, 1 mm ID, 3 µm; Axel Semrau GmbH & Co. KG, Sprockhövel, Germany). Using the area under the peak and an external standard curve, the dopamine content was determined. Detection limits for dopamine was 200 pM with a signal-to-noise ratio of 2.

3.6.2 RADIOMMUNIASSAY

Brains of post-dependent and control rats were microdissected and micropunches of the Acb, CPu, and VTA were prepared. These tissue samples were sent to Uppsala, Sweden, where they were further processed in the laboratory of Prof. Dr. Georgy Bakalkin (Department of Pharmaceutical Biosciences, Uppsala Universitet, Sweden).

Frozen tissue of microdissected brain regions (Acb, CPu, VTA) was finely powdered and 1M hot acetic acid was added. The samples were boiled for 5 min, ultrasonicated, and centrifuged. The tissue extracts were run through a SP-Sephadex ion exchange C-25 column and peptides were eluted and analyzed by RIA. The samples were then incubated with ^{125}I-labeled peptide and the primary antiserum over night at 4 °C. This was followed by 10 min centrifugation at

12 000 g. The pellet was then used for counting on a gamma counter. The method is described in detail elsewhere (153, 155, 259).

3.7 LOCOMOTOR ACTIVITY

Locomotor activity of control and post-dependent animals was measured in their homecage and in an OpenField experiment. These data were kindly provided by Dr. Marcus Meinhardt.

Homecage locomotion

For homecage locomotion, rats were single house 24 h prior to the experiment and an infrared sensor (Infra E-Motion GmbH, Henstadt-Ulzburg, Germany) was placed on top of the homecage. Body movements of every animal were monitored for 72 h starting on day 17 after the last alcohol exposure.

OpenField

OpenField locomotion was analyzed on day 25 after the last alcohol-exposure in an arena made of dark PVC (51 cm x 51 cm x 50 cm) at a light intensity of 50 lx. Over a period of 60 min, the distance traveled (cm) was recorded and the locomotor activity was analyzed with the observation program Viewer2 (Bioserve GmbH, Bonn, Germany).

3.8 ELECTROPHYSIOLOGY

Electrophysiological data were provided by the research group of Dr. Georg Köhr (Institute of Psychopharmacology, Central Institute of Mental Health Mannheim, Medical Faculty Mannheim, Heidelberg University, Germany) to give further insight into the functional consequences of the post-dependent state on the synaptic level. The results are part of a combined manuscript and support the interpretation of the data.

Patch-clamp whole-cell recordings

Coronal rat brain slices (300 µm) containing the AcbS were prepared using the HM 650 V microtome (Microm International, Walldorf, Germany) from four control and three post-

dependent rats at the age of 15-16 weeks. Rats were anesthetized by inhalation of isofluorane and brains were rapidly removed and placed in dissection buffer (220 mM sucrose, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM dextrose) at 4 °C. Individual slices were stored in artificial cerebrospinal fluid (ACSF) for at least 1.5 hours before recording. ACSF was similar to the dissection buffer except that sucrose was replaced by 124 mM NaCl, $MgCl_2$ and $CaCl_2$ were changed to 1.5 mM and 2.5 mM, respectively. Both dissection buffer and ACSF were saturated with 95 % O_2 / 5 % CO_2 (pH 7.4).

For the recordings, slices were transferred to a submerged recording chamber, perfused with ACSF at 2 ml/min and imaged using a Zeiss Axioskop 2 microscope (Carl Zeiss AG, Göttingen, Germany). Whole-cell recordings were performed at 30°C from medium spiny neurons (MSNs) located in the AcbS with the EPC-9 amplifier interfaced to Patchmaster software (HEKA Elektronik, Lambrecht, Germany). Borosilicate recording pipettes (outside-diameter, 1.5 mm; 2-4 MΩ) were pulled on the Flaming/Brown puller P-97 (Sutter Instruments, Novato, CA) and were filled with internal solution containing 130 mM K-Gluconate, 10 mM KCl, 0.2 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 0.5 mM Na-GTP and 10 mM Na-Phosphocreatine (pH 7.25, 280–290 mOsm). For electrical stimulation, borosilicate glass pipettes filled with ACSF were placed in the AcbS to evoke excitatory postsynaptic currents (EPSCs) in MSNs around 200 pA at the holding potential (V_h) of -80 mV. GABAergic transmission was antagonized by picrotoxin (1 mM) which was added to the internal solution (260). Electrophysiological data were filtered at 2 kHz and digitized at 10 kHz. Input resistance was monitored via hyperpolarizing pulses (-10 mV, 100 ms). Only cells with holding currents ≤100 pA at V_h = -80 mV and series resistance ≤20 MOhm were studied. Cells were discarded if any of these parameters changed by ≥20 % during the course of the experiment.

3.9 STATISTICS

Expression data from human post-mortem brain studies (complete sample set)

To examine differences in qRT-PCR and autoradiography data between the control, non-intoxicated, and intoxicated alcoholic groups an analysis of covariance was performed. Tissue pH-values, post-mortem interval, age, smoker state and RIN values (but only for the analysis of qRT-PCR data) were considered as candidate covariates. By a stepwise backward selection

procedure non-significant variables were sequentially removed. This was followed by a Fisher's LSD post-hoc test to determine which means differed between groups. In an analogous way the interaction effect of alcoholic group and A118G genotype was analyzed by stepwise analysis of covariance with factors alcoholic group (control, non-intoxicated, and intoxicated alcoholics), A118G genotype (AA and AG) and their interaction.

Parametric Pearson's analysis was used to correlate MOR binding sites and *OPRM1* mRNA to PMI, brain pH and RIN in NC and VS of alcoholics and controls.

Expression data from human post-mortem brain (core sample set) and animal studies

Expression data from human post-mortem "core samples" as well as post-dependent animals were statistically analyzed within a region by one-way ANOVA (treatment effect) followed by Fisher's PLSD post-hoc test.

For the analysis of NTX effects on expression levels (Study III), data of NTX-treated rats were normalized to their respective saline control and compared by group- and region-wise one-way ANOVA, i.e. saline treated not exposed animals vs. NTX treated not exposed animals and saline treated exposed vs. NTX treated exposed animals. For better visualization, exposed and not exposed animal groups are shown in the same graphs.

Clinical and PET data

Relapse data were analyzed according to an intention-to-treat analysis. A Kaplan Meier estimation of naltrexone response was performed comparing survival curves of relapse in naltrexone versus placebo groups.

For the PET scans, [^{11}C]-carfentanil BP_{ND} derived from pharmacokinetic analysis of regions of interest (ROI; ventral striatum, nucleus caudatus, putamen, and total striatum) were statistically analyzed using a Cox regression analysis to analyze the relation between MOR BP_{ND} and time until first relapse. Data were optionally censored with end of follow-up, for each ROI and the analysis was performed with SPSS22 and/or JMP 7 (SAS Institute, Inc). To control for the covariates age, sex, medication, tobacco use or *OPRM1* genotype additional cox analyses were performed.

Microdialysis and locomotion data

Data from the microdialysis experiment were analyzed by two-way ANOVA with repeated measures followed by Fisher's PLSD post hoc test. Data of the locomotion experiments were evaluated by two-way ANOVA followed by Fisher's PLSD post-hoc test.

Electrophysiology – Patch-clam whole-cell recordings

Statistical analysis during perfusion of drugs was performed for the last 10 min of every condition using two-way ANOVA followed by Bonferroni's post-hoc test and comparisons between control and dependent rats were performed using unpaired Student's t test.

All data are expressed as mean ± SEM. Throughout statistical significance was defined at the 0.05 level. Statistical analysis was carried out using the Statistica 10 (StatSoft, Tulsa, USA) or SPSS22 (IBM, Chicago, USA). For graphical representation of the data the Prism 5 (GraphPad, San Diego, USA) software was used.

3.10 META-ANALYSES

All meta-analyses were performed by Dr. Dr. Hamid R. Noori (Institute of Psychopharmacology, Central Institute of Mental Health Mannheim, Medical Faculty Mannheim, Heidelberg University, Germany) and his group and are part of two combined manuscripts. To support the interpretation do the data, they were kindly provided for this thesis.

Meta-analysis: *Oprm1* mRNA expression and Damgo binding in alcohol-dependent rats

A systematic selection of original research articles was performed using the online portal of the National Library of Medicine http://www.ncbi.nlm.nih.gov/pubmed/. The literature search was based on the general keywords: μ opioid receptor, expression, chronic and alcohol/ethanol. Based on a simultaneous search of those keywords 28 publications were found. Of these 28 articles, 12 using 233 rats provided complete sets of biological (gender, age, weight and strain) and procedural (measurement method, number of animals, alcohol administration paradigm in terms of dose of alcohol, duration of exposure, and alcohol

availability, length of withdrawal period) variables as well as control and ethanol-induced expression and binding values. The latter were used to calculate the relative changes in *Oprm1* expression and/or receptor binding properties following chronic alcohol intake.

Interestingly, all studies under investigation utilized a two bottle free-choice paradigm to induce alcohol dependence in male adult animals, which was defined by physical withdrawal symptoms. The duration of alcohol administration varied between 16 days and 10 months. Following studies were used for the meta-analysis (110-120, 261).

For the meta-analysis, a fixed effect model was used (13, 262) with respect to the variables "OPRM1 expression" or "Damgo binding" and analysed the time-dependent effects of abstinence following chronic alcohol exposure.

$$\bar{x} = \frac{1}{N} \sum_{i=1}^{k} n_i x_i$$

was used as the weighted average effect of chronic alcohol intake or withdrawal as the weighted sum of the products of the mean effects x_i from each experiment *i* and the number of animals used in that particular study. n_i , whereby

$$N = \sum_{i=1}^{k} n_i$$

denotes the total number of animals considered in the meta-analysis of the *k* studies. Data are taken from following publications.

Meta-analysis: Dopamine and its metabolites in alcohol-dependent rats

For the meta-analysis on dynamics of dopamine release during withdrawal and abstinence, the literature search was based on the keywords "alcohol/ethanol" AND "withdrawal/abstinence" AND "dopamine" AND "accumbens" OR "striatum" OR "Ventral Tegmental Area/VTA". Further selection criteria were (i) chronic administration of only alcohol (no other pharmacological interventions) and (ii) the presence of withdrawal symptoms. From approximately 225 publications, 29 (including 352 rodents chronically exposed to alcohol and 96 alcoholic individuals) fulfilled the abovementioned criteria. The subsequent variables (i.-vii.) were obtained from the publications and used for further analysis:

i. Weight, age, gender and consciousness (if anaesthetics applied: agent and dose).

ii. Exact method of measurement (*in vivo* microdialysis, patch-clamp recordings, tissue HPLC, PET etc.)

iii. Alcohol administration paradigm (self-administration, free-choice, i.p. injections etc.) and daily doses of alcohol in animals; the history of alcohol dependence and average daily alcohol consumption in humans

iv. Number of the alcoholic individuals and ethanol-exposed animals used in each experiment.

v. Extracellular and *in situ* dopamine, DOPAC and HVA concentrations, firing frequency and burst rates of dopaminergic neurons, availability of D1 and D2 receptors and dopamine transporter (DAT).

vi. Time of measurement after alcohol withdrawal

vii. Relative change (percentage) of the obtained variable (v) in comparison to the controls.

Based on the same fixed effect model as for the meta-analysis of MOR expression were employed (13, 262) with respect to the extracted variables (v) and analyzed the withdrawal interval of [0, 60] days.

$$\bar{x} = \frac{1}{N} \sum_{i=1}^{k} n_i x_i$$

represents the weighted average effect of the concentrations of dopamine and its metabolites respectively as the weighted sum of the products of the mean effects x_i from each experiment i and the number of animals used in that particular study n_i, whereby

$$N = \sum_{i=1}^{k} n_i$$

denotes the total number of animals considered in the meta-analysis of the k studies. If the amount of extracellular dopamine was not directly specified by the measurement (e.g. tissue punches), the ratio of DOPAC to dopamine was calculated as an estimate for active dopamine concentrations.

4 RESULTS

4.1 **STUDY I**: LOW μ-OPIOID RECEPTOR STATUS IN ALCOHOL DEPENDENCE ASSESSED BY COMBINED PET AND POST-MORTEM BRAIN ANALYSIS

A major hypothesis in the addiction research field states increased levels of MOR in the striatal brain regions of human alcoholics during abstinence as measured by [^{11}C]-carfentanil PET. The elevated MORs are thought to be blocked by the non-selective opioid receptor antagonist NTX. However, NTX is only effective in a subpopulation of patients. Moreover, the most commonly used radiotracer [^{11}C]-carfentanil is especially sensitive to levels of endogenous opioids.

To give more insight into the state of the MOR system during abstinence, post-mortem tissue samples of human alcoholics and control subjects were analyzed. Additionally, a PET study was performed in a subgroup of alcoholic patients.

4.1.1 POST-MORTEM STUDY: MOR EXPRESSION IS DECREASED IN STRIATAL BRAIN REGIONS OF ALCOHOLICS

For expression analysis of *OPRM1* mRNA and MOR binding sites, striatal brain tissue of deceased alcoholic (n=43) and control subjects (n=43) was investigated. Detailed information on each subject is shown in Suppl. Table 1. The group of alcoholics was further divided into subjects with positive blood alcohol levels at the time of death ("intoxicated", n=16) and subjects without detectable blood alcohol levels ("non-intoxicated", n=27).

A significant decrease of MOR binding sites, as assessed by [^{3}H]-Damgo autoradiography, was observed in the caudate nucleus (NC) and the ventral striatum (VS) in both the intoxicated and non-intoxicated alcoholics when compared to control subjects (NC: $F[2,77]=13.83$; $p<0.001$; VS: $F[2,75]=12.6$, $p<0.001$, Figure 10A, C, D). MOR binding sites in the NC were reduced by ~30 % in both alcoholic groups. Notably, there seems to be an up-regulation of MOR with age in the NC ($p<0.001$, $\beta = 0.337 \pm 0.093$). In the VS, however, non-intoxicated alcoholics showed a decrease by 23 % while the reduction in binding sites was even more pronounced in intoxicated alcoholics (51 %, Figure 10A). There was a significant difference in binding sites between non-intoxicated and intoxicated alcoholics in the VS.

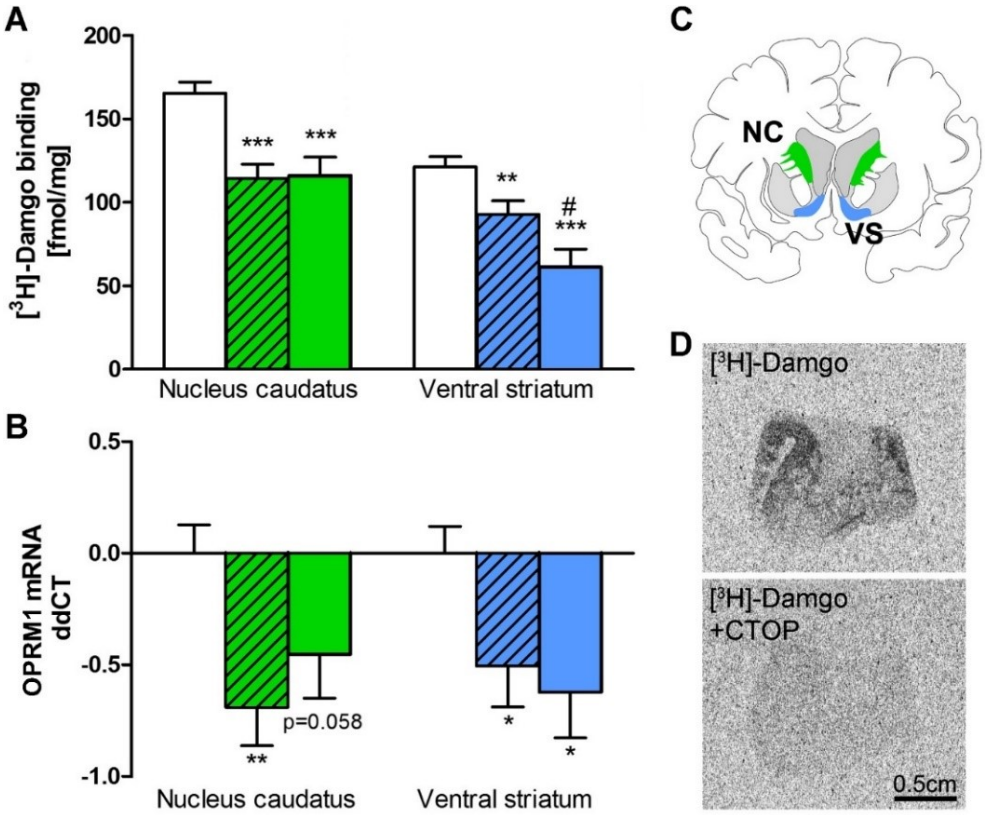

Figure 10: μ-opioid receptor (MOR) binding sites and *OPRM1* mRNA expression in the nucleus caudatus and ventral striatum of human striatal post-mortem tissue. Controls (white bars) are compared to non-intoxicated (lined and colored bars) and intoxicated (colored bars) alcoholic subjects. (**A-B**) Data show protein and mRNA expression of MOR on post-mortem brain sections measured by saturated [^{3}H]-Damgo receptor autoradiography (A) and qRT-PCR (B). Data are expressed as mean ± SEM (autoradiography n=14-40/group, *OPRM1* mRNA n=12-35/group). Statistical analysis was performed by analysis of covariance followed by Fisher's LSD post-hoc test, *p<0.05, **p<0.01, ***p<0.001 non-intoxicated/intoxicated alcoholics vs controls, #p<0.05 intoxicated alcoholics vs non-intoxicated alcoholics. (**C**) Schematic overview of the nucleus caudatus (NC) and ventral striatum (VS) on a coronal human brain section. (**D**) Representative [^{3}H]-Damgo receptor autoradiography on a frontal lobe section (upper panel) showing a specific pattern of MOR similar to Hiller and Fan (80) and Mathieu-Kia et al. (79). Non-specific signal was determined by [^{3}H]-Damgo binding in presence of the MOR antagonist CTOP (lower panel).

OPRM1 mRNA measured by qRT-PCR was also significantly reduced in both striatal brain regions of alcoholic subjects as compared to controls, mirroring the changes on binding site levels (NC: F[2,58]=5.65, p=0.006; VS: F[2,59]=4.76, p=0.012; Figure 10B). The decrease in transcripts of the intoxicated alcoholics in the NC did not reach significance but showed a trend towards a decrease (p=0.058) when compared to controls.

MOR expression can be affected by various confounding factors, i.e. nicotine use. For this reason, we included "smoking" into our statistical model. However, the statistical stepwise backward procedure excluded "smoking" as a non-significant co-variant.

Excluding additional confounding factors

In addition to nicotine use, various confounding factors can influence *OPRM1* mRNA and MOR binding site expression. For example, the single nucleotide polymorphism (SNP) *OPRM1*-A118G has been proposed to have an impact on receptor expression. Thus, a sensitivity analysis investigating a genotype x condition (controls, intoxicated, non-intoxicated alcoholics) interaction was performed including the A118G genotype but did not find a significant effect of the genotype on the expression levels of *OPRM1* mRNA or binding sites (Suppl. Table 2). However, number of G-allele carriers is low (n=4-13) and results have to be interpreted with caution.

The analyzed sample set of 43 controls and 43 alcoholics (Figure 10) also includes subjects which either committed suicide or were positive for substances known to alter MOR binding (i.e. benzodiazepines, opioids, cannabis) at the time of death. Therefore, these cases were excluded in an additional analysis resulting in group sizes of 30-38 controls and 21-31 alcoholics, depending on brain region and group. As reported above, lower MOR binding sites and transcript levels were detected in alcoholic subjects also in this sample set. In the NC ($F[2, 65]=11.892$, $p=0.00004$, controls: 166.08 ± 6.94, non-intoxicated: 119.01 ± 9.85, intoxicated: 110.86 ± 12.44), MOR binding sites were decreased in both alcoholic groups when compared to controls ($p<0.001$). In the VS ($F[2,64]=7.7430$, $p=0.001$, controls: 119.60 ± 6.40, non-intoxicated: 98.30 ± 9.29, intoxicated: 67.86 ± 11.89), there was only a trend towards a decrease in the not-intoxicated alcoholics ($p=0.06$) but a significant reduction in the intoxicated alcoholic subjects ($p=0.0002$ when compared to controls, $p=0.048$ when compared to non-intoxicated alcoholics).

Parametric Pearson's analysis was used to correlate MOR binding sites and *OPRM1* mRNA expression to PMI, brain pH and RIN values in the NC and VS of alcoholics and controls and no significant effects were found. This shows the decent quality of the post-mortem specimen.

Meta-analysis of MOR expression in rats

A meta-analysis in 233 rats to analyze striatal *Oprm1* mRNA and MOR binding sites during abstinence found decreased mRNA and receptor expression during the first three days of

abstinence (Table 9). This is in line with the human post-mortem data and further supports the existence of decreased MOR cell surface expression during alcohol abstinence

DAMGO binding (%)	Chronic alcohol effect	Abstinence day 1	Abstinence day 2	Abstinence day 3
Caudate putamen/Striatum	74 ± 4	73 ± 5	88 ± 7	73 ± 7
Nucleus accumbens core	84 ± 5	71 ± 10	79 ± 7	61 ± 7
Nucleus accumbens shell	86 ± 9	71 ± 10	61 ± 7	61 ± 7

Oprm1 mRNA (%)	Chronic alcohol effect	Abstinence day 3	Abstinence day 21
Caudate putamen/Striatum	80 ± 12	79 ± 10	138 ± 16
Nucleus accumbens	72 ± 10	-	-

Table 9: A meta-analysis found reduced mRNA levels especially during the first 3 days of alcohol withdrawal. A total of 233 rats were included in the meta-analysis. Performed by Dr. Dr. H.R. Noori.

4.1.2 [^{11}C]-CARFENATNIL PET STUDY: LOW BINDING POTENTIAL PREDICTS RELAPSE

The PET study was performed by Dr. Derik Herman (Department of Addictive Behavior and Addiction Medicine, Central Institute of Mental Health Mannheim, Medical Faculty Mannheim, Heidelberg University, Germany), Matthias Reimold and colleagues.

After three weeks of abstinence, a [^{11}C]-carfentanil PET scan was performed in medication-free alcohol dependent patients (n=38). One day afterwards, patients were assigned to a randomized placebo-controlled NTX treatment (n=20 NTX, n=18 placebo) for 90 days and were followed-up for 1 year. There were no significant differences in the abstinence rates between both groups neither after 90 days nor after 1 year (90 days: NTX 40 % vs. placebo 55 %; 1 year: NTX 30 % vs. placebo 17 %; log rank test p=0.8, chi square=0.07, df=1, Kaplan-Meier estimates are provided in Figure 11) allowing to further use all n=38 patients for the analysis of MOR BP_{ND} and time to relapse.

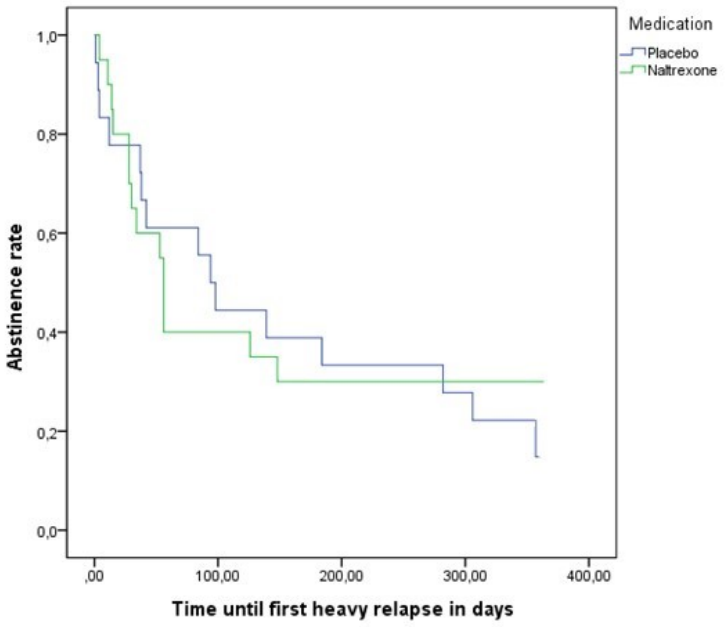

Figure 11: PET study – no significant differences in abstinence rates were observed between the NTX (blue) and placebo (green) group. Alcohol-dependent patients were treated with NTX (n=20) and placebo (n=18) for 90 days and relapse rates monitored over one year. Six of 20 NTX treated and three of 18 placebo treated patients remained abstinent over one year. This analysis was performed by Dr. D. Hermann, Dr. M. Reimold, and colleagues.

A cox regression analysis revealed an association of low MOR BP_{ND} in the putamen and an increased relapse risk during the 1 year follow-up (p=0.04, B= -2.05). In the ventral and total striatum a trend towards the same association was detected (ventral striatum: p=0.09, B= -1.15); total striatum: p=0.058, B= -1.7).

	MOR BP_{ND} and relapse risk (N=38)	
	B	P
Nucleus caudatus	-1.38	0.107
Putamen	-2.05	**0.040**
Ventral striatum	-1.15	0.093
Total striatum	-1.70	0.058

Table 10: PET study – a Cox regression found an association of low MOR BP_{ND} and with increased relapse risk in the putamen. This analysis was performed by Dr. D. Hermann, Dr. M. Reimold and colleagues.

Excluding confounding factors

Even though NTX treatment was ineffective in reducing relapse rates, medication effects cannot completely be excluded due to small sample size. However, an additional cox regression analysis of MOR BP_{ND} and relapse controlling for NTX/placebo treatment showed no influence of the medication (p=0.96) while the association of low MOR BP_{ND} in the putamen and increased relapse risk was replicated (p=0.04). Additionally, age, sex, tobacco use and the A118G genotype can influence MOR (132, 263). Therefore, additional cox regression analyses were performed controlling for these variable. They did not have a significant effect on BP_{ND} and the risk of relapse in all ROIs (Suppl. Table 3).

4.1.3 SUMMARY

Taken together, **Study I** shows a strong decreased of MOR binding sites in striatal post-mortem brain tissue of alcoholic subjects which appears to be independent of the *OPRM1* A118G genotype. Furthermore, the results suggest that patients with low MOR availability are more vulnerable to relapse.

4.2 **STUDY II:** NEUROADAPTATIONS IN THE ENDOGENOUS OPIOID SYSTEM IN PROTRACTED ABSTINENCE

4.2.1 MOR

Based on the findings in human post-mortem tissue, i.e. the strong down-regulation of MOR binding sites in alcoholic subjects, this thesis aimed to investigate the nature of the endogenous opioid system's regulation in alcohol dependence. Thus, the studies were extended to the established post-dependent animal model.

Alcohol dependence was induced in rats by seven weeks of CIE and brains were analyzed after three weeks of abstinence. Alcohol dependence-induced alterations of opioid receptor expression were studied on transcript, protein as well as functional levels (i.e. G-protein coupling) in striatal regions and the VTA. Furthermore, expression levels of the β-endorphin precursor *Pomc* provided evidence for a dysregulation of the endogenous MOR ligand. Transcripts of the adaptor protein β-arrestin2 were analyzed to suggest a possible mechanism of the regulation of MOR cell surface receptor availability.

In three weeks abstinent post-dependent animals, MOR binding sites as measured by [^{3}H]-Damgo receptor autoradiography were significantly reduced by about 10 % in both subregions of the ventral striatum, the AcbS and AcbC, as compared to controls. In the dorsal striatum as well as in the VTA no alterations have been detected (Figure 12A, E and Figure 13A). Accumulation of [^{35}S]-GTPγS, representing coupling of the receptor to intracellular G-proteins, was significantly upregulated by 67 % and 248 % in the AcbS and AcbC, respectively, while the dorsal striatum was unaffected. In the VTA, G-protein coupling was reduced by 13 % (Figure 12B, F). *Oprm1* transcript levels as measured by specific riboprobe *in situ* hybridization were not changed (Figure 12C, G). Statistical values are shown in Suppl. Table 4, Suppl. Table 5, Suppl. Table 6.

Transcript levels of the β-endorphin precursor *Pomc* were analyzed within the median eminence (ME, Figure 12D) and *Pomc* mRNA levels were significantly decreased by 17 % in post-dependent animals as compared to controls. F- and p-values are shown in Suppl. Table 7.

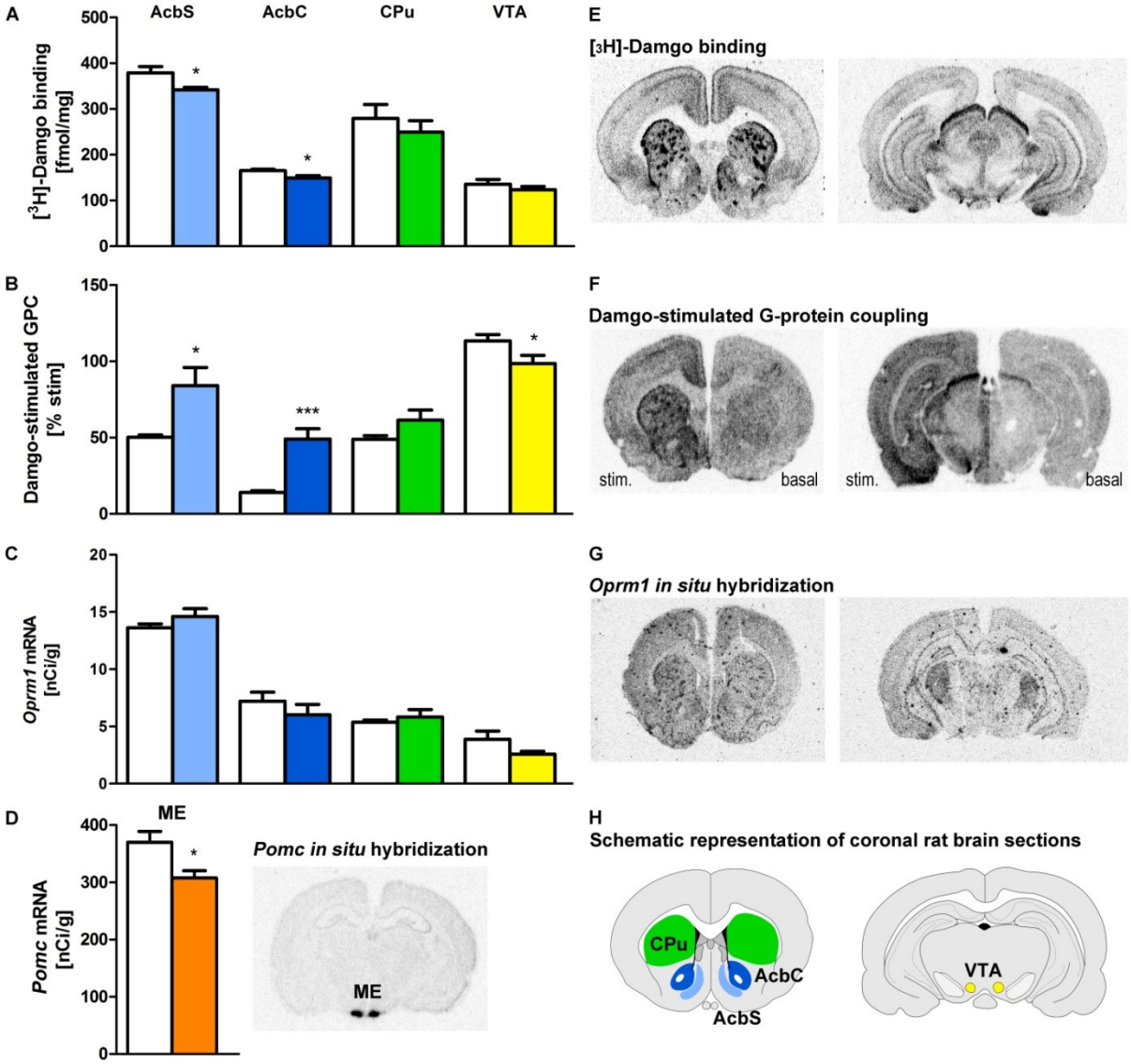

Figure 12: MOR binding sites are decreased in the ventral striatum but G-protein coupling is increased. (**A**) MOR binding sited were measured by [^{3}H]-Damgo receptor autoradiography in the ventral striatum (AcbS – light blue, AcbC – dark blue), dorsal striatum (CPu – green), and ventral tegmental area (VTA – yellow) of post-dependent animals as compared to controls (white). G-protein coupling of the MOR (**B**) and *Oprm1* mRNA levels (**C**) were analyzed in the same regions. MOR binding sites as well as G-protein coupling is changed in the ventral striatum while the dorsal striatum is unaffected by the induction of alcohol-dependence. (**D**) *Pomc* mRNA, the precursor of the MOR ligand β-endorphin, was measured by *in situ* hybridization in the median eminence (ME, orange) and its specific expression pattern is displayed. The reduction of *Pomc* suggests a decrease in the endogenous opioid β-endorphin. The specific distribution of MOR binding sites (**E**), [^{35}S]-GTPγS accumulation in presence/absence Damgo stimulation (**F**), and *Oprm1* mRNA (**G**) in the striatum (left image) and midbrain (right image) is shown in representative autoradiograms. (**H**) Schematic illustration of coronal rat brain sections with regions analyzed according to (246).

4.2.1.1 ELEVATED B-ARRESTIN2 LEVELS

Since the adaptor protein β-arrestin2 is known to influence MOR function and trafficking and has been shown to be involved in the rewarding effects of alcohol (98, 101), *bArr2* mRNA levels were studied by *in situ* hybridization. Transcripts of *bArr2* were specifically in regions with decreased MOR binding sites (Figure 13A, B). In the AcbS and AcbC, *bArr2* mRNA was upregulated by 52 % and 54 %, respectively (Statistics are shown in Suppl. Table 8).

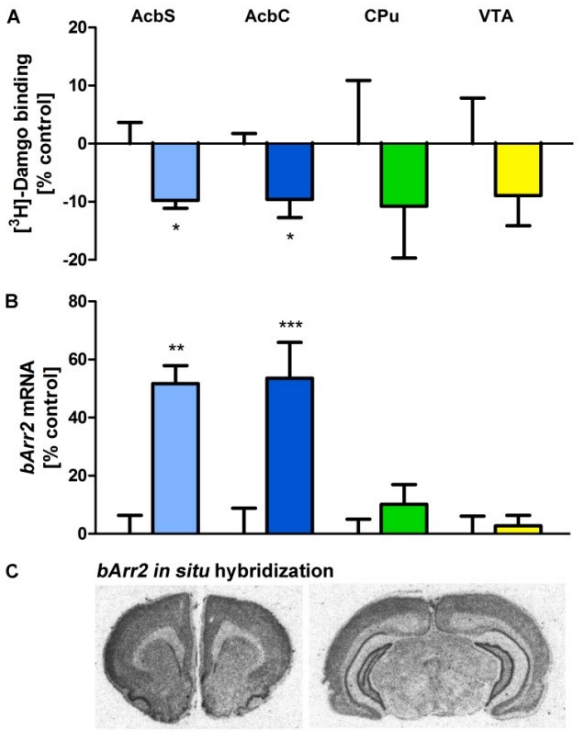

Figure 13: *bArr2* transcript levels are significantly increased in regions with decreased MOR binding sites. (**A**) MOR bindings sites were measured by [^{3}H]-Damgo receptor autoradiography and are here represented as normalized data as compared to air exposed control animals (% control ± SEM). Data in fmol receptor/mg tissue are displayed in Figure 12A. (**B**) *bArr2* mRNA levels were measured by *in situ* hybridization and are presented as % control ± SEM. Levels are strongly increased in the AcbS and AcbC of post-dependent animals. In the same region, a significant reduction of MOR was detected indicating a role of β-arrestin2 in MOR regulation. (**C**) Representative autoradiograms of the expression pattern of *bArr2* mRNA in striatal and midbrain regions.

4.2.1.2 SUMMARY: MOR IN ALCOHOL DEPENDENCE

In post-dependent animals, MOR binding sites were significantly reduced mirroring the situation in human post-mortem tissue. G-protein coupling was increased. The increased *bArr2* mRNA levels in regions with decreased MOR indicates a role in receptor regulation. *Pomc* transcript levels, the precursor of β-endorphin, were significantly decreased.

4.2.2 DOR

Even though the importance of the DOR for alcohol intake behavior and dependence has been established, the state of the DOR during protracted alcohol abstinence is relatively understudied. Several preclinical reports proof the ability of DOR antagonists to reduce alcohol consumption and increased DOR activity is hypothesized to contribute to alcohol dependence. To broaden our knowledge on the role of DOR during alcohol abstinence, this study analyzed this receptor in both human post-mortem tissue and post-dependent animals.

The RIA analysis of endogenous opioids was performed by the lab of Dr. Bakalkin in Sweden.

4.2.2.1 DOR HUMAN POST-MORTEM BRAIN TISSUE

Measurement of DOR binding sites in human striatal post-mortem tissue by [^{3}H]-DPDPE receptor autoradiography revealed increased DOR binding sites by 57 % in the ventral striatum (VS) of alcoholic subjects as compared to controls (one-way ANOVA: F[1,17]=0.045, p=4.69, Figure 14A, n=9-10/group). In the NC, DOR availability was numerically increased. However, this effect did not reach significance (one-way ANOVA: F[1,17], p=0.463, Figure 14A, n=9-10/group).

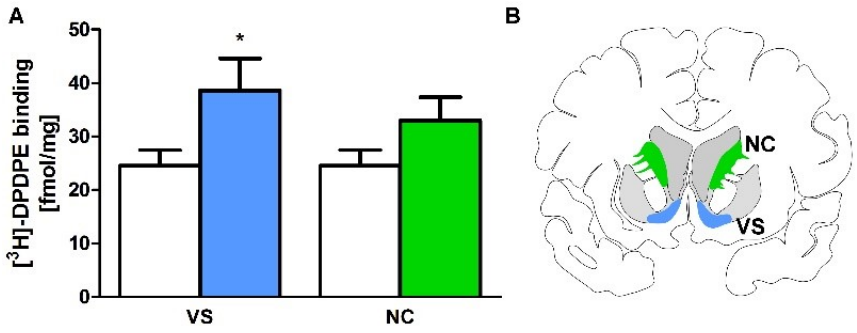

Figure 14: DOR binding sites are increased in the ventral striatum of human alcoholic subjects. (**A**) Analysis of DOR binding sites by [^{3}H]-DPDPE receptor autoradiography in the ventral striatum (VS, blue) and nucleus caudatus (NC, green). (**B**) Schematic representation of a coronal section of the human brain. The ventral striatum (VS, blue) and nucleus caudatus (NC, green) are highlighted. Statistical analysis was performed by one-way ANOVA and data are shown as mean ± SEM.

4.2.2.2 DOR IN POST-DEPENDENT RATS

In post-dependent rats, DOR binding sites were increased in the striatum as well as in the VTA as compared to control animals. In the AcbS, there was a trend towards an increase (p=0.06). In the AcbC and CPu, binding sites were significantly upregulated by 36 % and 14 %, respectively. An increase of 34 % was detected in the VTA of post-dependent rats (Figure 14A, Suppl. Table 9). Receptor functionality, as analyzed by [^{35}S]-GTPγS accumulation representing G-protein coupling of the receptor, was significantly decreased in all striatal brain regions by 60 % to 64 % but increased in the VTA by 90 % (Figure 14B, Suppl. Table 10). Transcript levels of *Oprd1* were non-significantly decreased in the ventral striatum (AcbS, AcbC). In the dorsal striatum (CPu) and VTA, however, Oprd1 mRNA was significantly reduced by 29 % and increased by 26%, respectively (Figure 14C, Suppl. Table 11).

Messenger RNA levels of the enkephalin precursor *Penk*, were increased in the AcbS (by 26 %) and AcbC (by 24 %). In the CPu, *Penk* mRNA levels were unaltered and decreased by 21% within the VTA (Figure 14, Suppl. Table 12). The changes in *Penk* transcripts, however, do not appear to translate into protein since no changes in Met-enkephalin-Arg-Phe (MEAP) levels were detected by a radioimmunoassay (Acb: $F[1,11]=2.912$, $p=0.116$; CPu: $F[1,10]=3.680$, $p=0.084$; VTA: $F[1,13]=0.104$, $p=0.752$, Figure 16). Peptide levels of Leu-enkephalin-Arg (Arg6-Leu), which is actually a marker for *Pdyn* expression and the dynorphin system but binds to DOR and MOR (155), was increased in the Acb of post-dependent rats ($F[1,13]=8.072$, $p=0.014$) but not in the CPu or VTA (CPu: $F[1,14]=0.421$, $p=0.527$; VTA: $F[1,14]=0.293$, $p=0.597$).

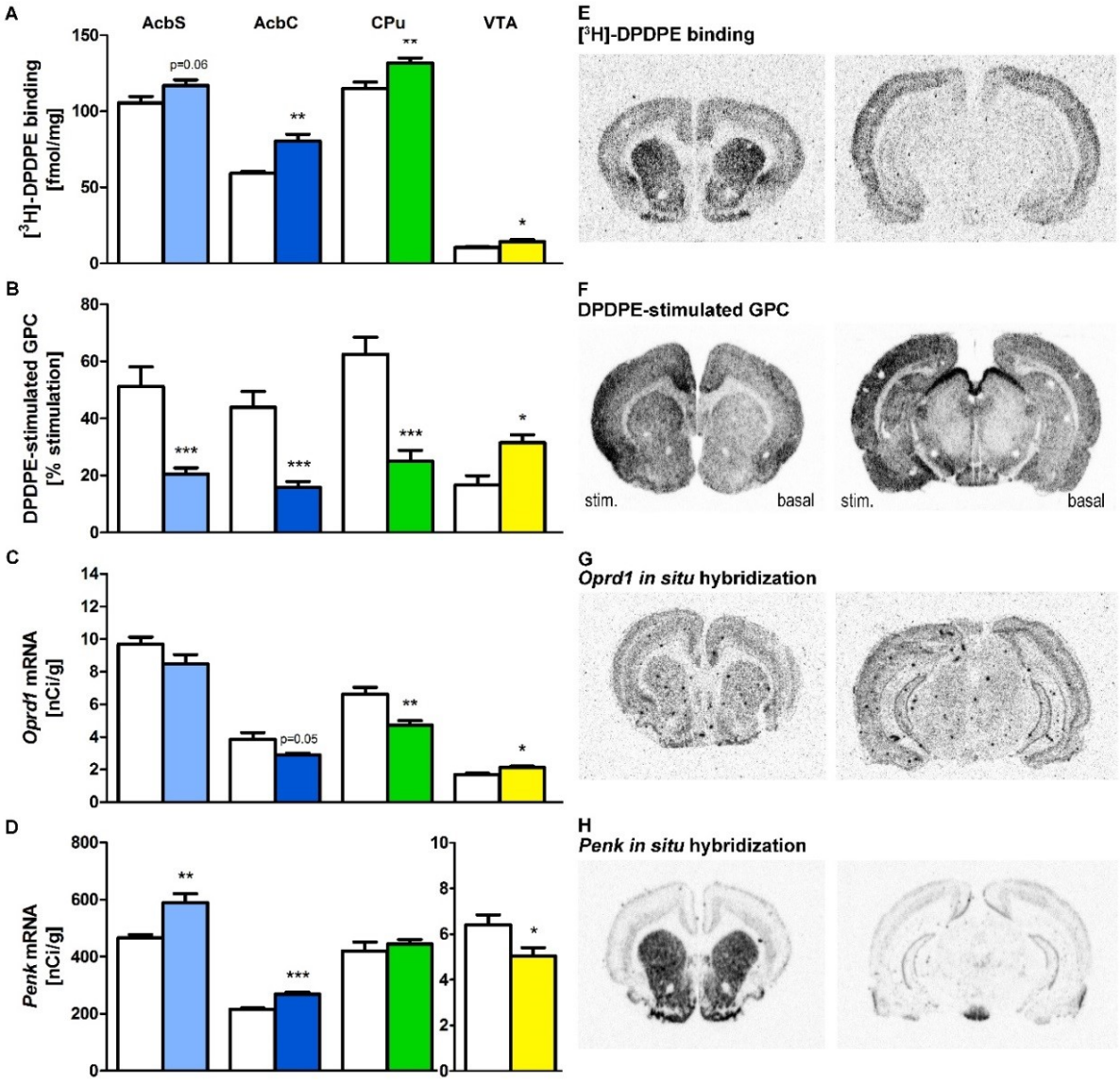

Figure 15: DOR binding sites are increased in the striatum of post-dependent rats but G-protein coupling is significantly decreased. Bar graphs show DOR binding sites analyzed by [^{3}H]-DPDPE receptor autoradiography (**A**), DPDPE-stimulated G-protein coupling (**B**), *Oprd1* (**C**) and *Penk* (**D**) mRNA in post-dependent animals in the AcbS (light blue), AcbC (dark blue), CPu (green) and VTA (yellow) as compared to control animals (white). DOR Binding sites are increased in the striatum of post-dependent rats while G-protein coupling of the receptor is decreased. Within the VTA binding sites as well as G-protein coupling is increased. (**E-H**) Representative autoradiograms on coronal striatal and midbrain sections.

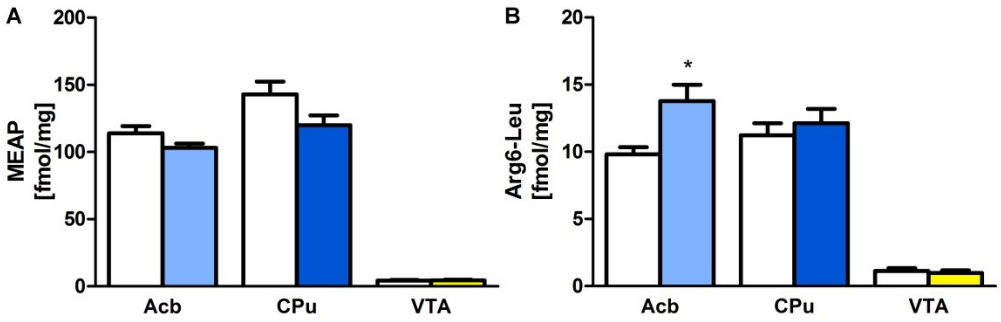

Figure 16: Radioimmunoassay for Met-enkephalin (**A**, MEAP) and Leu-Enkephalin (**B**, Arg6-Leu) in controls (white) post-dependent rats (colored). The RIA experiment was performed by the group of Dr. G. Bakalkin.

4.2.2.3 SUMMARY: DOR IN ALCOHOL DEPENDENCE

In human alcoholics and post-dependent animals, DOR binding sites were increased. DOR function is significantly decreased in the striatum of post-dependent rats. In the VTA, both DOR binding sites and G-protein coupling are increased. Peptide levels of the DOR-ligand Met-enkephalin were unchanged. Leu-enkephalin was increased only in the Acb. Please note, Leu-enkephalin is derived from *Pdyn* rather than *Penk*.

4.2.3 KOR

The KOR is known to mediate the negative, anhedonic effects of alcohol consumption. It is hypothesized that the KOR/dynorphin system is upregulated during alcohol dependence. So far, information on this system during protracted abstinence is missing.

4.2.3.1 KOR IN HUMAN POST-MORTEM TISSUE

KOR binding sites were analyzed in human striatal post-mortem tissue of alcoholic and control subjects by [^{3}H]-U69,593 binding that was specifically blocked in the presence of norbinaltorphimine (nor-BNI). In the VS, KOR availability was slightly increased. However, this was not significant (one-way ANOVA: $F[1,15]=2.1086$, $p=0.17$, n=8-9/group). In the NC, no differences have been observed ($F[1,13]=0.3573$, $p=0.56$, n=7-8/group) (Figure 17A).

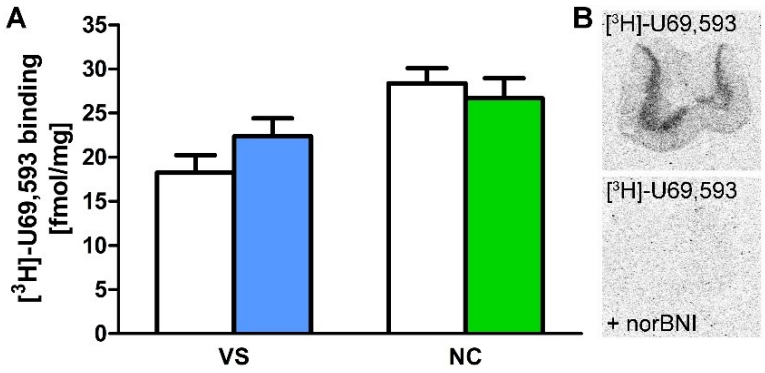

Figure 17: KOR binding sites a s measured by [^{3}H]-U69,593 autoradiography were measured on brain sections of the ventral striatum (VS) and caudate nucleus (NC) of human control (white bars) and alcoholic (colored bars) subjects. (**A**) KOR binding sites are numerically increased in the ventral striatum of human alcoholics; however, this is not significant. In the NC, binding sites are unchanged. Statistical analysis was performed by one-way ANOVA and data are shown as mean ± SEM. (**B**) Representative KOR autoradiography on a human frontal lobe brain section. When adding nor-BNI, a selective KOR antagonist, [^{3}H]-U69,593 binding is blocked proofing the specificity of the experimental approach.

4.2.3.2 KOR IN POST-DEPENDENT RATS

KOR binding sites were assessed in post-dependent and control rats by [^{3}H]-U69,593 receptor autoradiography. In the ventral as well as dorsal striatum, KOR availability was increased by 22 % – 37 % (Figure 18A, Suppl. Table 13). This was accompanied by an increase of [^{35}S]-GTPγS accumulation by 153 % in the AcbS of alcohol-dependent rats indicating increased signaling via the KOR in this region. No significant effects were detected in the other regions (Figure 18B, Suppl. Table 14). *Oprk1* transcripts were only changed in the CPu and increased by 11% (Figure 18C, Suppl. Table 15).

Transcript levels of the dynorphin A and B precursor *Pdyn* were unchanged in either region. These results were confirmed on the peptide level by a radioimmunoassay showing unaltered dynorphin A and dynorphin B levels in post-dependent rats when compared to control animals (Dynorphin A: Acb: $F[1,12]=0.065$, $p=0.854$; CPu: $F[1,14]=0.890$, $p=0.362$; VTA: $F[1,14]=0.216$, $p=0.649$; Dynorphin B: Acb: $F[1,14]=0.029$, $p=0.868$; CPu: $F[1,14]=1.175$, $p=0.297$; VTA: $F[1,14]=0.308$, $p=0.588$, Figure 19).

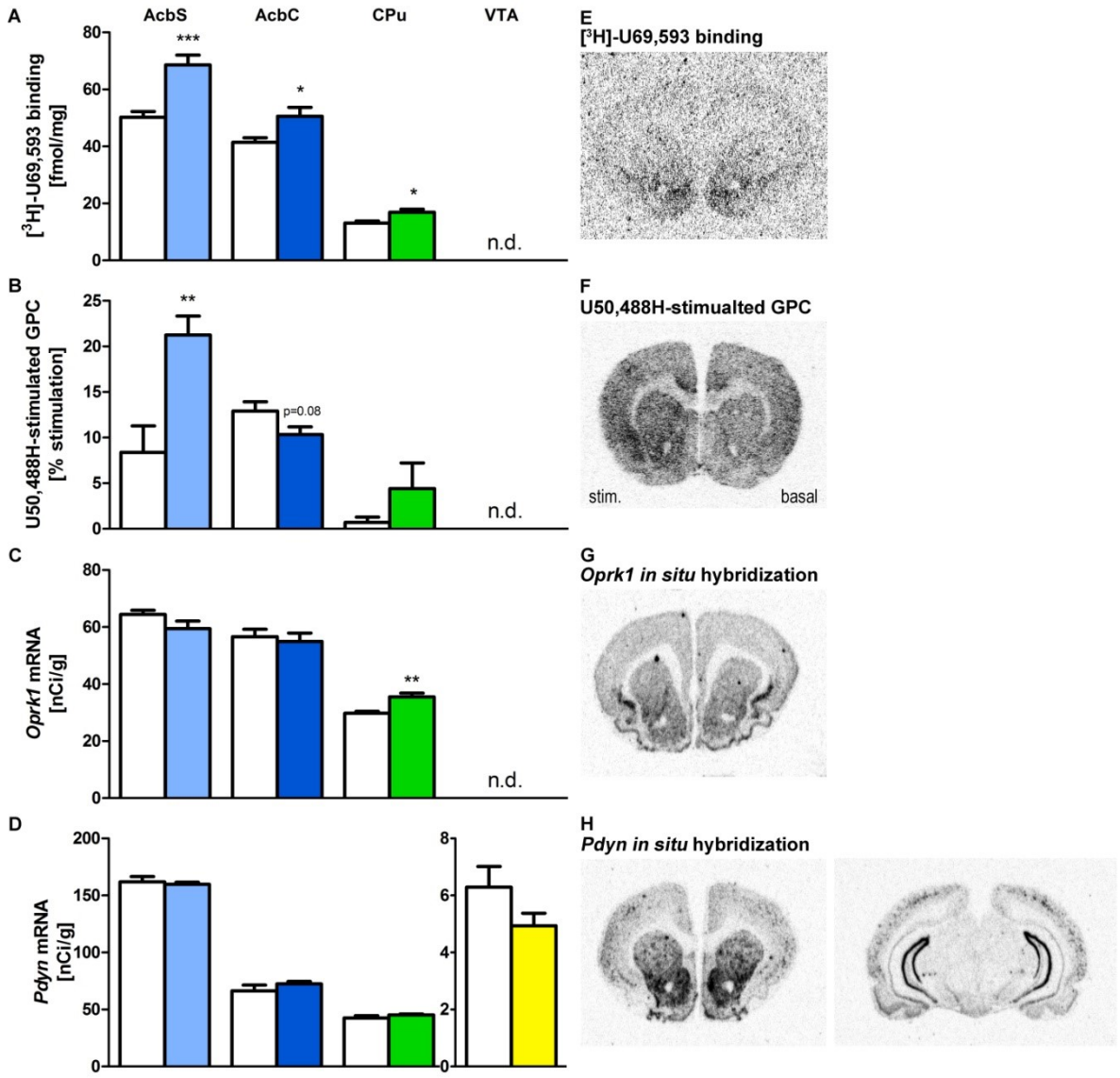

Figure 18: KOR binding sites are increased in the striatum of post-dependent rats and indicate increased signaling via this receptor. KOR binding sites measured by [^{3}H]-U60,593 receptor autoradiography (**A**), KOR G-protein coupling (**B**), *Oprk1* (**C**) and *Pdyn* (**D**) mRNA analyzed by *in situ* hybridization in the AcbS (light blue), AcbC (dark blue), CPu (green) and VTA (yellow). Neither KOR binding sites nor KOR G-protein coupling nor *Oprk1* mRNA were detectable within the VTA (n.d. – not detectable). (**E-H**) Representative autoradiograms of the regions analyzed on coronal rat brain sections of the striatum and midbrain.

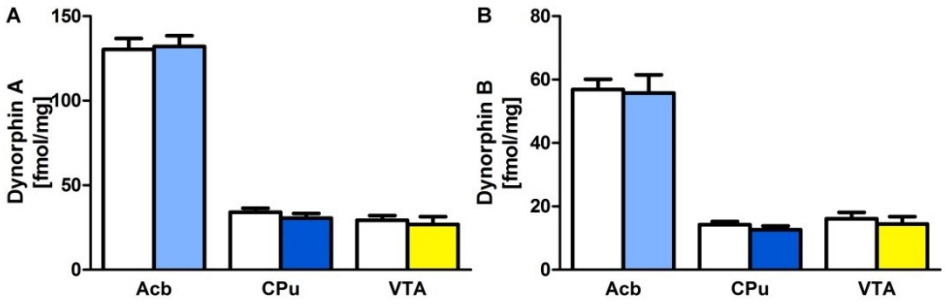

Figure 19: Radioimmunoassay for Dynorphin A (**A**) and Dynorphin B (**B**) in controls (white) post-dependent rats (colored). RIA experiment was performed by the group of Dr. G. Bakalkin.

4.2.3.3 SUMMARY: KOR IN ALCOHOL DEPENDENCE

In human alcoholics, KOR binding sites were non-significantly increased in the VS. In post-dependent rats, however, KOR expression as well as function was elevated indicating augmented activity of this system. Dynorphin A and B peptides, the endogenous ligands of the KOR, were unchanged in all regions.

4.2.4 OVERALL SUMMARY

Taken together, the data of **Study II** show a similar regulation of the MOR, DOR, and KOR in human striatal post-mortem tissue and the striatum of post-dependent rats. MOR binding sites are significantly decreased while receptor functionality is upregulated. In contrast, DOR density is increased but its functionality decreased. The KOR system appears to be hyperfunctional in post-dependent rats.

These results suggest a severe dysregulation of the endogenous opioid system with a decrease in MOR/DOR but an increased in KOR signaling. This might cause increased aversive states in alcohol withdrawal and abstinence contributing to relapse.

4.3 **STUDY III**: IMPACT OF CHRONIC NALTREXONE ON THE ENDOGENOUS OPIOID SYSTEM IN ALCOHOL DEPENDECE

The opioid receptor antagonist NTX is one of the few approved pharmacotherapies for alcohol dependence. As an MOR antagonist it is thought to counteract increased MOR receptor densities to reduce alcohol craving and relapse. However, the above results proof consistently, in human alcoholic subjects as well as post-dependent rats, that the availability of cell surface MORs is strongly reduced in the striatum. Even though opioid antagonists, including NTX, have been shown to increase opioid receptor densities, the results of this study have news value by reporting on changes induced by chronic NTX treatment (14 days, 2.5mg/kg, i.p.) in alcohol-dependent abstinent rats, here referred to as "alcohol exposed". So far, the focus has been set on actively drinking rats subjected to (mostly) short-time NTX. The applied dose of NTX in the here presented study was comparably high and sufficient to inhibit alcohol self-administration in rats. However, it did not block alcohol intake the day after cessation of NTX treatment (personal communication with Dr. Wolfgang Sommer). After seven weeks of CIE and three weeks of abstinence, animals were daily injected with 2.5 mg/kg naltrexone (NTX) or saline (i.p.) for 14 days (Figure 20E). Three to four hours after the last injection the animals were sacrificed in their active phase and the brains analyzed. Results are normalized to the respective saline control and compared by group- and region-wise one-way ANOVA. For a detailed description of the statistics please see "Materials and Methods (3.9)".

MOR binding sites - Chronic NTX treatment caused a comparable increase of MOR binding sites in the AcbS of not alcohol exposed and exposed animals by 16 % and 22 %, respectively. In the AcbC, by contrast, only a trend towards an elevation ($p=0.06$) was observed in exposed rats. No effects were observed in the CPu. The strongest effects were observed within the VTA. Here, MOR binding sites were increased by 61 % and 120 % in not exposed and exposed animals, respectively (Figure 20A). For statistical details see Suppl. Table 17.

Pomc - Transcript levels of *Pomc* in the median eminence were strongly increased following daily NTX injections in both groups of animals with an increase by 45 % in not exposed rats and by 13 % alcohol exposed rats (Figure 20 D, Suppl. Table 19).

bArr2 - Transcripts were mainly unaffected by chronic NTX-treatment with an increase only in the CPu of not exposed rats (for raw data and statistics see Suppl. Table 18).

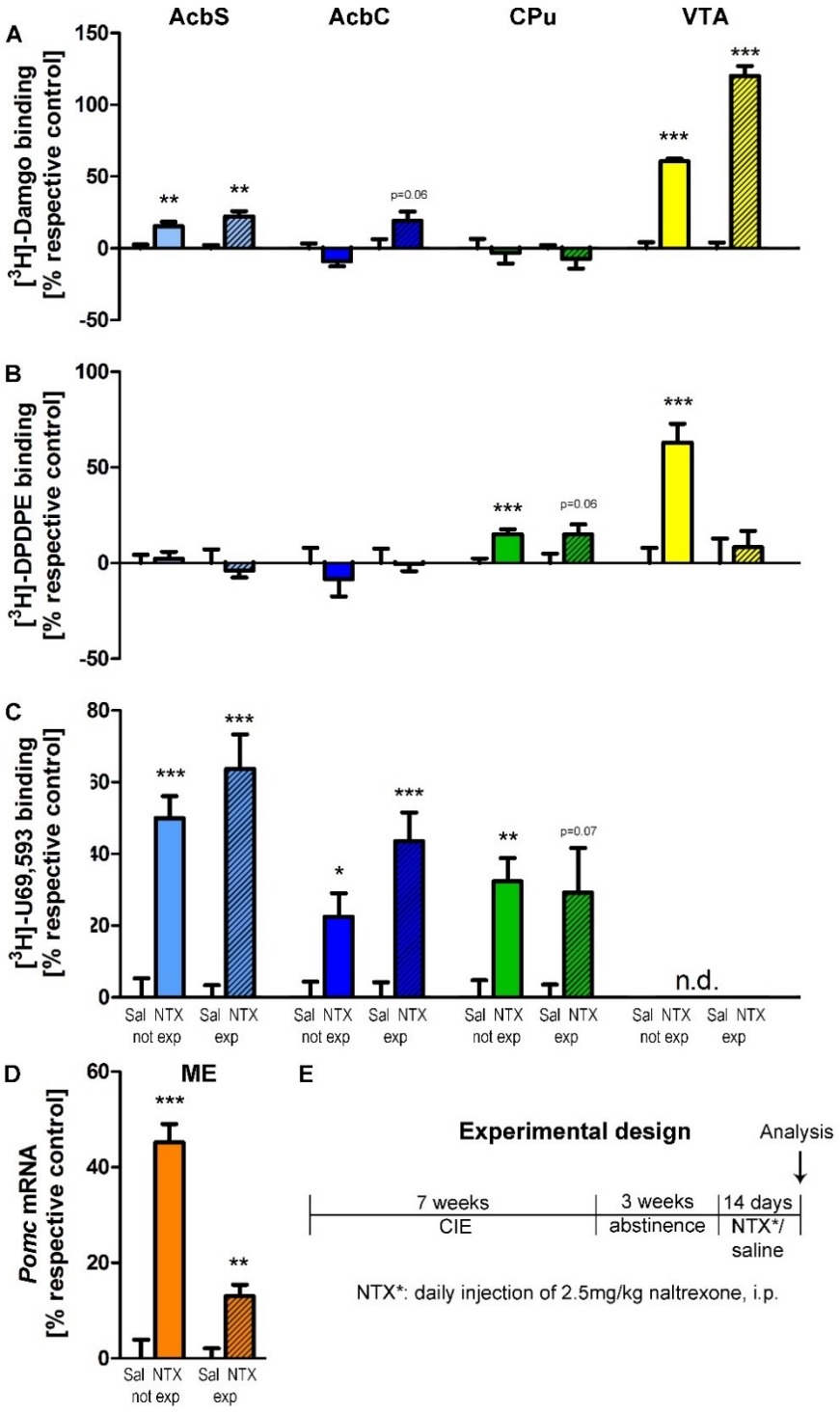

Figure 20: Chronic naltrexone (NTX) increases MOR and KOR binding sites and *Pomc* expression. Alcohol exposed (lined bar graphs) and not exposed (not lined) animals were treated with daily injections of NTX (2.5 mg/kg, i.p.) or saline for 14 days. Expression data of NTX-treated animals were normalized to their respective saline control (NTX not exposed to saline not exposed; NTX exposed to saline exposed). The effect of NTX treatment in each group (not exposed or exposed) was analyzed by region-wise one-way ANOVA. Effects of chronic NTX on MOR (**A**), DOR (**B**), and KOR (**C**) binding sites as well as *Pomc* (**D**) mRNA expression are shown. Data are expressed as mean ± SEM in % of respective saline control. (**E**) Timeline of the experiment. After chronic intermittent alcohol exposure (CIE, 7 weeks) and abstinence (3 weeks), rats were subjected to daily injections of NTX or saline. AcbS – nucleus accumbens shell, AcbC – Acb core, CPu – caudate putamen, VTA – ventral tegmental area, ME – median eminence

DOR binding sites - Chronic treatment with the opioid antagonist NTX did not affect DOR binding sites in the ventral striatum. However, DOR availability was significantly increased by 15 % in the CPu of not exposed animals after NTX treatment. In alcohol exposed rats, the increase only reached trend level (p=0.06). The strongest upregulation of DOR binding sites by 63 % was detected in the VTA of not alcohol exposed rats while no changes were observed in alcohol exposed rats (Figure 20B). Moreover, NTX appears to affect DOR binding sites exclusively in non-dependent animals. Statistical values are presented in Suppl. Table 20.

KOR binding sites - KOR binding sites were significantly increased in response to chronic NTX treatment in the ventral and dorsal striatum of alcohol exposed and not exposed rats. In the AcbS, binding sites increased by 50 % in not exposed animals and by 64 % in alcohol exposed animals. An elevation of 22 % and 44 % in the AcbC of not exposed and exposed rats, respectively, after NTX treatment has been found. In the CPu of both groups of animals, KOR expression was elevated by ~30 %, respectively, when compared to their saline control (Figure 20 C, Suppl. Table 21).

4.3.1 SUMMARY

Study III identified the MOR and KOR system as main targets of NTX treatment in the post-dependent animals. Significant increases of the densities of both receptors have been observed. While the main effects on the MOR were observed within the VTA, KOR binding was strongly altered in the striatum. This corresponds well with the knowledge of the role of the receptors in the regulation of rewarding striatal dopamine release.

4.4 **STUDY IV**: CONVERGENT EVIDENCE FROM ALCOHOL DEPENDENT HUMANS AND RATS FOR A HYPERDOPAMINERGIC STATE DURING ABSTINENCE

Human brain imaging studies (PET) using the radiotracer [^{11}C]-raclopride, report reduced D2-like receptor availability in alcoholic subjects. Based on these data, the hypothesis of a hypodopaminergic state during abstinence developed and this is seen as a driving force for the relapsing course of the disorder. However, the interpretation of PET data is challenging as the used radiotracers compete with the endogenous ligand dopamine. Thus, a decrease in PET signal can reflect both, reduced receptor density or increased endogenous ligand.

The following study aimed to clarify the state of the dopamine system during alcohol withdrawal and abstinence in human post-mortem tissue of alcoholic and healthy subjects and in the post-dependent animals to provide support for the interpretation of PET data and the development of therapeutic targets.

This study is the joint work of a group of researchers. Dr. Dr. Hamid R. Noori performed the meta-analysis. Dr. Marcus Meinhardt and Dr. Stéphanie Perreau-Lenz provided *in vivo* microdialysis and locomotion data. The group of Dr. Georg Köhr performed the electrophysiology experiments. I am very grateful for the possibility to present this work in my thesis to support the interpretation of my results.

4.4.1 POST-MORTEM BRAIN ANALYSIS SUGGESTS A HYPERDOPAMINERGIC STATE IN HUMAN ALCOHOLICS

Ten alcoholic and ten control subjects ("core samples") were included in this study and all subjects were free of positive blood alcohol levels at the time of death. Sections of the VS and NC were analyzed for D1 receptor ([^{3}H]-SCH23390), D2-like receptor ([^{3}H]-raclopride) and dopamine transporter (DAT, [^{3}H]-mazindol) expression by autoradiography.

D1 receptor binding sites were significantly reduced in both striatal brain regions (VS: 59%, [$F_{1,15}$=31.7], p<0.001; NC: 61%, [$F_{1,16}$=104.2], p<0.001, Figure 21A) as compared to controls. In contrast, D2-like receptor density was unchanged (VS: [$F_{1,16}$=0.005], p>0.5; NC: [$F_{1,15}$=1.3], p>0.5., Figure 21B). For DAT, a similar decrease in transporter density was detected (VS: 62%, [$F_{1,14}$=139.8], p<0.001; NC: 56%, [$F_{1,14}$=65.4], p<0.001; Figure 21C).

Additionally to the analysis of the D1 receptor by [^{3}H]-SCH23390 autoradiography in a small samples set of n=10/group (Figure 21), the investigation was extended to a large sample set (controls vs. non-intoxicated and intoxicated alcoholics, n=9-26/group). Tissue pH, post-mortem interval, age and smoker state were considered as candidate covariates but a stepwise analysis of covariance did exclude them as non-significant. In both regions analyzed, the NC and VS, non-intoxicated as well as intoxicated alcoholics displayed significantly reduced D1 binding sites (NC: [$F_{2,43}$=7.62], p=0.001, controls: 34.78 ± 2.54, non-intox: 21.47 ± 4.02, intox: 19.05 ± 3.83 fmol/mg ± SEM; VS: [$F_{2,43}$=10.36], p=0.0002, controls: 37.36 ± 2.39, non-intox: 24.38 ± 3.16, intox: 19.93 ± 3.50 fmol/mg ± SEM). No significant differences were observed between non-intoxicated and intoxicated alcoholics. In the NC, D1 binding sites were decreased by 38% and 45% in non-intoxicated and intoxicated alcoholics, respectively. Similar effect sizes (reduction by 35% in non-intoxicated and 47% in intoxicated alcoholics) were found in the VS.

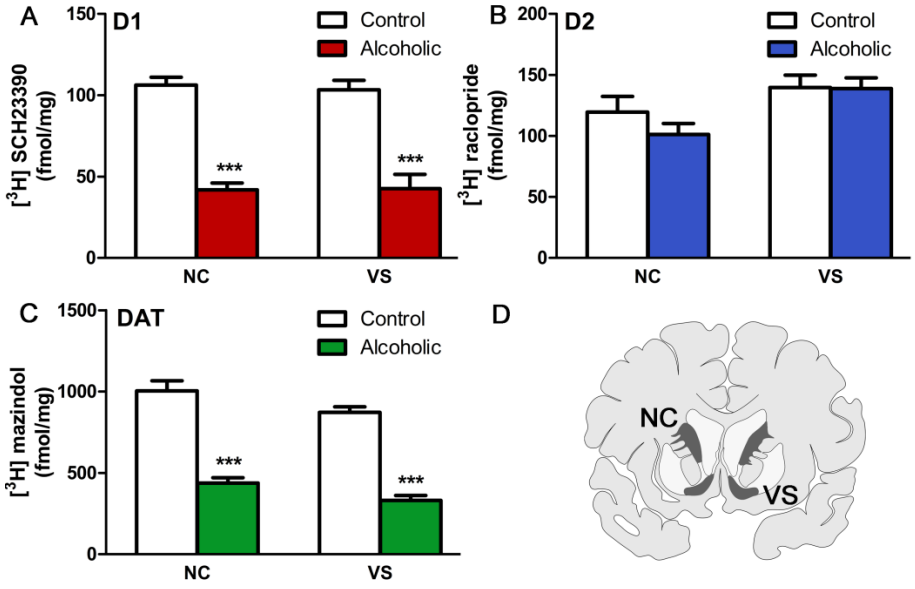

Figure 21: Autoradiography in human striatal post-mortem tissue suggests a hyperdopaminergic state. Bar graphs show expression of D1 (red, **A**), D2 (blue, **B**) and DAT (green, **C**) binding sites in human post-mortem tissue of alcoholic subjects as compared to controls (white). D1 and DAT are significantly decreased in the striatum of human alcoholics. Data were analyzed by one-way ANOVA and are represented as mean ±SEM, n=8-9/group. (**D**) Schematic presentation of a coronal section of a human brain with regions used for analysis highlighted (NC – nucleus caudatus, VS – ventral striatum).

Transcript levels as assessed by quantitative real-time PCR did not show any differences between groups for *DRD1* and *DRD*2 (Table 6). *SLC6A3* mRNA encoding for the DAT was

not determined as transcripts are mostly located in cell bodies of nigrostriatal projections and VTA neurons but not in the striatum. These data suggest that the changes observed on the protein levels are not caused by alterations on the transcriptional level but rather by other mechanisms such as receptor internalization or degradation.

Transcript	Region	Controls dCt	Alcoholics dCt	ddCt	F	p
DRD1	VS	4.91 ± 0.17	5.03 ± 0.30	-0.12	[1,18] 0.13	0.73
	NC	4.37 ± 0.10	4.35 ± 0.11	0.02	[1,18] 0.02	0.89
DRD2	VS	0.60 ± 0.30	0.84 ± 0.31	0.24	[1,18] 0.30	0.60
	NC	3.99 ± 0.10	4.23 ± 0.22	-0.24	[1,18] 0.93	0.35

Table 11: No changes of dopamine *D1* and *D2* receptor mRNA expression in post-mortem striatal tissue of heavy alcoholics. QRT-PCR for *DRD1* and *DRD2* mRNA; data are expressed as mean ± SEM, n = 9-10/group. *GAPDH* Ct values in NC, alcoholics: 22.0 ± 0.3, controls: 21.6 ± 0.1; *GAPDH* Ct values in VS, alcoholics: 23.6 ± 0.3, controls: 23.2 ± 0.2; NC, nucleus caudatus; VS, ventral striatum.

The data from this post-mortem striatal brain tissue analysis indicate reduced dopamine signaling via the D1 receptor accompanied by potentially higher extracellular dopamine levels due to decreased DAT and unaltered D2-like receptor levels.

To provide convergent evidence for these surprising findings, a systematic meta-analysis on dopamine concentrations and its metabolites during abstinence was performed. Additionally, the dopaminergic system was examined at different time points during alcohol abstinence in post-dependent rats.

4.4.2 ALCOHOL-DEPENDENT RATS MIRROR THE HYPERDOPAMINERGIC STATE OBSERVED IN HUMAN ALCOHOLICS: META-ANALYSIS ON DOPAMINE RELEASE DURING ABSTINENCE

Neither human imaging (204-207, 210, 264) nor animal studies using electrophysiological methods on D1 and D2 receptor or DAT availability (214, 217, 265-269) provide sufficient data for a robust meta-analysis of the dynamics of dopaminergic processes. Therefore, the analysis was focused on alterations of concentrations of dopamine and its metabolites. The presented meta-analysis on dopamine release and its metabolites in the Acb during abstinence is based on 16 published studies on rats including a total of 192 animals chronically exposed to ethanol (214, 216, 270-283). The time course of dopamine, DOPAC and HVA

concentrations in Acb (Figure 22) was obtained by continuous interpolation of the averages of experimental values with respect to the time of measurement after alcohol withdrawal. While the paradigm to history (pattern) of ethanol intake did not affect the analysis, the withdrawal period may be considered as a vanishing swing between two states.

While the studies showed increased dopamine release on day 0 of abstinence, a decrease followed on day 1 to 3. Within the first 6 days of withdrawal, dopamine levels declined about 30% below baseline reflecting a hypodopaminergic state. However, afterwards levels again increased and the system moves to a hyperdopaminergic state reaching its peak during the second and third week of abstinence (Figure 22 and Figure 23A). The concentration changes of dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) followed a similar pattern as observed for dopamine.

The data suggest a hypodopaminergic state during early abstinence which is followed by a hyperdopaminergic state during protracted abstinence.

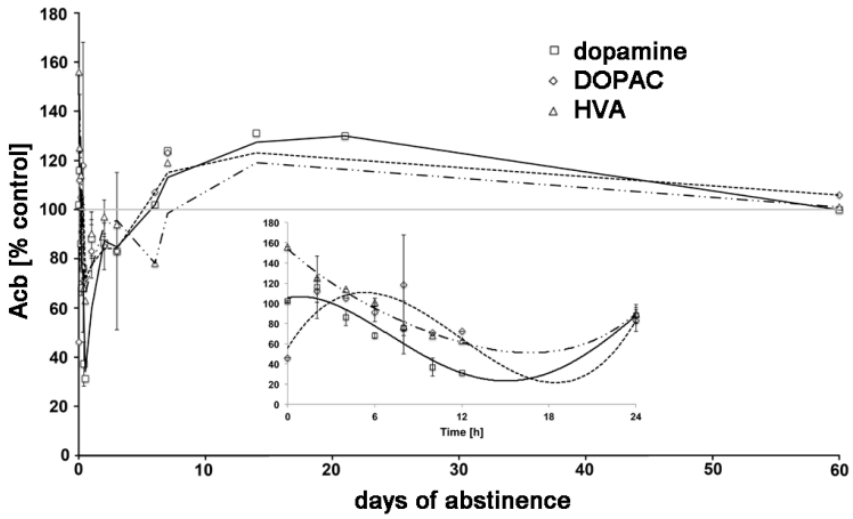

Figure 22: Dopamine and its metabolites in the Acb were investigated during alcohol abstinence by a meta-analysis (performed by Dr. Dr. H. R. Noori). During the first six days of abstinence, dopamine, HVA (homovanillic acid), and DOPAC (3,4-dihydroxyphenylacetic acid) concentrations decline up to 30 % of the baseline condition (hypodopaminergic state). Afterwards, concentrations rise above baseline levels (hyperdopaminergic state). The inlet shows the dynamic regulation of dopamine and the two metabolites during the first 24 hours (h) of abstinence.

4.4.3 DYNAMIC REGULATION OF DOPAMINE RECEPTORS AND TRANSPORTER DURING ABSTINENCE

To extent the knowledge on the dopamine system obtained in the human post-mortem tissue, a time course experiment to analyze D1, D2 receptors and DAT at different time points during abstinence in post-dependent rats was performed. After seven weeks of CIE the animals were sacrificed at day 0, 1, 3, 7, and 21 after the last alcohol exposure according to previous studies (236, 284). On day 0, the animals were sacrificed immediately after the last alcohol exposure still having positive blood alcohol levels of 273 ± 52 mg/dl. Dopamine receptors and transporters in the AcbS, AcbC, and CPu were analyzed by quantitative receptor autoradiographies and are presented as normalized data to respective control group at every time point. Raw data of controls are summarized in Suppl. Table 22.

In the AcbS (Figure 23B), binding sites of D1 and DAT varied as a function of time from alcohol exposure (two-way ANOVA treatment x time; D1: [$F_{4,54}$=4.6], p<0.01; DAT: [$F_{4,54}$=4.8], p<0.01). On day 0, D1 receptors were significantly reduced by 11 % but reached controls levels one day later (day 1). After three days of abstinence (day 3), a slight increase was observed (10 %, p=0.07) that reached significance on day 7. After three weeks of complete abstinence (day 21), dopamine D1 receptors were decreased by 14%. Expression of DAT binding sites is regulated differently with a trend towards an increase on day 0 (22 %, p=0.07). One day afterwards (day 1), DAT was decreased by 33 % and returned to control levels on day 7. On day 21, DAT was again significantly reduced by 35 %.

In the AcbC (Figure 23C), the regulation of D1 receptor and DAT binding sites followed a similar pattern as in the AcbS (two-way ANOVA treatment x time; D1: [$F_{4,58}$=7.9], p<0.001), DAT: ($F_{4,61}$=6.2], p<0.001). D1 was reduced by 15 % on day 0 but increased on day 7 by 30 % and reduced again on day 21 by 15 %. DAT binding sites were significantly increased by 24 % on day 0 and returned to controls levels on days 1 to 7. On day 21, there was a trend towards an increase by 11 % (p=0.05).

In the CPu, alterations in D1 receptor and DAT binding sites paralleled the regulation in the AcbS (two-way ANOVA treatment x time; D1: [$F_{4,58}$=10.8], p<0.001, DAT: [$F_{4,55}$=25.2], p<0.001, Figure 23D). However, D1 receptors were not reduced on day 21. On day 0, D1 receptors were decreased by 14 % but increased on day 3 (by 8 %) and day 7 (11 %). DAT was significantly increased on day 0 by 34% and decreased on day 1 (by 9 %) and day 21 (by

13 %). In contrast to D1 receptor and DAT binding sites, D2-like receptors were not changed at any time point in any region (Figure 23B-D).

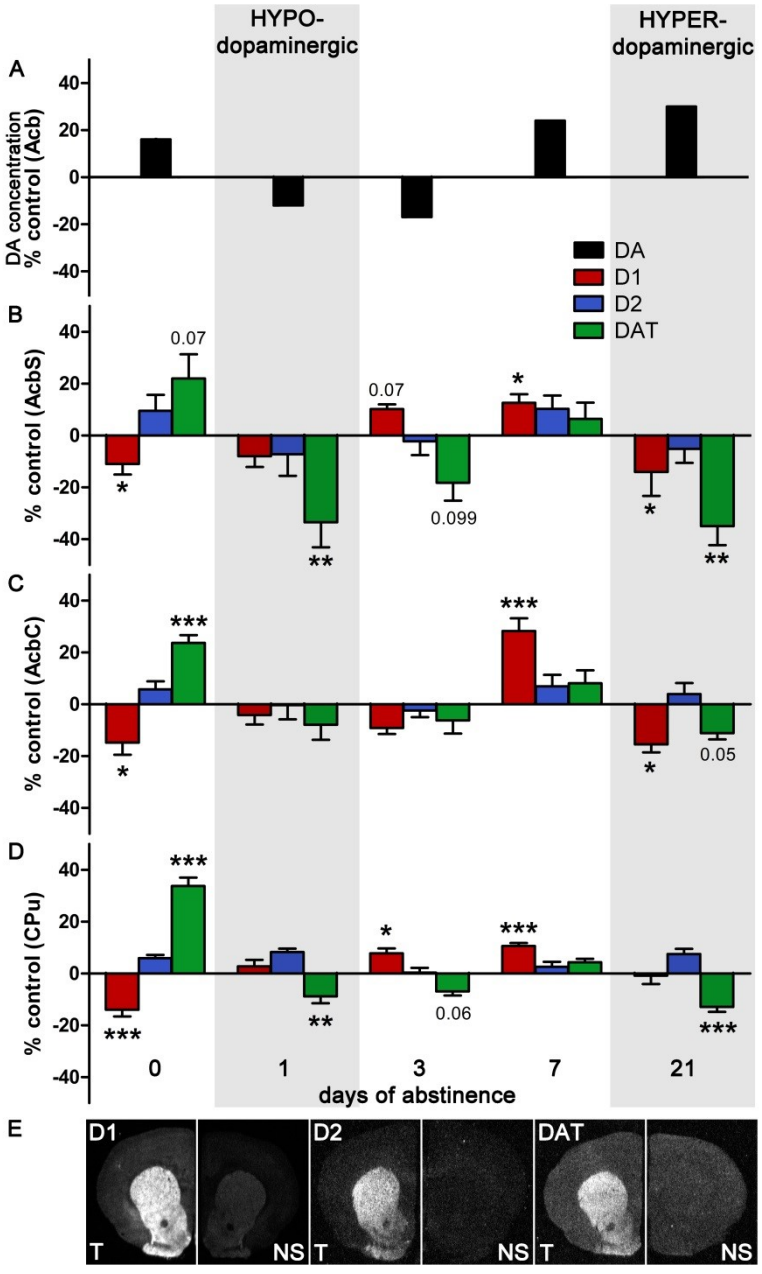

Figure 23: Analyses of the dopaminergic system in alcohol-dependent rats reveals a hyperdopaminergic state in protracted abstinence. (**A**) The time course of dopamine (DA) release in the nucleus accumbens (Acb) was modeled by a meta-analysis from 16 animal studies. During the first 6 days of withdrawal, the dopamine concentrations decline to 30 % of the baseline concentrations (hypo-dopaminergic state) but increase again afterwards to a hyperdopaminergic state. (**B-D**) Regulation of D1- (red bars), D2-like receptors (blue) and dopamine transporter (DAT, green) binding sites during different days of abstinence in (**B**) nucleus accumbens shell (AcbS), (**C**) nucleus accumbens core (AcbC) and (**D**) caudate putamen (CPu) of alcohol dependent rats vs. control rats (set as 0 % baseline at each time point). After seven weeks of CIE, rats were sacrificed immediately after the last exposure cycle (day 0) and on 1, 3, 7 and 21 days of abstinence. D1 and DAT are dynamically regulated at different times of abstinence, while D2-like binding levels remain unaffected. Statistical analysis was performed by two-way ANOVA followed by Fisher's post-hoc test. Data are expressed as percent of controls ± SEM, n=4-8/group. For expression levels in controls of each time point see Suppl. Table 22. Gray shaded areas in A-D indicate a hypo- or a hyperdopaminergic state during abstinence. (**E**) Representative images showing total (T) D1 ([^{3}H]SCH23390), D2-like ([^{3}H]raclopride) and DAT ([^{3}H]mazindol) binding on a coronal striatal rat brain section. Non-specific (NS) binding was determined on adjacent section by adding flupenthixol (D1), sulpiride (D2-like) and nomifensine (DAT) to radioligand.

4.4.4 ELEVATED EXTRACELLULAR DOPAMINE LEVELS AND HYPERLOCOMOTION DURING PROTACTED ABSTINENCE

Extracellular levels of dopamine were measured in the AcbS region of post-dependent rats after 21 days of abstinence via *in vivo* microdialysis and basal dialysate dopamine concentrations were found to be significantly elevated in dependent rats ([$F_{1,26}$=2.7], p<0.05; Figure 24A). Subsequently to baseline measurements, different doses of ethanol (0 (saline), 1, 2 g/kg, i.p.) were injected. Application of saline did not show significant differences between control vs. post-dependent rats (p>0.5). However, injection of 2g/kg ethanol increased extracellular dopamine levels in control animals by 49 % ± 33 % as compared to baseline. Post-dependent rats displayed a blunted response to the same treatment with a non-significant increase of 9 % ± 49 % from baseline levels (Figure 24B). Repeated measurement ANOVA revealed a significant effect of alcohol injections ([$F_{1,14}$=7.1], p<0.05), a trend for treatment (dependent vs. control, [$F_{1,14}$=3.8], p=0.07), but no interaction effect ([$F_{1,14}$=0.8], p>0.5). A significant increase of *TH* mRNA levels in the substantia nigra pars compacta of three weeks abstinent post-dependent rats by 31 % that was measured by *in situ* hybridization gives support for an increase in accumbal dopamine release (Figure 24C).

Additionally, locomotion of control and post-dependent animals was assessed in an OpenField experiment and in the homecage. In the OpenField (Figure 24D) during first 20 min, under the conditions of novelty, no differences in total distance traveled were detected between control and post-dependent animals (post-dependent 5417.1±405.2 cm vs. control 5234.3±419.6 cm; p>0.5). When the animals habituated to the OpenField (after the first 20 min) basal locomotor activity was assessed. Post-dependent animals traveled significantly higher track lengths as compared to controls (post-dependent 753.2±72.6 cm vs. controls 420.8±49.9 cm; mean per 5 min, Figure 24D). In the homecage, post-dependent rats displayed hyperlocomotion with a significantly increased total sum of body movements (post-dependent 45928.7±1206.1 cm vs. controls 40838.4±1294.8 cm, Figure 24E).

To consider functional consequences of the hyperdopaminergic state on the synaptic level in control and post-dependent rats, glutamatergic inputs to medium spiny neurons (MSNs) of the AcbS in brain slices were examined during alternative, electrical stimulation in the AcbS (Figure 24F-H). Ethanol perfusion (25 mM; for 25 min) increased excitatory post-synaptic currents (EPSC) in both groups of rats (n=12 MSNs from 4 control rats; n=7 MSNs from 3 dependent rats). Subsequent perfusion of the D1 agonist SKF81297 (5 µM; for 20 min) in the presence of ethanol further enhanced the EPSCs in control but not post-dependent rats

(F[2,20]=2115, p<0.001; dependent, F[2,20]=270, p<0.001 and p>0.05 when adding SKF81297), also apparent from the EPSC difference between control vs. dependent rats in ethanol plus SKF81297 (p=0.019).

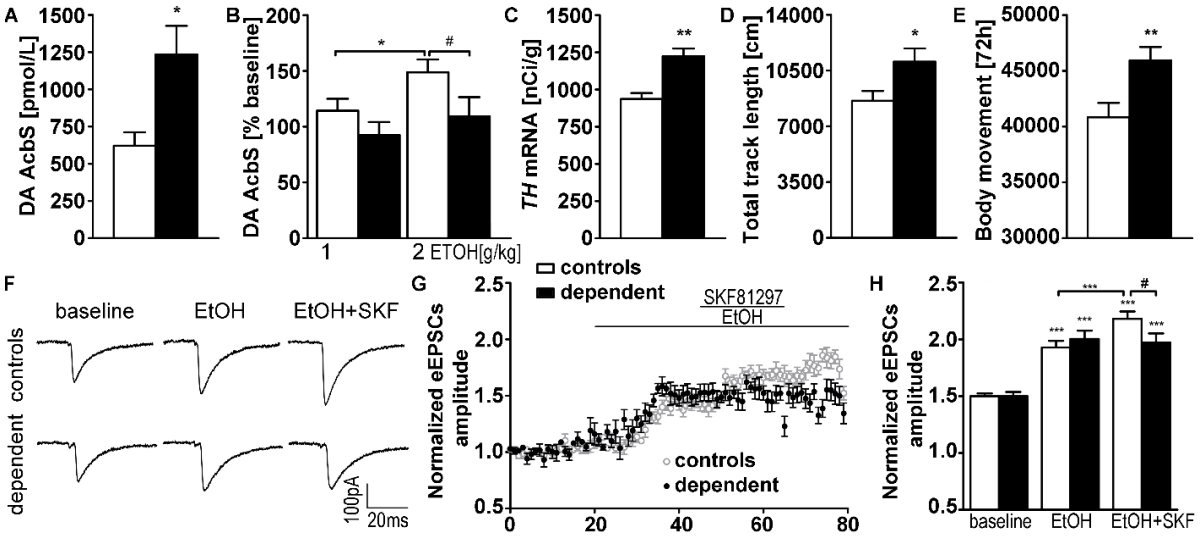

Figure 24: Hyperdopaminergic state in three weeks alcohol abstinent rats. (**A-B**) A microdialysis displays increased dopamine levels and blunted response to ethanol treatment in alcohol-dependent rats. (**A**) Basal extracellular dopamine levels within the AcbS are markedly increased in dependent rats (n=15/group). (**B**) AcbS dopamine levels after application of consecutive doses of ethanol (1 and 2 g/kg, i.p.). Control animals show increase of extracellular dopamine levels after ethanol (2 g/kg, i.p.), whereas dependent rats show a blunted response to the treatment (n=8/group). (**C**) Elevated *TH* mRNA levels in the substantia nigar pars compacta as measured by *in situ* hybridization. (**D-E**) Three weeks abstinent rats display hyperlocomotion as detected by locomotor activity records in the homecage assessed with a homecage e-motion system for 72 hrs (**D**) and OpenField for 60 min (**E**). (**F**) Representative EPSCs recorded at -80 mV in MSNs were evoked by electrical stimulation in the Acb cortex before (baseline) and during perfusion of ethanol (25 mM) and ethanol (25 mM) plus SKF81297 (5 µM). Current traces represent the average of ten sweeps. (**G**) Time courses of the effects shown in (F) for normalized EPSCs. (**H**) Summary of the effects on EPSCs (control: n=12; dependent: n=7). For detailed statistics, see text. These data are joint work of N. Hirth, M. W. Meinhardt, H. Salgado, O. Torres-Ramirez, S. Perreau-Lenz, G. Köhr.

4.4.5 SUMMARY

In summary, these data show a dynamic regulation of dopamine transporter and receptor D1 expression during alcohol withdrawal and abstinence as measured by autoradiography experiments. Dopamine D2 receptors are unchanged in all regions at any time point. An increase in dopamine function and a hyperlocomotion in protracted abstinence is suggested due to increased midbrain *TH* mRNA and accumbal extracellular dopamine levels. Hence, a hyperdopaminergic state in protracted abstinence was observed.

5 DISCUSSION

5.1 **DISCUSSION STUDY I:** ALCOHOLISM INDUCED DOWN-REGULATION OF MU OPIOID RECEPTORS PREDICTS RELAPSE BEHAVIOUR: POST-MORTEM AND PET RESULTS

Pharmacological therapies using the opioid antagonists naltrexone (NTX) or nalmefene to treat alcohol dependence by reducing alcohol reward, craving, and relapse, are based on the theory of increased MORs in the reward system. This hypothesis has been established according to previously published PET studies (106-108). Additionally, it has been shown that elevated MOR BP_{ND} correlates with alcohol carving (107). However, PET data are difficult to interpret. Elevated MOR BP_{ND} can either be caused by higher receptor expression or/and by low availability of endogenous ligands that compete with the PET tracer. This is particularly important as [^{11}C]-carfentanil – the only MOR selective PET ligand established to date – is an agonist and thus especially sensitive to synaptic levels of endogenous ligands due to G-protein-mediated internalization (285, 286).

In contrast to the above mentioned view, the here presented post-mortem study shows a clear reduction of *OPRM1* mRNA as well as MOR binding sites in the NC and VS of alcoholic subjects. The finding of reduced MOR binding sites in the brain of alcoholics is consistent with the animal literature. Here, a meta-analysis on published rodent studies including 233 rats found reduced mRNA levels and binding sites especially during the first 3 days of alcohol withdrawal. The reduction in MOR binding sites is independent of the A118G genotype (Suppl. Table 2). This is in line with a study in the mouse line carrying the human *OPRM1* gene with the A118G genotype (127). In contrast, other studies reported decreased MOR expression in G-allele carries. However, they did not analyze striatal brain tissue but the global brain (PET) (132), pons tissue of post-mortem specimen (125), and *in vitro* systems (131).

As shown in Figure 25, a new model of dynamic alterations of the MOR system is proposed based on the combined post-mortem brain and *in vivo* PET data. The endogenous opioid β-endorphin and other opioids are released upon alcohol consumption (104), thereby mediating positive feelings.

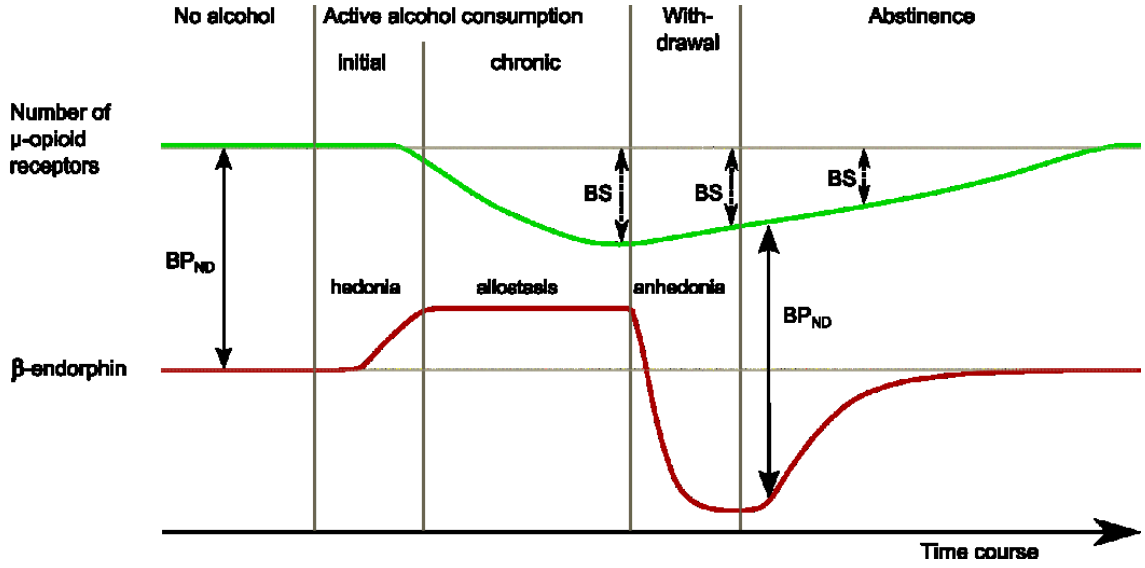

Figure 25: Initial and repeated alcohol consumption results in release of endogenous opioids by progressively enhancing frequency and strength of opioidergic neurotransmission. This is accompanied by reward and hedonia. As an adaptive mechanism MOR expression gets reduced and a new allostatic state develops. In alcohol withdrawal and early abstinence, which are characterized by anhedonia, alcohol-induced release of endogenous opioids is suddenly stopped while MOR expression is strongly diminished. During protracted abstinence the responsiveness of the opioidergic system is still diminished. In order to recover, the system has to adapt to less intense natural rewards in comparison to alcohol. [^{11}C]-carfentanil PET assesses MOR availability which depends on the absolute number of MOR binding sites and endogenous ß-endorphin levels. Therefore, PET data can only be correctly be interpreted if additional data on either of these measures is available. The binding potential (BP$_{ND}$, solid black arrows) is the specific-to non-specific equilibrium partition coefficient that is determined by the concentration of the endogenous ligand ß-endorphin and the absolute number of MOR binding sites. Saturated [^{3}H]-Damgo autoradiography in post-mortem tissue are measures of number of available MOR binding sites (BS), which are reduced in alcoholics at different times of abstinence in relation to controls (dashed black arrows).

Chronic repeated alcohol intake results in increased opioidergic neurotransmission and MOR expression is downregulated to compensate for this. Diminished surface density of MORs may contribute to tolerance to the rewarding effects of alcohol, driving further enhanced alcohol consumption. When alcohol use is discontinued, i.e. during acute withdrawal and early abstinence, the release of endogenous opioids is suddenly stopped while MOR expression is strongly reduced. This combination of low opioids and low MORs might contribute to the anhedonic state during early abstinence that is characterized by dysphoria, increased anxiety, and depressiveness (35). Reduced peripheral β-endorphin levels have been consistently observed in rats as well as alcohol dependent patients during at least the first month of alcohol withdrawal (147, 287, 288). Together with the here presented data, this suggests a diminished responsiveness of the opioidergic system during alcohol abstinence. The system has to recover its ability to react appropriately to less intense natural rewards than

alcohol, e.g. social interaction, and release endogenous opioids in response. The reduction in endogenous opioids during the recovery phase might constitute the neurochemical stimulus to increase and progressively normalize the surface density of MORs. This is supported by the finding of an even stronger reduction of MOR binding sites in alcoholic subjects that were drinking until death.

Previous PET studies report on increased MOR BP_{ND} in alcoholics during early abstinence (106, 107). Together with the knowledge of significantly reduced MOR binding sites in post-mortem tissue and decreased plasma β-endorphin levels (147, 289), increased PET signals suggest reduced endogenous opioid levels (Figure 25).

Considering the decrease in MOR binding sites, pharmacological blockade of the remaining receptors by antagonists such as naltrexone or nalmefene would be expected to worsen the condition of alcohol dependence and induce relapse. Thus, there must be other modes of action of this pharmacotherapy such as enhanced signaling at the MOR. However, NTX therapy was not effective in the here presented sample which is in line with results from the previously published *PREDICT* study (37, 184, 223). In the PREDICT study, a total of 426 alcohol-dependent patients did not benefit from NTX when compared to placebo treatment. One explanation might be the degree of alcohol dependence severity as a critical factor influencing NTX efficiency study (37, 184, 223). In patients with high severity, as included into this study, the main target of NTX as well as nalmefene – the MOR – is reduced as the results of the post-mortem study show.

In addition to strongly reduced MORs in the reward system, a second key finding is that low MOR BP_{ND} in the putamen of alcoholics may predict the risk for alcohol relapse. The here proposed model of regulation of the MOR system suggests low MORs to be accompanied by a state of anhedonia. Patients suffering from this negative mood state are more susceptible to relapse (35). Thus, low MOR BP_{ND} may potentially be used as a biomarker for relapse prediction.

The investigation of post-mortem tissue can be confounded by various factors, such as suicide, pre-mortem medication, smoking and abuse of illicit drugs. In a sensitivity analysis, samples displaying such confounding factors were excluded which did not change the main findings. **Study II** gives further proof of the quality of the post-mortem tissue as analysis of the DOR and KOR did not show a down-regulation but rather an upregulation or unaltered

expression. Although a previous study showed remarkable stability of mRNA and proteins in post-mortem tissue – independent of duration of the post-mortem interval – this issue was addressed by correlating levels of *OPRM1* mRNA and MOR binding sites with PMI, tissue pH, RIN in each group and found no significant effects. Overall, the post-mortem tissue samples used display a decent quality and were obtained from one of the best providers of post-mortem specimens of human alcoholics and healthy controls, the New South Wales Tissue Resource Center, University of Sidney, Australia. These samples have already been used successfully in numerous studies (173, 290).

5.1.1 SUMMARY

In summary, reduced MOR is suggested to be a neuroadaptation on response to alcohol-induced released of endogenous ligands and may explain the low efficiency of naltrexone therapy in a subset of severely diseased alcoholic patients. Furthermore, a decreased in MOR is proposed to be a molecular marker for a negative disease course. The combination of post-mortem brain and PET analysis allows the characterization of a receptor status, i.e. the number of cell surface receptor. Therefore, it provides more certainty in the interpretation of PET results that otherwise is challenging. The data show a strong decrease in MOR binding sites in striatal post-mortem tissue. The PET study shows low MOR availability to be associated with increased relapse risk. The combined approach of post-mortem and PET analysis has led to the development of a new model showing the dynamics of the endogenous MOR system during alcohol dependence.

5.2 **DISCUSSION STUDY II**: DIFFERENTIAL REGULATION OF OPIOID RECEPTORS IN ALCOHOL DEPENDENCE: EVIDENCE FROM THE HUMAN AND RAT BRAIN

The results presented in **Study I** provide evidence for strongly reduced MOR binding sites in severely diseased human alcoholic subjects and proposes a new model for the dynamic alterations in the MOR system during alcohol dependence. Moreover, these findings suggest that treatment with opioid antagonists such as NTX or nalmefene would worsen the situation of alcoholic patients leading to relapse. Nevertheless, many patients profit from this pharmacological therapy and it is effective in reducing alcohol consumption in humans and post-dependent animals. Together, this indicates that there are additional/other mechanisms mediating opioid antagonist action in alcohol dependence. Research opportunities are undoubtedly limited in living human subjects. Thus, for this study human post-mortem tissue and the post-dependent animal model were used to elucidate the regulation of the opioid system during alcohol dependence. Dependence was induced in rats by seven weeks of CIE followed by three weeks of abstinence. Moreover, the DOR and KOR play a critical role in the development and maintenance of alcohol dependence and also NTX shows affinity for these receptors. Therefore, they were also included in this investigation.

5.2.1 MOR

Similar to the situation in human post-mortem tissue, MOR binding sites were significantly reduced in the ventral striatum (AcbS) of post-dependent rats. In the CPu, in contrast, no changes have been detected (Figure 12A) while in the human post-mortem tissue MOR binding sites were strongly reduced in the VS and NC. This most likely reflects the different anatomy of the human and rodent brain. In humans, the caudate and putamen are anatomically divided by a fiber bundle, the internal capsule, while they are one combined structure in rodents. Notably, effect sizes are smaller in post-dependent rats as compared to human alcoholics. This may be caused by the higher severity of alcohol dependence and duration of heavy alcohol administration in humans. Human alcoholics are likely to consume high amounts of alcohol over a period of months up to several years resulting in a stronger disruption of homeostasis of brain neurotransmitter systems and more severe neuroadaptations. The post-dependent rats were exposed to CIE for seven weeks, reaching blood alcohol levels of 150-250 mg/dl and showing withdrawal signs after alcohol administration was discontinued (235). Nevertheless, the post-dependent rats are a valid, well

established animal model to study alcohol dependence displaying good predictive, face, and construct validity (235). As already mentioned in the meta-analysis of MOR regulation during withdrawal and abstinence in **Study I** and in Table 4, most animal studies used a two bottle free choice paradigm or alcohol-containing liquid diet for chronic alcohol self-administration. Rat strains, duration of alcohol access and abstinence as well as method of quantitative analysis varies among studies. For this reason, it is not surprising that variable results on MOR status have been reported. Nonetheless, the meta-analysis in **Study I** found decreased MOR binding in the striatum during the first three days of abstinence (Table 9). Investigations during protracted abstinence in severely dependent animals were missing so far. This thesis aimed to fill this gap and shows a reduction of MOR binding sites in the ventral but not dorsal striatum of post-dependent rats and no regulation within the VTA. This decline in striatal binding sites appears not to be caused by transcriptional mechanisms since *Oprm1* mRNA is unaltered in all brain regions (Figure 12C). On the other hand, accumulation of Damgo-stimulated [^{35}S]-GTPγS, an indicator of receptor G-protein coupling and, thus, of MOR signaling, is strongly elevated in the ventral striatum (Figure 12B). As reduced MOR bindings sites are an unlikely target of NXT therapy, blocking the alcohol-induced enhanced MOR signaling could be a possible target of NTX action to prevent alcohol relapse.

Although there is no significant change in MOR binding sites within the VTA, G-protein coupling appears to be reduced in this region. In combination with reduced β-endorphin levels during abstinence as proposed by several investigators (287-289), by **Study I**, and indicated by reduced *Pomc* mRNA levels (Figure 12D). This may result in reduced disinhibition of dopaminergic activity of neurons projecting to the striatum. Consequently, firing of these dopaminergic neurons may be reduced resulting in a blunted dopamine response to alcohol/drug administration in alcohol dependent animals as shown in **Study IV** and human alcoholics (210).

A variety of proteins, such as RanBP or RGS-proteins (291-293), can regulate MOR internalization and de-/sensitization processes as well as signaling. Another important adaptor protein is β-arrestin2 that is known to be linked to MOR signaling and alcohol reward (98, 101). In case of MOR activation, G-protein signaling cascades are initiated, the receptor is phosphorylated by G-protein receptor kinases and affinity for β-arrestin2 is enhanced. In post-dependent rats, *bArr2* mRNA levels are significantly enhanced in regions with decreased MOR binding sites but increased Damgo-stimulated [^{35}S]-GTPγS accumulation suggesting a role of β-arrestin2 in the development and maintenance of these neuroadaptations (Figure 13).

Importantly, Björk et al. (97) showed that elevated *bArr2* transcript levels potentially translate into increased *bArr2* mRNA. This suggests that in the post-dependent animals β-arrestin2 proteins also are increased. Elevated β-arrestin2 availability might facilitate rapid MOR desensitization and internalization upon receptor activation, thereby resulting in reduced cell surface receptors. Indeed, overexpression of β-arrestin2 in cell culture has been shown to decrease cell surface localization of G-protein coupled receptors (99, 102). Increased MOR internalization has also been linked to elevated β-arrestin2 immunoreactivity in rats after natural reward (100). However, β-arrestin2 regulation of receptors belonging to several neurotransmitter systems including both the opioid and dopamine system is complex (294).

5.2.1.1 SUMMARY: MOR IN ALCOHOL DEPENDENCE

Taken together, the data show a potential role of β-arrestin2 in the significant reduction of striatal MOR binding sites. G-protein coupling of the MOR is significantly increased in the striatum, most likely to compensate for reduced binding sites, and this could be the target of NTX therapy. However, in severely diseased patients, MOR diminution appears to be more pronounced (**Study I**) and compensatory processes are unlikely to be effective.

5.2.2 DOR

In contrast to MOR, DOR binding sites were increased in the ventral striatum of human alcoholic subjects as measured by [^{3}H]-DPDPE receptor autoradiography (Figure 14). In the NC, binding sites were numerically increased but this did not reach significance. This is mirrored by the results in the post-dependent animal model where elevated DOR binding sites have been found in the striatum and VTA (Figure 15A). In contrast, *Oprd1* mRNA levels are decreased which may represent a compensatory mechanism to counteract the increased cell surface density of the receptor protein (Figure 15C). Even though transcript levels of the enkephalin precursor *Penk* are increased in the ventral striatum this does not translate into elevated enkephalin peptide levels as measured by RIA (Figure 15D). Levels of MEAP (Met-enkephalin-Arg-Phe) are unchanged in all analyzed regions including the ventral striatum. Previous studies reported increased enkephalin levels after acute (295, 296) and chronic alcohol intake (297) in animals. For instance, post-dependent rats that were subjected to a two-bottle free choice paradigm after CIE, showed elevated Met-enkephalin levels 72 hours after access to alcohol (298). However, enkephalin levels tend to decline back to control

levels after the first week of alcohol exposure (299) and no changes have been reported during acute withdrawal and protracted abstinence of 21 days (158, 300). Importantly, this is in line with human data where plasma enkephalin levels were unchanged during acute withdrawal and abstinence (147). In contrast, a decline in plasma β-endorphin during withdrawal has been observed that normalized after five weeks of abstinence (147). The RIA for Leu-enkephalin-Arg6 showed a small increase in the Acb of post-dependent animals. Even though this peptide binds to the DOR it rather is a marker for the dynorphin system as it is synthesized from the precursor *Pdyn* indicating an activation of the KOR/dynorphin system in protracted abstinence.

In a previous PET study employing [^{11}C]-carfentanil and [^{11}C]-methylnaltrindole to assess MOR and DOR availability, respectively, in human alcoholics, MOR was found to be increased while DOR seemed only numerically but non-significantly elevated (106). On the first view this appears to be in contrast with the results presented in this thesis. However, the increase in measured [^{11}C]-carfentanil PET signal (MOR) can be explained by a deficiency of β-endorphin as it is discussed in detail in **Study I** and, thus, actually is in line with the results of decreased MOR and *Pomc* in human alcoholics and post-dependent rats. The elevation in [^{11}C]-methylnaltrindole signal, although not significant, is comparable to the here presented observations of increased DOR in human as well was rodent brain tissue. Even though the DOR possess affinity for β-endorphin, its main endogenous ligands are enkephalins. Since the levels of enkephalins have been shown to be unchanged or slightly increased in this thesis and by others (147, 158, 300), the [^{11}C]-methylnaltrindole signal in controls and alcoholics is expected not to be significantly influenced by endogenous ligands.

In the post-dependent animals, DPDPE-stimulated DOR G-protein coupling is decreased in the striatum of post-dependent animals by more than 50 % when compared to control animals indicating decreased DOR activity in this region (Figure 15 B). The decrease in DOR signaling might increase anxiety and thereby result in elevated alcohol-seeking. DOR knockout mice display high innate anxiety and alcohol consumption. Because alcohol intake reduced anxiety levels, this is thought to be a self-medication approach (149, 301). Additionally, decreased DOR G-protein coupling has been linked to anxiety during cocaine withdrawal (302).

Moreover, *in vitro* as well as *in vivo* data suggest that DOR activity requires functional MOR expression to form MOR-DOR heteromers (148). However, the MOR and DOR are mainly

localized in separate neurons in the rodent forebrain (303) suggesting that the two receptors do not interact on the cellular level in these regions. The observations presented in this thesis show an opposite regulation of DOR and MOR expression and coupling in the striatum (compare Figure 12 and Figure 14). This opposing regulation of receptor availability and G-protein coupling could be a compensatory mechanism. In post-dependent rats, the opioid system might still be flexible enough to counterbalance dependence-induced changes, at least in part. For instance, the increase in MOR G-protein coupling and DOR binding sites may be an attempt to compensate for decreased MOR binding sites and DOR G-protein coupling. In individuals suffering from severe alcohol dependence as most likely is the case in the human post-mortem tissue, neuroadaptations are much more pronounced and a compensation of, e.g. decreased MOR binding sites by elevated G-protein coupling, is unlikely to be sufficient.

5.2.2.1 SUMMARY: DOR IN ALCOHOL DEPENDENCE

In summary, the DOR availability is increased but its function significantly decreased in alcohol dependence. The increase in DOR binding sites might be a compensatory process to counteract strongly diminished G-protein coupling. However, this mechanisms appears no to be sufficient and activity of the DOR system is reduced. Functionally, this may be linked to increased anxiety as observed in post-dependent rats and, thus, contribute to elevated alcohol intake.

5.2.3 KOR

Acute alcohol releases endogenous opioids β-endorphin and enkephalin that are involved in mediating the positive reinforcing effects of alcohol. Furthermore, dynorphins are responsible for the negative reinforcing aspects via activating KORs. Higher doses of alcohol cause increased dynorphin release, possibly reflecting the anhedonic sensations in response to ingestion of large amounts of alcohol. Even though the KOR/dynorphin system is evolving as potential target for pharmacological interventions, a detailed description of this system during protracted alcohol abstinence is still lacking.

The analysis of KOR binding sites by [^{3}H]-U69,593 autoradiography in striatal post-mortem tissue of human alcoholics revealed numeric but non-significant increases within the VS when compared to controls. No effect was detected within the NC (Figure 17). In the post-

dependent animals, the increases in KOR binding sites reached significance within the ventral and dorsal striatum (Figure 18A). Furthermore, G-protein coupling of the receptor was increased in the AcbS (Figure 18B). These data show enhanced activity of the KOR system in the striatum of post-dependent animals. *Pdyn* mRNA as well as dynorphin A and B peptide levels were unchanged in the same regions and within the VTA.

A previous post-mortem brain tissue study demonstrated an upregulation of the KOR/dynorphin system in the prefrontal cortex and hippocampus of alcoholic subjects (154). A more recent analysis of the striatum showed unchanged dynorphin A and B in the nucleus caudatus and a decrease of dynorphin A in the putamen (155). The authors state that the striatal KOR/dynorphin system is downregulated in alcohol dependence. However, they did not analyze KOR mRNA or protein levels and, thus, no complete picture of the system is provided. In the post-dependent animals, dynorphin levels were unchanged and, thus, correspond well to the previously reported post-mortem data. As the receptor and its G-protein coupling are significantly increased, the KOR/dynorphin system appears to be rather up- than downregulated. This assumption is supported by the fact that blockade of the KOR by the specific antagonist nor-BNI reduces alcohol consumption selectively in dependent animals (40, 41, 156, 164).

5.2.3.1 SUMMARY: KOR IN ALCOHOL DEPENDENCE

The upregulation of KOR density and signaling, suggest increased sensitivity of the KOR/dynorphin system in alcohol dependence that is, at least in part, responsible for alcohol intake behavior and the negative subjective aspects of alcohol withdrawal and abstinence. This makes the striatal KOR/dynorphin system a useful target for pharmacotherapies aiming to decrease alcohol intake in dependent individuals.

5.2.4 SUMMARY OF THE COMBINED EFFECTS OF OPIOID RECEPTORS IN ALCOHOL ABSTINENCE

The findings of **Study II** coincide with the "opponent process theory" model (304) that has previously been applied for the regulation of the endogenous opioid system in alcohol dependence (163).

This theory implies that a positive hedonic state as induced by alcohol intake is automatically opposed by a negative state (Figure 26). After chronic drug exposure the positive proportion would be diminished while the negative would be enhanced. When alcohol intake is ceased, this results in enhanced negative emotional states that force the dependent individuals to excessively seek and use alcohol (163). Alcohol-evoked MOR and DOR activation by β-endorphin and enkephalins produces positive hedonic states that are followed by negative sensations mediated by the KOR/dynorphin system. In line with this hypothesis, several studies report decreased MOR- and DOR-signaling in alcohol dependence and acute withdrawal (112, 113, 116) but increased KOR (158-160).

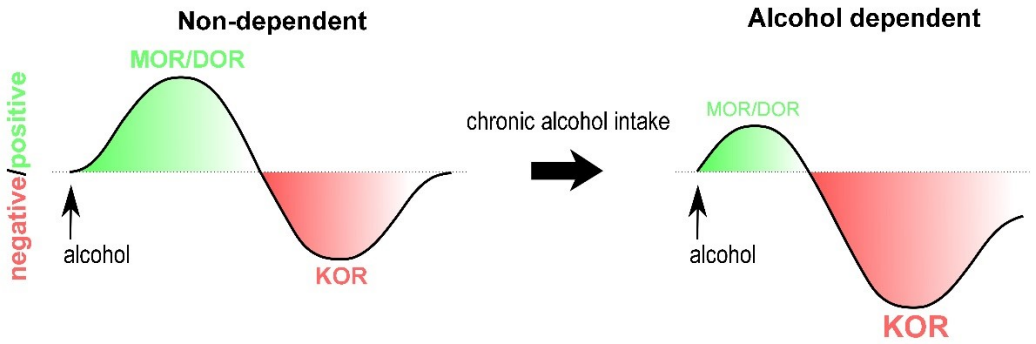

Figure 26: The opponent process theory applied for the regulation of the endogenous opioid system in alcohol dependence. In a non-dependent state, alcohol consumption results in a positive hedonic emotional state that is mediated by the MOR/DOR system. Subsequently, the stimulation of the KOR/dynorphin system automatically opposes this by a negative state. In an alcohol dependent individual, the positive component diminishes since MOR/DOR signaling is decreased and the negative state is more pronounced since the KOR/dynorphin system is supersensitive (adapted from (163)).

The results of **Study II** are extending our knowledge on the endogenous opioid system in protracted abstinence. In post-dependent animals, MOR signaling is attenuated as reflected by decreased striatal MOR availability and reduced *Pomc* levels. DOR signaling is strongly diminished as reflected by significantly reduced G-protein coupling. In contrast, DOR binding sites are elevated. However, it is questionable if the comparably small increase in binding sites, as observed in post-dependent rats (Figure 15) and human brain tissue of severely diseased alcoholics (Figure 14), has functional impact compared to the pronounced reduction in G-protein coupling.

Thus, the positive effects of alcohol intake are diminished. The negative component mediated by the KOR/dynorphin system, in contrast, is augmented. This is reflected by increased expression and functionality of the KOR and can even be enhanced by elevated anxiety induced by decreased DOR G-protein coupling (302).

In summary, **Study II** provides evidence for a severe dysregulation of the endogenous opioid system during alcohol dependence and abstinence. The alterations observed in the post-dependent animals are – to a certain degree – transferrable to the human situation and, thus, the post-dependent animal model provides a good tool to investigate mechanisms underlying alcohol dependence in detail.

5.3 **DISCUSSION STUDY III**: IMPACT OF CHRONIC NALTREXONE ON THE ENDOGENOUS OPIOID SYSTEM IN ALCOHOL DEPENDECE

The results of **Study I**, **II** and **IV** as well as the literature (for a summary see (235)) have proven the post-dependent animals to be a valid tool to study alcohol dependence and translate the results onto the human situation. Therefore, the impact of chronic NTX treatment on the endogenous opioid system has been investigated in this model.

The unspecific opioid antagonist NTX displays the highest affinity for the MOR (Ki=0.37 nM), followed by KOR (Ki=4.8 nM) and DOR (Ki=9.4 nM) (305). In alcohol-dependent patients, usually daily doses of 50 mg are recommended by the FDA. Even though half-life of NTX and its metabolite β6-naltrexol in humans is only four and eleven hours, respectively (176), a single of 50 mg NTX is sufficient to block the MOR for 48 - 72 hours (306). Doses that occupy about 80 – 90 % of the MOR occupy 50 - 80 % of KOR (307). DOR blockade is expected to be lower as NTX's affinity is lower. In Wistar rats, NTX's half-life is only about one hour and β6-naltrexol is not detectable (177). In the here presented study, a comparably high dose of NTX (2.5mg/kg, daily i.p. injections) was applied that was sufficient for blocking alcohol self-administration in rats but did not block alcohol intake the day after cessation of NTX treatment (personal communication Dr. Wolfgang Sommer).

MOR expression was upregulated in the ventral striatum following chronic NTX in alcohol exposed (=alcohol-dependent) and not exposed (non-dependent) rats but the main effects were found in the VTA. *Pomc* mRNA levels, the precursor of β-endorphin, were strongly upregulated in the median eminence, the main region of *Pomc* synthesis. The DOR showed an upregulation in the dorsal striatum. Strong effects were also found in the VTA, however, only in non-dependent animals. KOR binding sites were increased in the dorsal and ventral striatum of both not exposed and exposed animals.

To date, no studies on NTX effects on the molecular level in alcohol-dependent long-term abstinent rats have been published. NTX-induced alterations of opioid receptors and their ligands have mainly been investigated in actively drinking animals (111, 118, 268) focusing on the MOR. To better mimic the human situation where often detoxified alcoholics are treated by daily doses of NTX (see (37) and **Study I**), we chronically administered daily injections (2.5 mg/kg, i.p.) to alcohol abstinent rats. Several studies have shown that chronic treatment with opioid antagonists including NTX causes an increase of opioid receptors (111,

308-311) but no changes in affinity for the radioligand [^{3}H]-Damgo (312). However, this thesis is the first study to report on the expression levels of all opioid receptors by saturated receptor autoradiographies in alcohol-dependent long-term abstinent rats receiving chronic NTX-treatment.

Interestingly, the brain regions and opioid receptors analyzed appear to be differentially affected by NTX. MOR binding sites are increased in the ventral striatum but the main effects are observed within the VTA (Figure 20A). Investigating striatal brain regions, Oliva et al. (118) reported the strongest NTX effects on MOR function within the AbcS of actively drinking animals and suggested this might be the target region of NTX treatment. A series of studies proposed that the ventral striatum in alcohol dependent patients but not healthy controls developed increasing tolerance to the ability of alcohol to activate this region (239, 313, 314) which may be explained by the observed dependence-induced decreases in MORs. NTX elevates MOR density which could be the rational for increased striatal activity after NTX treatment as reported by others (313). Moreover, NTX is thought to remove the inhibitory tone on the VS that is exerted by KORs by blocking those receptors.

Within the VTA, MOR binding sites were increased by 61 % in not exposed and by 120 % in exposed rats after chronic NTX treatment. Activation of VTA MORs results in disinhibition of dopaminergic projection neurons and causes dopamine release in the ventral striatum (see feedback loop Figure 8). Blocking those receptors might be the mechanism by which NTX further attenuated alcohol-induced dopamine release in the striatum (174, 175).

Since *bArr2* mRNA levels are neither changed in the AcbS nor VTA of exposed and not exposed rats, the strong increase of receptor densities observed in those regions appears to be mediated by other mechanisms. For example, increases in MOR binding sites evoked by NTX and naloxone, a broad spectrum opioid antagonist, were accompanied by decreases in trafficking proteins G-protein receptor kinase 2 (GRK-2) and dynamin (DYN-2) (310, 315).

The involvement of DOR in NTX-treatment outcome in the alcohol-dependent animals is debatable. Although NTX displays the lowest affinity for the DOR (as compared to MOR and KOR), a comparably high dose of NTX (2.5mg/kg) was applied. Thus, this dose is assumed to be sufficient to also affect DORs. Animal studies found heterogeneous results of DOR blockade on alcohol intake and reward are heterogeneous (316-321). This and the strong down-regulation of DOR G-protein coupling in the striatum of alcohol exposed rats (**Study II**) might indicate only a small impact of DOR in the treatment outcome of NTX therapy.

The KOR, on the other hand, seems to be a useful target for the treatment of alcoholism as antagonizing the receptor results in a reduction of alcohol self-administration specifically in alcohol-dependent animals (40, 41, 156). Naltrexone is effective in blocking alcohol self-administration in alcohol-dependent rats but does so also in non-dependent animals (41). NTX and nalmefene show equal affinity for the MOR but nalmefene has a higher affinity to KOR. Equivalent low doses of both compounds reduce alcohol consumption in non-dependent animals, probably due to binding to MOR. However, the same dose of NTX was not sufficient to reduce alcohol intake in alcohol-dependent rats while nalmefene was efficient (41). This suggests that the compounds do not primarily exert their effects via the MOR in alcohol-dependent animals at low doses. As the results of **Study II** show, MOR binding sites are significantly reduced in post-dependent animals and the low doses of NTX might not be sufficient to occupy the remaining receptors. The superiority of nalmefene in reducing alcohol consumption in dependent animals most likely can be assigned to its higher activity at the KOR/dynorphin system which is upregulated during alcohol dependence and, thus, provides a good target.

Furthermore, NTX appears to be differently effective in increasing MOR binding sites in alcohol exposed than in not exposed animals which is especially marked within the VTA. Here, MOR binding sites were increased by 61 % in not exposed and by 120 % in exposed rats after chronic NTX treatment. Behaviorally, NTX dose-dependently decreased alcohol self-administration in alcohol exposed and control rats but might be more efficient in dependent animals (41). Differences in effect sizes most likely are due to differences in the basal state of the opioid system at which NTX therapy exert its effects. **Study II** revealed differences in opioid receptor expressions between control and post-dependent animals which were also observed in this study when comparing saline treated not exposed and saline treated exposed animals (see raw data in Suppl. Table 17, Suppl. Table 20, Suppl. Table 21). Thus, NTX most likely acts differentially in alcohol-dependent and non-dependent animals as baseline levels of opioid receptors are different. This shows the necessity to study NTX effects in alcohol dependent individuals and not healthy controls as outcomes can be different.

5.3.1 SUMMARY

In conclusion, the MOR and KOR were the main target of NTX treatment in the here applied experimental setup. More precisely, the MOR within the VTA and the KOR within the striatum appear to play a major role in NTX pharmacology. This suggests that NTX exerts its effects by affecting midbrain MORs and striatal KORs to induce its anti-relapse effects.

5.4 **DISCUSSION STUDY IV**: CONVERGENT EVIDENCE FROM ALCOHOL DEPENDENT HUMANS AND RATS FOR A HYPERDOPAMINERGIC STATE DURING ABSTINENCE

In this study, evidence is provided for a hyperdopaminergic state in protracted alcohol abstinence in humans as well as rats. Receptor autoradiographies in human post-mortem tissue of alcoholic subjects show significant reductions in striatal D1 receptors and DAT while D2 receptor levels are unchanged. In an additional analysis of D1 receptors in a larger, more heterogeneous sample of human alcoholics and controls, D1 downregulation is independent of the smoking state of the subjects or active alcohol consumption (positive blood alcohol levels at the time of death). These results are supported by a dynamic regulation of D1 and DAT in alcohol dependent animals with a pronounced reduction of both proteins after three weeks of alcohol abstinence. On a functional level, a lack of glutamatergic modulation upon stimulation of D1 was observed. Furthermore, substantia nigra pars compacta *TH* mRNA levels as well as basal extracellular dopamine in the AcbS is increased which is supported by a meta-analysis. Accumbal dopamine shows a blunted response to alcohol challenges. Behaviorally, post-dependent rats display hyperactivity. Taken together, all findings provide conclusive evidence for a hyperdopaminergic state during protracted abstinence. In accordance with the literature, the time course study suggests a dynamic regulation of the mesolimbic dopamine system during abstinence with a hypodopaminergic state during acute withdrawal (213, 322) and a hyperdopaminergic state that is characteristic for protracted abstinence (Figure 23 and Figure 27).

A major hypothesis in the alcohol research field proposes a hypodopaminergic state as driving force for alcohol relapse (322, 323). This view is supported by animal experiments (213) and PET studies that report a reduction of striatal D2-like receptor availability in alcoholic patients (204-210). However, other PET studies provide incoherent results (211, 212, 324). Therefore, saturated receptor autoradiographies were performed in this thesis to assess the number of dopamine receptors and the DAT. Interestingly, D1 receptors as well as DAT are significantly reduced in striatal human post-mortem tissue of human alcoholics as compared to controls while D2 receptor levels are unaltered. The reduction of D1 receptors is independent of "smoking" and alcohol consumption shortly before death. This implies rather a hyper- than a hypodopaminergic state and shows that the interpretation of PET results is challenging. The commonly used low-affinity radiotracers can easily be displaced by competing endogenous dopamine. Hence, a decreased PET signal does not necessarily result

from reduced receptor levels but can be caused by increased endogenous ligands. In fact, it has been shown that pharmacological manipulation of dopamine levels alters striatal D2 receptor availability as assessed by the widely used low affinity tracer [^{11}C]-raclopride (325). A recent study using the PET ligand [^{18}F]-fallypride which is less sensitive to endogenous dopamine levels (326) reports unaltered D2 receptor availability in abstinent alcoholics when compared to healthy controls (211).

A previous post-mortem study found a similar reduction of D1 binding sites as the present study (327). Furthermore, they observed a reduction of D2 receptors that is in apparent contrast to our data. This inconsistency can be caused by various factors. Some samples in the study of Tupala et al. (327), display high levels of alcohol or medication at the time of death. Although we show with our additional analysis in an extended sample set that D1 binding sites are not significantly changed by active alcohol use, this is not necessarily true for D2 binding sites. The reduction of DAT is in line with our and other observations in human *in* vivo studies and post-mortem tissue (264, 324, 328, 329). Moreover, increased dopamine synthesis was observed in *in vivo* human imaging studies by assessing the uptake of [^{18}F]-DOPA, an immediate precursor of dopamine synthesis (330).

Animal studies can provide further insight into alterations of the mesolimbic dopamine system in alcohol dependence. The focus of preclinical studies has been set on the withdrawal period where reward deficits associated with suppression of accumbal dopamine release has been observed (12, 216, 331). Remarkably, fewer efforts have been made to elucidate adaptations in the dopamine system in protracted abstinence which is the most relevant clinical condition in alcohol and other substance use disorders (12, 35, 223). To fill this gap, a meta-analysis of the existing rodent literature on concentrations of dopamine and its metabolites in the AcbS at different time points during abstinence was performed. We found evidence for increased concentrations during active alcohol consumption (day 0) that was followed by a decline during acute withdrawal. Around the 6th day of abstinence an increase in dopamine and its-metabolites has been found which is augmented in protracted abstinence. The general pattern of dopamine and its metabolites appears to be robust and seems to be regulated in an oscillatory-like manner over time, even though the method of dependence induction in rats varies among studies.

To confirm this pattern of regulation at the membrane level, autoradiographies were performed for the dopamine receptor D1 and D2 and the DAT in the AcbS, AcbC as well as

the CPu of alcohol dependent rats at different time points of abstinence according to previous studies (236, 332). A similar regulation of the dopamine receptors/DAT was found in all regions analyzed with no alterations of the D2 at any time point. Under conditions of alcohol load (day 0), D1 receptors are significantly decreased in response to increased availability of extracellular dopamine at this time point. This is followed by an increase of D1 until day 7 of abstinence which may be caused by the decrease of dopamine during acute withdrawal. At this time, dopamine concentrations are rising again ending the need for elevated D1 binding sites. When dopamine concentrations are high on day 21 of protracted abstinence, D1 binding sites are decreased again (Figure 27). This downregulation of D1 receptors in response to elevated dopamine levels is further supported on a functional level by electrophysiological data. Upon D1 stimulation a blunted modulation of glutamatergic transmission was observed in accumbal MSNs in the presence of alcohol.

The most prominent alterations were observed for DAT. On day 0, when alcohol is still present and dopamine levels are high, DAT levels are increased. Most likely plasma membrane recruitment and transport of DAT is increased to compensate for increased extracellular dopamine. Dopamine concentrations decline during acute withdrawal which is the followed by a reduction of DAT as part of a feedback regulation (333). The mechanisms underlying the regulation of the DAT at the different time points of abstinence remain unknown. During protracted abstinence, however, dopamine concentrations increase again which induces a decrease in DAT (Figure 27). This downregulation of DAT may reflect an important vulnerability factor for a 'relapse-prone' state of the reward circuitry in abstinence.

Furthermore, *TH* mRNA expression was increased during protracted abstinence suggesting increased dopamine synthesis. Indeed, an *in vivo* microdialysis experiment showed elevated extracellular dopamine levels within the AcbS. This is further supported by increased locomotor activity in abstinent post-dependent rats. In addition to increased dopamine levels in the AcbS, the microdialysis experiment showed a blunted accumbal dopamine response to acute alcohol injections in post-dependent rats in protracted abstinence which coincides with previous studies (207, 210). This is in line with reports from human PET studies after psychostimulant challenges in alcohol dependent patients. There are two possible interpretations for this lack of responsiveness: either response dynamics are state specific dependent on low or high extracellular dopamine levels or it reflects a relative dopamine deficit due to high chronic demands that have exhausted compensatory mechanisms.

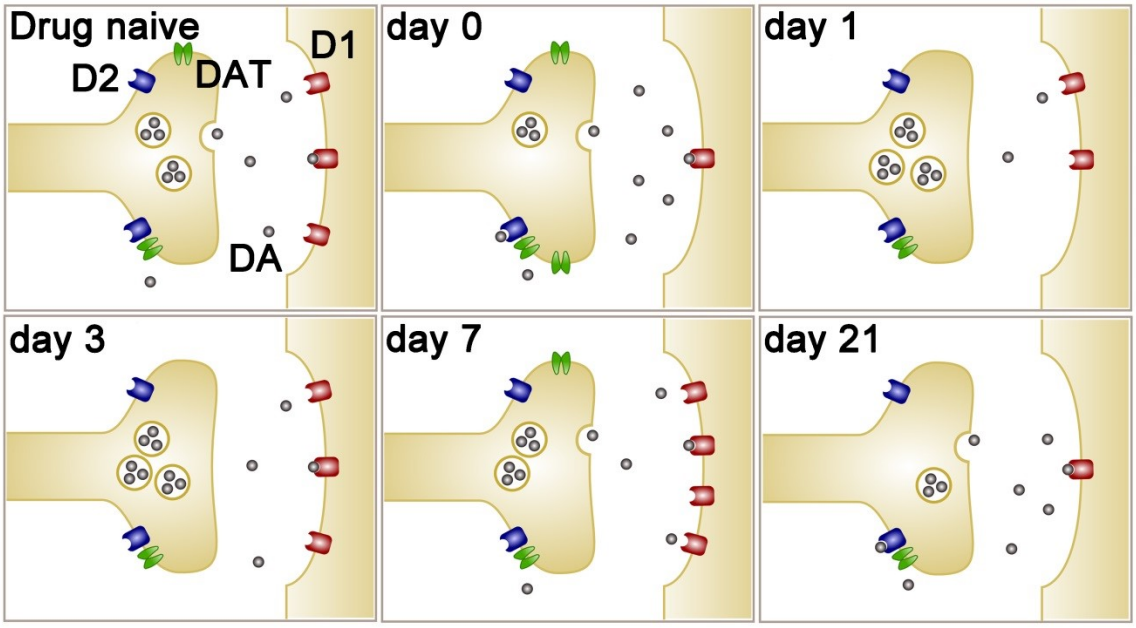

Figure 27: Schematic illustration of a dopaminergic synapse during the addiction cycle. Within the striatum of healthy individuals, D1 (red color) and D2 (blue color) receptors mainly are distributed onto different types of neurons to the post- and presynaptic site, respectively (192). DAT (green) is localized to the presynaptic site, where it is crucial for the termination of dopamine (DA, black circles) transmission and the maintenance of presynaptic dopamine storage (drug-naïve). Chronic alcohol exposure induces increases in extracellular dopamine resulting in decreased D1 and increased DAT (day 0). Cessation of alcohol exposure inhibits dopamine release causing a hypodopaminergic state with a compensatory increase of D1 and decrease of DAT during the first three days of acute withdrawal (day 1-3). After that, dopamine release is increasing, and subsequently D1 and DAT are increasing on the post- and presynaptic site (day 7). In protracted abstinence, extracellular DA concentrations are high (hyperdopaminergic state) which causes a reduction of both D1 and DAT (day 21). This state mirrors our post-mortem data from heavy alcoholics. D2 is not changed at any time point (days 0-21).

On a mechanistic level, the here reported decrease in D1 and DAT binding sites in alcoholic subjects as well as post-dependent rats on day 21 of abstinence can be explained in several ways. Repeated alcohol intoxication and subsequent chronic stimulation of the D1 may result in internalization and degradation of the receptor. Such a mechanism has been demonstrated after repeated administration of dopamine agonists and produced a lack of sensitivity to subsequent administration of dopamine agonists on a behavioral, biochemical, and electrophysiological level (334, 335). Moreover, there is an intrinsic relationship between DAT and D1 expression as DAT knockout mice display less D1 binding sites (336). Alternatively, the decrease of D1 could be associated with the hyperactive corticotropin-releasing hormone system (337). Indeed, increased activity of the extra-hypothalamic CRH system is well established in the post-dependent model (237).

During both, acute withdrawal and protracted abstinence, a high risk for alcohol relapse exists. According to the presented results, this increased vulnerability can be linked to either a hypo- or hyperdopaminergic state. In a hypodopaminergic state, relapse risk might be increased due to reward deficiency while in a hyperdopaminergic state hyperactivity and poor impulse control may cause vulnerability to relapse. Many biological functions dependent on a homeostatic regulation whereby too low as well as too high levels impair performance (338).

5.4.1 SUMMARY

In summary, this study shows the dynamic regulation of the mesolimbic dopamine system during acute alcohol exposure, withdrawal and protracted abstinence to extent our knowledge of the neurobiology of alcohol dependence and establishes the concept of a hyperdopaminergic state during alcohol abstinence. Enhanced dopaminergic activity during acute alcohol exposure is followed by a hypodopaminergic state that is characteristic for the first few days of alcohol withdrawal. Subsequently, counteradaptive changes involving D1, DAT and dopamine releasing properties result in a hyperdopaminergic state during protracted abstinence. To identify whether this hyperdopaminergic state is a vulnerability factor for craving and relapse in alcohol dependence clinical studies are warranted and may provide a window for specific interventions.

5.5 **GENERAL DISCUSSION:** THE INTERACTION OF THE ENDOGENOUS OPIOID AND DOPAMINE SYSTEM

In summary, this thesis gives conclusive evidence for a dysregulation of the dopamine and endogenous opioid system during abstinence in alcohol dependent humans and rats. The dopamine system is dynamically regulated and is characterized by a hypodopaminergic phase during acute withdrawal but by hyperdopaminergia during protracted abstinence (**Study IV**). The endogenous opioid system is closely associated with the dopamine system and modulates dopamine release in the Acb. The results show a significant reduction of MOR densities in the striatum of human alcoholics as well as post-dependent animals (**Study I** and **Study II**). Within the VTA, functionality of the MOR is decreased. The reduction in MOR density is counteracted by chronic naltrexone treatment (**Study III**). DOR binding sites are increased in all analyzed regions while coupling of the receptor to intracellular G-proteins is strongly reduced (**Study II**). The KOR system is upregulated (**Study II**).

Various studies revealed an interaction of the mesolimbic dopamine and opioid system. Opioid receptors are involved in the modulation of accumbal dopamine release, thereby contributing to the rewarding and reinforcing effects of alcohol (220, 222, 230, 339-342).

The VTA is mainly composed of dopaminergic (60-65 %) and GABAergic (30-35 %) neurons that project to various brain areas, including the striatum and amygdala (202). A simplified scheme of the neurocircuitry between VTA and Acb is shown in Figure 28A. Accumbal GABAergic medium spiny neurons (MSNs), the major striatal cell type (90-95 %, (202, 343)), project back to the VTA via a direct or indirect pathway. The direct pathway is characterized by D1 expressing neurons that co-localize dynorphin and substance P (192, 232, 344). MSNs of the indirect pathway are expressing D2 and enkephalin. They innervate the pallidum which in turn sends projections to the midbrain. Even though the distinction of D1-rich direct and D2-rich indirect pathways is more pronounced in the dorsal striatum, it is also observed in the ventral striatum/Acb (202). Activation of striatal D1 receptors facilitates signaling via the direct pathway through induction of long-term potentiation (LTP) on glutamatergic synapses (345, 346). In contrast, D2 stimulation blocks signaling via the indirect projections by induction of long-term depression (LTD) (347). These processes are crucial for reward- and aversion learning, respectively (348-351).

MSNs of the direct pathway synapse onto non-dopaminergic neurons in the VTA and their terminals have been proven to be sensitive to MOR agonists (352). Presynaptic activation of

these MORs results in hyperpolarization via G-protein dependent voltage-gated potassium channel pathways (353, 354). This removes GABAergic inhibition on dopaminergic neurons. The activity of MORs is crucial for the maintenance of baseline dopamine levels as well as firing in response to activating cues (220). In a non-dependent individual, alcohol intake results in activation of MORs by endogenous opioids and, thereby, increased striatal dopamine release (223).

Based on the results of this thesis and the literature a new model is proposed to describe the link between the observed hyperdopaminergic state and the strong reduction of MOR binding sites in alcohol abstinence.

In the ventral striatum of human alcoholics as well as long-term abstinent post-dependent rats, MOR binding sites are significantly decreased (Figure 28B). Striatal MORs have been shown to be involved in dopamine release in the striatum as intra-accumbens application of the MOR agonists fentanyl or Damgo increase accumbal dopamine (355). However, another study did not observe this effect (220). These differences might be due to different agonist concentrations used and other methodological differences (355). In the Acb, MORs are expressed, among others, on corticostriatal terminals, extrasynaptically on MSN dendrites (356, 357), and presynaptically on GABAergic afferents (357, 358). Thus, they are ideally located to modulate the activity of striatal neurons.

Acute alcohol consumption releases endogenous opioids, including β-endorphin (104, 231), and the presynaptic activation of MOR on GABAergic afferents might result in elevated activity of MSNs and disinhibition of dopaminergic neurons. Consequently, extracellular dopamine release is transiently increased within the striatum and induces reward-learning by activating the direct pathway. In contrast, the indirect pathway, and thus aversion-learning, remains silent as elevated dopamine concentrations act on the D2 receptor (Figure 28B).

The proposed model (Figure 28C) suggests that a decrease in MORs in the striatum during abstinence results in reduced inhibition of GABAergic MSNs. This might be due to diminished MOR-mediated inhibition of cortical glutamatergic inputs to the striatum (359-361). Consequently, MSN efferents to the VTA are increasingly active and exert inhibition on GABAergic VTA neurons. Since these VTA neurons regulate the activity of dopaminergic afferents to the striatum, this causes elevated striatal dopamine release as measured as a hyperdopaminergic state by *in vivo* microdialysis (**Study IV**).

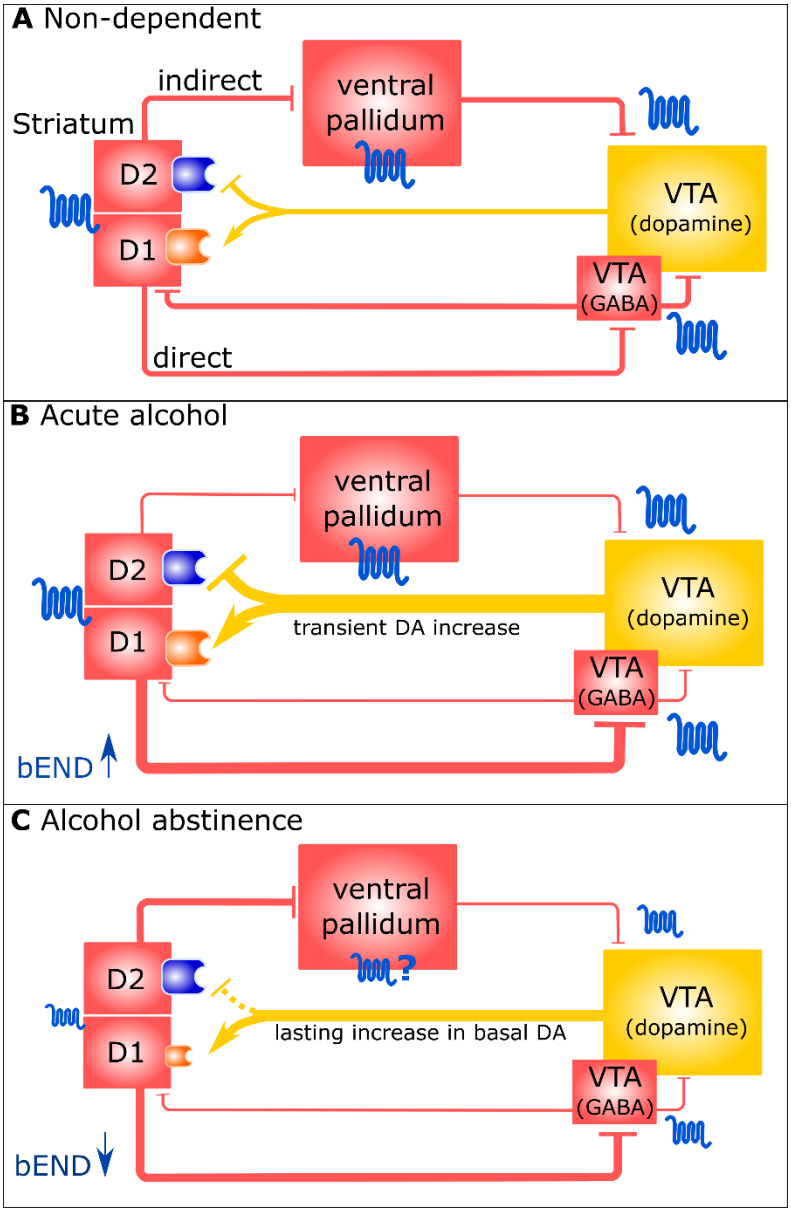

Figure 28: Feedback loop between the ventral striatum and the VTA. D1 expressing striatal GABAergic medium spiny neurons (MSNs) innervate GABAergic VTA neurons via the direct pathway. D2 expressing MSNs project via an indirect pathway to the VTA. (**A**) Dopaminergic VTA neurons are under inhibitory control by GABAergic inputs. Disinhibition of the dopamine neurons, i.e. by activation of MOR, results in striatal dopamine release. Activation of striatal D1 receptors facilitates signaling through the direct pathway which is associated with reward learning. D2 stimulation blocks the indirect pathway and, thereby, aversion-learning. (**B**) Acute alcohol intake activates the MOR in the striatum and VTA resulting in disinhibition of dopamingeric VTA neurons and dopamine release in the striatum is transiently increased. By stimulating D1 receptors the direct pathway is activated causing reward learning. (**C**) In alcohol dependence, MORs are strongly decreased in the striatum resulting in elevated GABAergic input on VTA GABA neurons. Thus, dopaminergic neurons are disinhibited and a hyperdopaminergic state can be observed within the striatum. Due to elevated basal dopamine levels, the direct pathway might be continuously activated and the system decreases D1 receptors to counteract. The indirect pathway may be activated as well since D2 receptors might be desensitized by long-term elevated dopamine levels (362). DA – dopamine, bEND – β-endorphin, inhibitory projections (GABAergic) – red, modulatory (dopaminergic) – yellow

The hyperdopaminergic state in post-dependent animals is accompanied by decreased striatal D1 and unchanged D2 binding. Densities of the D1 receptor most likely are decreased to counteract the elevated dopamine concentrations (334, 335). As compared to the situation of acute alcohol administration, the direct pathway might still be activated but to a lesser degree. Consequently, the rewarding effects of alcohol would be diminished. Although D2 receptor densities are unaltered in post-dependent animals, the proposed model suggests increased activation of the indirect pathway during alcohol abstinence (Figure 28C). Most likely, a new allostatic state developed in the dopamine and opioid system in alcohol dependence and the system adapts to these new conditions. The elevated dopamine concentrations in the striatum can lead to desensitization of the D2 receptor, e.g. by functional uncoupling of the receptor (362), which might be linked to elevated β-arrestin2 levels as found in **Study II** (363). The D2 would now need even higher dopamine levels to effectively block the "aversion"-pathway. Alcohol challenges, however, showed only blunted dopamine responses. Thus, the hypothetical combination of a decrease in activity of the direct ("reward") and increase in the indirect ("aversion") pathway may contribute to the elevated negative emotional states and vulnerability to relapse in alcohol abstinence.

In the post-dependent animals, an increase in striatal MOR G-protein coupling has been found and is interpreted as a compensatory mechanism. However, this is unlikely to be sufficient to counteract the significant reduction in MOR density levels as the endogenous ligand, β-endorphin, is reduced during abstinence as well (**Study I**, **II**, and (147, 287, 288)). Furthermore, in heavy human alcoholics the reduction in striatal MOR binding sites is even more pronounced suggesting that an increase in G-protein coupling of the remaining receptors would not be sufficient to restore MOR function.

Within the VTA, MOR binding sites are unchanged and the proposed model (Figure 28C) assumes that VTA MORs do not considerably contribute to the hyperdopaminergic state in alcohol abstinence. However, G-protein coupling is slightly decreased in this area indicating disruptions in MOR signaling. In combination with diminished alcohol-induced β-endorphin release this might lead to a dysfunctional disinhibition of dopaminergic neurons upon alcohol intake. This may be reflected by the blunted dopamine release in response to an alcohol challenge (**Study IV**) and might contribute to a reward deficit.

The anti-relapse pharmacotherapy NTX has been proven to be efficient in post-dependent animals (235). In abstinent rats subjected to seven weeks of CIE, 14 days of daily NTX injections resulted in strongly increased MOR binding sites in the AcbS and VTA. Thus, the NTX-induced increase in MOR density and *Pomc* is suggested to counteract the alcohol induced receptor/ligand losses and restore normal receptor function. However, based on the here presented data it cannot be explained how NTX counteracts alcohol-induced dopamine release (174, 175). To answer this question, more studies have to be conducted resolving the precise localization of elevated MOR in the involved brain regions. Moreover, analyzing the effect of NTX on the dopamine receptors and dopamine release in the post-dependent animals would be helpful.

Certainly, the proposed model has limitations. First, the analysis of binding sites by autoradiography methods is not suitable to distinguish cell types expressing the MOR. For this reason, it is unclear if the reduction of striatal MOR is found mainly on dendrites or terminals. Depending on cell type and cellular localization activation of MOR can have inhibitory or excitatory effects. Second, the model only considers the ventral striatum and VTA. The feedback loop including the dorsal striatum and substantia nigra is excluded. However, it might be assumed that similar processes are involved in the opioid and dopamine systems in those regions. **Study IV** reveals a comparable regulation of the dopamine receptors in the dorsal striatum suggesting that a hyperdopaminergic state is also present there. Moreover, no data are available on the ventral pallidum and should be collected to strengthen the model. Third, additional MOR-sensitive GABAergic terminals arising from cell populations outside the VTA to modulate dopamine output that have not been studied in this thesis. This, for example, includes GABAergic neurons directly projecting from the rostral medial tegmental nucleus onto dopaminergic neurons within the VTA (364, 365). It has also been proposed that projections from the CeA modulate the activity of GABAergic neurons within the VTA and this can result in disinhibition of dopaminergic projection neurons to the striatum (366). Moreover, agonist-stimulation (Damgo) of MORs can directly excite VTA neurons, including dopaminergic neurons via opening of $Ca_V2.1$ channels. This effect is independent of GABA or glutamate signaling (367). Fourth, the opioid receptors DOR and KOR also modulate striatal dopamine release but have not been included in the model.

Agonist stimulation of DORs but not KORs results in elevation of dopamine in the striatum (220, 222, 368). DORs are predominantly expressed presynaptically and localized on axons and axon terminals to regulate dopaminergic and glutamatergic activity (369-371). Although to a lower extent, DORs are also expressed postsynaptically to regulate the responses of MSNs (370). In the VTA, the DOR is expressed at significantly lower levels than MOR. However, receptor density as well as signaling is increased in post-dependent abstinent rats. This upregulation might be a compensatory mechanism to counteract diminished MOR functionality and maintain dopaminergic signaling at least at lower levels.

KORs within the striatum are expressed on dopamine terminals, on GABAergic inputs to the AcbS, and, to a lesser extent, on presumably excitatory synapses (163, 372, 373). Importantly, agonist-activation of KORs in the striatum reduces basal dopamine release while antagonists, such as nor-BNI, enhance dopamine (220, 230). In this thesis, it was methodologically not possible to detect KOR within the VTA. Nevertheless, KORs are expressed in this brain region, e.g. presynaptically on glutamatergic inputs to the VTA and directly on VTA dopamine neurons (163, 374, 375). However, stimulation of KORs within this brain region does not modulate striatal dopamine responses but rather decreases prefrontal dopamine release (220, 222, 375).

5.5.1 SUMMARY

Together, this provides evidence for an opposing regulation of dopamine by the opioid receptors MOR/DOR and KOR. Furthermore, the observations that KOR agonists produce aversive and dysphoric effects implies that a blunted dopamine release is responsible for the behavioral and emotional changes (340). The increase in KOR signaling within the striatum of post-dependent rats (**Study II**) may contribute to the blunted dopamine response to an alcohol challenge (**Study IV**) and contribute to anhedonic emotional states that further enhance drug-seeking.

6 SUMMARY AND OUTLOOK

By combining the analysis of human post-mortem tissue of alcoholics and an animal model of alcohol dependence this thesis gives important insight into the regulation of the endogenous opioid and dopamine system. Dependence-induced neuroadaptations in these neurotransmitter systems are characterized in detail and new models of the time course and consequences of these neuronal changes are proposed.

The analysis of the endogenous opioid system revealed reduced functionality of the MOR/DOR but an increase in the KOR system. It is suggested that these alterations are, at least in part, responsible for the elevated anhedonic states during alcohol abstinence and are a vulnerability factor for alcohol relapse. Furthermore, the data indicate a disruption of opioidergic modulation of dopamine transmission during alcohol abstinence. This might result in increased basal dopamine levels, blunted dopamine release in response to alcohol cues and anhedonic states. NTX may counteract these neuroadaptations.

Based on the time-dependent regulation of the dopamine system during alcohol withdrawal and abstinence, it can be hypothesized that neurotransmitter systems do not assume a static new state but rather show an oscillatory-like behavior. The deviations from the state in healthy subjects, either increased or decreased activity of the system, lead to phases of vulnerability which are interspersed with phases of higher stability when the state of the system resembles more the state of a healthy subject.

However, further studies are needed to investigate these hypotheses in detail.

The findings of the applied translational approach provide a solid basis for the design of future investigations and suggest a reinterpretation of previous PET results. It is proposed that PET studies ideally should be combined with the analysis of human post-mortem tissue to optimize the gain of knowledge and achieve more precise pictures of neuronal changes underlying diseases.

Taken together, this thesis is an important step towards the development and/or improvement of pharmacological therapies for alcoholism by providing a detailed picture of dependence-induced neuroadaptations in protracted abstinence that are relapse-preventing targets.

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9 APPENDIX

Suppl. Table 1: Demographic data and tissue characteristics of human post-mortem subjects.

Age	PMI	Brain pH	Clinical cause of death	Toxicology	DSM IV Alcohol class	Smok-ing	A118G
34	8.5	6.61	Hanging	Blood alcohol 0.341g/100ml	Alcohol abuse Chronic > 80g	Yes	AG
54	17	6.41	Chest and abdominal injury	Blood alcohol 0.016g/100ml	Alcohol abuse Chronic > 80g	Yes	AA
46	24	6.51	Alcohol toxicity	Blood alcohol 0.315g/100ml, Nordiazepam 0.2mg/l	Alcohol abuse Chronic > 80g	?	AA
51	27	5.58	Gastrointestinal haemorrhage	Blood alcohol 0.119/100ml	Alcohol abuse Chronic > 80g	Yes	AG
50	24	6.59	Gastrointestinal haemorrhage, cirrhosis	Blood alcohol 0.241g/100ml	Alcohol abuse Chronic > 80g	Yes	AG
73	24	6.3	Cirrhosis	Blood alcohol 0.118g/100ml	Alcohol abuse Chronic > 80g	No	AA
56	45	6.51	Bleeding oesophageal varices	Blood alcohol 0.283g/100ml	Alcohol abuse Chronic > 80g	?	AA
37	17	6.33	Acute alcohol poisoning	Blood alcohol 0.479g/100ml Carbamazepine 1mg/l	Alcohol abuse Chronic > 80g	No	AA
25	44	6.7	Carbon monoxide and alcohol intoxication	Blood alcohol 0.193g/100ml CO saturation >80 %	Alcohol abuse Chronic > 80g	?	AA
61	21	6.93	Hypertensive heart disease and chronic alcoholism	Blood alcohol 0.020g/100ml Metoprolol 0.5mg/l	Alcohol abuse Chronic > 80g	Yes	AA
42	41	6.5	Combined bromoxynil and alcohol toxicity	Blood alcohol 0.174g/100ml CNS Drugs (DL:01mg/l), Bromoxynil 1.5mg/l	Alcohol abuse Chronic > 80g	No	AG
60	17	6.48	Alcoholism liver cirrhosis and drug toxicity	Blood alcohol: 0,017g/100ml, Codeine	Alcohol abuse Chronic > 80g	Yes	AA
55	48	7.02	Ischaemic heart disease	Blood alcohol 0.246g/100ml, Diazepam 0.8mg/l, Nordiazepam 0.5mg/l	Alcohol-dependent Chronic > 80g	Yes	AA
64	39	6.76	Acute alcohol toxicity	Alcohol 0.293g/100ml	Alcohol-dependent Chronic >80g	Yes	AA
55	17	6.85	Asphyxia due to choking with food	Alcohol 0.206g/100ml, Amiodarone 1.2mg/l, Nordiazepam 0.1mg/l, Paracetamol 4mg/l, valporic acid <10mg/l	Alcohol abuse Chronic > 80g	No	AG
59	35	6.57	Coronary artery thrombosis	Alcohol 0.063g/100ml	Harmful Heavy 50-80g	Yes	AA
61	28	5.29	Multiple organ failure	-	Alcohol abuse Chronic > 80g	?	AA
67	48	6.4	Acute bronchopneumonia, morphine toxicity	Morphine 3mg/l, Nordiazepam 0.2mg/l, paracetamol 5mg/l	Alcohol abuse Chronic > 80g	Yes	AA
53	57	6.75	Chronic airflow limitation		Alcohol abuse Chronic > 80g	Yes	AA
41	54	6.7	Epilepsy, chronic alcoholism	Δ-9-THC acid 0.01mg/l, Δ-9-THC 0.005mg/l, Phenytoin 0.1mg/l	Alcohol abuse Chronic > 80g	Yes	AA
60	51	6.7	Hepatic cirrhosis	Paracetamol 22mg/l	Alcohol abuse Moderate 20-50g	No	AG

Suppl. Table 1 (continued)

73	44	6.59	Coronary artery atheroma		Alcohol abuse Chronic > 80g	No	AA
54	27	6.16	Ischaemic heart disease	Δ-9-THC acid 0.01mg/l, Amiodarone 5.0umol/l, marijuana breakdown product	Alcohol abuse Chronic >80g	Yes	AG
56	67	6.47	-	-	Alcohol abuse Chronic >80g	Yes	AG
58	45	6.47	-	-	Alcohol abuse Heavy 50-80g	Yes	AA
65	72	6.88	Acute intracerebral haemorrhage (right caudate), cerebral vascular malformation	-	Alcohol-dependent Heavy 50-80g	Yes	AA
41	39	6.55	Alcohol related	-	Alcohol abuse Heavy 50-80g	Yes	AG
69	22		Prescription drug overdose	-	Alcohol-dependent Chronic > 80g	Yes	AA
63	26	6.21	Combined effects of ischemic heart disease and chronic lung disease	Paracetamol < 3mg/l	Alcohol abuse Chronic >80g	Yes	AA
70	32	6.05	Sepsis, alcohol liver disease	-	Alcohol abuse Chronic > 80g	?	AA
65	32	5.66	Complication of chronic alcoholism	Moclobemide: 17mg/l, , Phenytoin 6mg/l, Paracetamol7mg/l, Quinine 0.4mg/l	Alcohol abuse Chronic > 80g	?	AA
52	46	6.78	Lobar pneumonia and chronic alcoholism	-	Alcohol abuse Chronic > 80g	Yes	AG
61	28	5.87	Liver failure	Metoclopramide <0.1mg/l,	Alcohol-dependent Chronic >80g	Yes	AG
66	12	6.14	Pneumonia	-	Alcohol abuse Chronic >80g	Yes	AA
39	24	6.56	Aortic stenosis	-	Chronic >80g	Yes	AG
70	34	6.24	Respiratory failure	-	Alcohol abuse Heavy 50-80g	Yes	AG
56	15	6.66	Ischaemic heart disease and emphysema		Alcohol abuse Chronic >80g	?	AG
50	17	6.3	Ischaemic heart disease	-	Alcohol abuse Chronic >80g	?	AA
58	20	6.64	Ischaemic heart disease, cirrhosis	Guaiphenesis 8.5mg/l, ibuprofen 3.5 mg/l, paracetamol 16 mg/l	Alcohol abuse Chronic >80g	Yes	AG
43	29	6.29	Intra-abdominal haemorrhage, complications of sepsis, multiple abdominal surgeries, massive hepatic necrosis, chronic hepatitis, chronic cholecystitis	-	Alcohol abuse Chronic >80g	Yes	AG

Suppl. Table 1 (continued)

58	22	6.65	Focal acute and chronic pancreatitis	-	Alcohol abuse Chronic >80g	Yes	AA
73	19	6.84	Ischeamic bowl, atherro-sclerotic cardio-vascular disease	-	Alcohol abuse Heavy 50-80g	Yes	AG
45	19	6.57	-	-	Alcohol dependence Chronic >80g	Yes	AG
43	13	6.43	Thrombotic coronary artery occlusion	-	Control <20g	Ex-	AA
51	20	5.88	Cardiac tamponade	-	Control <20g	?	AA
46	25	6.65	Mitral valve prolapse	-	Control <20g	?	AA
44	50	6.6	Ischaemic heart disease	-	Control <20g	Ex-	AA
63	72	6.9	Severe coronary artery atherosclerosis	-	Control < 20g	Ex-	AA
69	16	6.6	Atherosclerotic cardiovascular disease	Paracetamol 23mg/l, 1% blood saturation of CO (low)	Control 20-50g	Yes	AA
63	24	6.94	Atherosclerotic cardiovascular disease	Atenolol <1mg/l	Control 20-50g	Yes	AA
73	48	6.8	Dilated cardiomyopathy, ischaemic heart disease	-	Control <20g	Yes	AG
64	9.5	6.94	Ischaemic heart disease	-	Control 20-50g	Yes	AA
73	51	6.82	Congestive cardiac failure, atrial fibrillation, ischemic heart disease	-	Control 20-50g	Yes	AA
53	27	6.64	Acute myocardial infarct of the anterolateral wall of the left ventricle, atherosclerotic coronary artery disease with 90% stenosis of the left marginal artery	-	Control <20g	?	AA
24	43	6.27	Idiopathic cardiac arrhythmia	-	Control < 20g	Yes	AG
55	39	6.89	Coronary artery atherosclerosis	Irbesartan 0.4mg/l	Control <20g	No	AG
64	40	6.68	Coronary artery thrombosis	-	Control 20-50g	No	AA
59	43	6.69	Atherosclerotic cardiovascular disease		Control 20-50g	Yes	AA
68	46	6.12	Ischaemic heart disease	Amiodarone 0.7mg/l, Paracetamol 3mg/l	Control <20g	No	AG
59	40	6.53	Ischaemic heart disease, coronary atherosclerosis	Amiodarone 1.9mg/l	Control <20g	Ex	AA
55	12	6.39	Hypertensive heart disease	-	Control 20-50g	No	AA
73	39	6.28	-	-	Control <20g	Ex	AA
66	63	6.91	-	-	Control <20g	No	AA
62	46	6.95	-	-	Control <20g	Ex	AA
36	34	6.67	-	-	Control <20g	Ex	AA

Suppl. Table 1 (continued)

60	25	6.7	Bacterial peritonitis, ascites, carcinomatosis, gastrointestinal stomach tumor	-	Control 20-50g	No	AA
37	21	6.64	Ischaemic heart disease	-	Control <20g	?	AA
47	38	6.74	Dilated Cardiomyopathy, morbid obesity	Blood alcohol 0.029g/100ml	Control <20g	Yes	AA
50	29	6.68	Ischeamic heart disease	-	Control <20g	No	AA
55	8	6.9	Atherosclerotic cardiovascular disease	Amphetamines positive, THC positive	Control <20g	?	AA
68	22	6.59	Suicide by hanging. Asphyxia	Citalopram 0.4mg/l, Thioridazine 0.6mg/l, Mianserin <0.1mg/l	Control <20g	Yes	AA
59	20	6.56	Coronary thrombosis	-	Control <20g	Yes	AA
56	37	6.76	Ventricular scarring, hypertension and cardiomegaly	-	Control <20g	Yes	AA
60	28	6.8	Ischaemic heart disease	-	Control 20-50g	No	AA
69	19	6.34	Cardiac tamponade, acute myocardial infarction	-	Control <20g	No	AA
54	28	-	Cardiac arrest	-	Control <20g	Ex	AA
38	14	6.26	Atherosclerotic cardiovascular disease	-	Control <20g	Yes	AA
53	16	6.84	Dilated cardiomyopathy	Lignocaine 0.9mg/l, Sotalol 3.8µmol/l	Control< 20	No	AA
48	24	6.73	Ischaemic heart disease	-	Control <20g	Yes	AG
57	18	6.6	Ischaemic heart disease	-	Control <20g	Ex	AA
66	23	6.74	Ischaemic and hypersensitive heart disease	Irbesartan 0.6mg/l, Sulphapyridine detected	Control <20g	Ex	AA
56	19	6.9	Atherosclerotic coronary artery disease	-	Control <20g	No	AA
60	22	6.66	Ischaemic heart disease	-	Control <20g	No	AA
50	19	6.26	Ischaemic heart disease	-	Control <20g	Ex	AA
34	21	6.73	Acute exacerbation of asthma	-	Control <20	Yes	AA
58	12	6.46	Ischeamic heart disease	-	-	Yes	AA

'Core samples' of alcoholics and controls are highlighted in grey.

Study I

Suppl. Table 2: *Post-mortem study* - Results genotype x condition interaction analysis in striatal post-mortem brain tissue. No significant influence of the A118G genotype (rs1799971) on OPRM1 transcript (qRT-PCR) or MOR binding sites expression (autoradiography) was detected.

Nucleus caudatus		**qRT-PCR** F[2,55]=1.08, p=ns			**Receptor autoradiography** F[2,74]=1.11, p=ns		
Group	**Genotype**	Mean (ddCt)	SEM	N	Mean (fmol/mg)	SEM	N
Control	AA	0.00	0.14	27	168.75	7.39	34
	AG	0.00	0.36	4	145.71	17.58	6
Non-intoxicated	AA	-0.59	0.25	8	109.15	12.63	12
	AG	-0.91	0.24	9	119.02	11.94	13
Intoxicated	AA	-0.63	0.24	9	123.94	13.00	11
	AG	-0.15	0.36	4	98.65	19.66	5

Ventral striatum		**qRT-PCR** F[2,56]=2.24, p=ns			**Receptor autoradiography** F[2,72]=0.28, p=ns		
Group	**Genotype**	Mean (ddCt)	SEM	N	Mean (fmol/mg)	SEM	N
Control	AA	0.00	0.13	31	122.14	6.83	34
	AG	0.00	0.35	4	115.70	16.25	6
Non-intoxicated	AA	-0.32	0.27	8	101.75	11.04	13
	AG	-1.15	0.25	7	82.17	12.00	11
Intoxicated	AA	-0.41	0.24	9	69.06	12.59	10
	AG	-1.58	0.41	3	42.13	19.90	4

Suppl. Table 3: *PET study* - Cox regression of the association of μ-opioid receptor (MOR) availability and relapse risk controlling for OPRM1 genotype, sex, age, smoking and medication (naltrexone /placebo). NC – nucleus caudatus, Put – putamen, VS – ventral striatum, ST – total striatum, Cov – covariate, FTND – Fagerström Test for Nicotine Dependence

MOR -BP_{ND} and relapse risk		**controlling for OPRM1 genotype**			**controlling for *sex***			**controlling for age**			**controlling for *smoking (FTND)***			**controlling for medication (*nal/pla)***	
		B	**P**		**B**	**P**		**B**	**P**		**B**	**P**		**B**	**P**
NC	NC	-1.4	0.09	NC	-1.6	0.07	NC	-1.7	0.07	NC	-1.4	0.12	NC	-1.3	0.11
Cov	gene	-0.5	0.34	Sex	0.4	0.37	Age	-0.0	0.18	FTND	0.5	0.44	Med	-0.0	0.97
Put	Put	-2.1	0.03	Put	-2.1	0.04	Put	-2.2	0.04	Put	-2.0	0.06	Put	-2.1	0.04
Cov	gene	-0.6	0.26	Sex	0.2	0.62	Age	-0.0	0.26	FTND	0.01	0.86	Med	0.2	0.96
VS	VS	-1.2	0.07	VS	-1.2	0.08	VS	-1.4	0.04	VS	-1.1	0.12	VS	-1.2	0.10
Cov	gene	-0.6	0.29	Sex	0.3	0.49	Age	-0.0	0.14	FTND	0.03	0.63	Med	-0.0	0.93
ST	ST	-1.8	0.04	ST	-1.8	0.04	ST	-1.9	0.04	ST	-1.6	0.08	ST	-1.7	0.06
Cov	gene	-0.6	0.29	Sex	0.3	0.49	Age	-0.4	0.18	FTND	0.03	0.67	Med	0.0	1.0

Study II

μ-opioid receptor

Suppl. Table 4: [^{3}H]-Damgo receptor autoradiography in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	**F-value**	**p-value**
AcbS	378.96 ± 13.83	6	342.06 ± 5.19	6	[1,10]=6.24	0.032*
AcbC	165.20 ± 2.90	6	149.36 ± 5.16	6	[1,10]=7.15	0.023*
CPu	279.52 ± 30.46	6	249.47 ± 24.94	6	[1,10]=0.58	0.462
VTA	135.58 ± 10.63	5	123.47 ± 7.01	6	[1,9]=0.96	0.352
CeA	54.36 ± 5.68	6	92.79 ± 9.74	6	[1,10,]=11.61	0.007**
BLA	922.76 ± 52.62	6	668.05 ± 53.17	5	[1,9]=11.41	0.008**

Suppl. Table 5: Damgo-stimulated [^{35}S]GTPgS autoradiography

Region	**[^{35}S]GTPgS baseline** not exposed	**[^{35}S]GTPgS % stimulated** not exposed	**n**	**[^{35}S]GTPgS baseline** exposed	**[^{35}S]GTPgS % stimulated** exposed	**n**	**F-value**	**p-value**
AcbS	584.14 ± 17.99	50.31 ± 1.54	6	656.09 ± 21.73	84.12 ± 11.87	8	[1,12]=5.90	0.032*
AcbC	517.03 ± 27.94	14.05 ± 1.11	7	649.03 ± 28.90	49.04 ± 6.82	5	[1,10]=36.35	0.000***
CPu	534.53 ± 34.01	48.95 ± 2.32	5	657.32 ± 20.74	61.43 ± 6.57	7	[1,10]=2.36	0.155
VTA	376.33 ± 16.21	113.43 ± 4.13	8	387.68 ± 23.04	98.53 ± 5.42	7	[1,12]=4.79	0.049*

Suppl. Table 6: *Oprm1 in situ* hybridization in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	13.63 ± 0.32	6	14.61 ± 0.68	6	[1,10]=1.69	0.22
AcbC	7.20 ± 0.78	6	6.03 ± 0.89	6	[1,10]=0.98	0.35
CPu	5.37 ± 0.46	6	5.83 ± 0.65	6	[1,10]=0.46	0.51
VTA	3.87 ± 0.73	5	2.58 ± 0.24	5	[1,8]=2.81	0.13

Suppl. Table 7: *Pomc in situ* hybridization in alcohol-dependent rats

	Not exposed		**Exposed**			
ME	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
active cycle	369.97 ± 18.80	8	307.67 ± 12.42	7	[1,13]=7.17	0.019*

Suppl. Table 8: *bArr2 in situ* hybridization in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	7.27 ± 0.46	8	11.02 ± 0.45	7	[1,13]=33.41	0.00006***
AcbC	6.07 ± 0.53	8	9.32 ± 0.75	7	[1,13]=12.97	0.003**
CPu	4.30 ± 0.22	7	4.74 ± 0.29	8	[1,13]=1.39	0.26
VTA	8.72 ± 0.53	7	8.96 ± 0.31	7	[1,12]=0.16	0.70

δ-opioid receptor

Suppl. Table 9: [^{3}H]-DPDPE receptor autoradiography in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	**F-value**	**p-value**
AcbS	105.62 ± 4.17	8	116.91 ± 3.73	8	[1,14]=4.07	0.06
AcbC	59.21 ± 1.28	6	80.33 ± 4.66	8	[1,12]=14.51	0.002**
CPu	115.18 ± 4.05	8	131.82 ± 3.28	8	[1,14]=10.19	0.007**
VTA	10.61 ± 0.46	8	14.27 ± 1.26	8	[1,14]=7.45	0.02*

Suppl. Table 10: DPDPE-stimulated [^{35}S]GTPgS autoradiography

Region	**[^{35}S]GTPgS baseline** not exposed	**[^{35}S]GTPgS % stimulated** not exposed	**n**	**[^{35}S]GTPgS baseline** exposed	**[^{35}S]GTPgS % stimulated** exposed	**n**	**F-value**	**p-value**
AcbS	313.47 ± 19.95	51.16 ± 6.93	6	416.61 ± 18.03	20.58 ± 2.04	7	[1,11]=20.58	0.0009***
AcbC	312.83 ± 20.59	43.88 ± 5.54	8	392.75 ± 16.11	15.88 ± 2.05	7	[1,13]=20.10	0.0006***
CPu	294.01 ± 12 01	62.48 ± 5.97	8	377.90 ± 17.25	25.05 ± 3.76	8	[1,14]=28.16	0.0001***
VTA	240.01 ± 6.46	16.64 ± 3.33	4	279.15 ± 21.94	31.50 ± 2.81	5	[1,7]=11.78	0.01*

Suppl. Table 11: *Oprd1 in situ* hybridization in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	9.69 ± 0.43	6	8.49 ± 0.56	6	[1,10]=2.95	0.12
AcbC	3.85 ± 0.41	6	2.89 ± 0.11	6	[1,10]=4.93	0.05
CPu	6.63 ± 0.41	6	4.73 ± 0.28	6	[1,10]=14.79	0.003**
VTA	1.68 ± 0.08	6	2.11 ± 0.10	4	[1,8]=10.71	0.01

Suppl. Table 12: *Penk in situ* hybridization in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	467.27 ± 589.26	6	589.26 ± 32.06	6	[1,10]=13.11	0.005**
AcbC	216.62 ± 3.68	6	269.31 ± 6.76	6	[1,10]=46.85	0.00004***
CPu	420.37 ± 31.45	6	444.17 ± 16.96	16.96	[1,10]=0.44	0.52
VTA	6.40 ± 0.44	6	5.03 ± 0.37	5	[1,9]=5.35	0.046*

κ-opioid receptor

Suppl. Table 13: [^{3}H]-U69,593 receptor autoradiography in alcohol-dependent rats

	Not exposed		**Exposed**			
Region	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	**F-value**	**p-value**
AcbS	50.20 ± 2.02	7	68.56 ± 3.47	8	[1,13]=19.34	0.0007***
AcbC	41.42 ± 1.61	8	50.57 ± 3.14	8	[1,14]=6.75	0.02*
CPu	13.12 ± 0.74	7	16.93 ± 1.04	8	[1,13]=8.48	0.01*
VTA	n.d.	-	n.d.	-	-	-

Suppl. Table 14: U50,488H-stimulated [^{35}S]GTPgS autoradiography

Region	**[^{35}S]GTPgS baseline** not exposed	**[^{35}S]GTPgS % stimulated** not exposed	**n**	**[^{35}S]GTPgS baseline** exposed	**[^{35}S]GTPgS % stimulated** exposed	**n**	**F-value**	**p-value**
AcbS	287.49 ± 10.86	8.40 ± 2.88	7	288.73 ± 8.16	21.24 ± 2.08	7	[1,12]=13.03	0.004**
AcbC	284.57 ± 15.02	12.90 ± 1.02	6	279.17 ± 10.13	10.33 ± 0.85	7	[1,11]=3.80	0.08
CPu	259.70 ± 11.06	60.70 ± 0.58	5	264.75 ± 7.80	4.41 ± 2.81	8	[1,11]=1.03	0.33
VTA	n.d.	n.d.	-	n.d.	n.d.	-	-	-

Suppl. Table 15: *Oprk1 in situ* hybridization in alcohol-dependent rats

	Not exposed		Exposed			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	64.44 ±.1.45	7	59.42 ± 2.70	6	[1,11]=2.91	0.12
AcbC	56.64 ± 2.60	7	54.96 ± 2.97	5	[1,10]=0.18	0.68
CPu	29.78 ± 0.67	5	35.48 ± 1.36	7	[1,10]=10.94	0.008**
VTA	n.d.	-	n.d.	-	-	-

Suppl. Table 16: *Pdyn in situ* hybridization in alcohol-dependent rats

	Not exposed		Exposed			
Region	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n	**F-value**	**p-value**
AcbS	161.93 ± 4.63	6	159.67 ± 1.96	6	[1,10]=0.20	0.66
AcbC	66.47 ± 4.99	6	72.34 ± 2.25	5	[1,9]=1.00	0.34
CPu	42.60 ± 1.96	6	45.26 ± 0.85	5	[1,9]=1.35	0.27
VTA	6.29 ± 0.72	6	4.93 ± 0.44	5	[1,9]=2.29	0.16

Study III

μ-opioid receptor

Suppl. Table 17: [^{3}H]-Damgo binding in saline/NTX treated animals

Region	**Saline not exposed** [fmol/mg]	**NTX not exposed** [fmol/mg]	**F-value**	**p-value**	**Saline exposed** [fmol/mg]	**NTX exposed** [fmol/mg]	**F-value**	**p-value**
AcbS	601.64 ± 15.47 n=7	694.62 ± 18.28 n=8	[1,13]= 14.580	0.002**	590.65 ± 13.41 n=6	721.88 ± 22.11 n=8	[1,12]= 21.62	0.001**
AcbC	312.58 ± 10.79 n=7	284.23 ± 11.13 n=6	[1,11]= 3.32	0.096	264.22 ± 17.05 n=6	315.17 ± 16.82 n=8	[1,12]= 4.35	0.059
CPu	499.96 ± 32.88 n=8	484.66 ± 37.71 n=8	[1,14]= 0.09	0.764	500.27 ± 11.21 n=7	462.84 ± 32.72 n=8	[1,13]= 1.04	0.326
VTA	191.18 ± 8.22 n=6	307.05 ± 3.60 n=6	[1,10]= 166.70	0.000***	175.57 ± 7.17 n=7	386.73 ± 12.00 n=5	[1,10]= 184.07	0.000***

Suppl. Table 18: *bArr2 in situ* hybridization in saline/NTX treated animals

Region	Saline not exposed [fmol/mg]	NTX not exposed [fmol/mg]	F-value	p-value	Saline exposed [fmol/mg]	NTX exposed [fmol/mg]	F-value	p-value
AcbS	8.31 ± 0.46 n=7	8.73 ± 0.46 n=8	[1,13]=0.40	0.54	9.90 ± 0.81 n=7	9.74 ± 0.63 n=7	[1,12]=0.02	0.88
AcbC	8.19 ± 0.40 n=8	7.80 ± 0.61 n=8	[1,14]=0.29	0.60	9.00 ± 0.27 n=7	9.08 ± 0.87 n=6	[1,11]=0.01	0.92
CPu	6.22 ± 0.23 n=8	6.30 ± 0.21 n=7	[1,13]=8.80	0.01*	5.72 ± 0.40 n=8	5.34 ± 0.24 n=7	[1,13]=0.58	0.46
VTA	9.26 ± 0.20 n=6	8.54 ± 0.50 n=8	[1,12]=1.41	0.26	9.07 ± 1.01 n=7	8.69 ± 0.35 n=7	[1,12]=0.12	0.73

Suppl. Table 19: POMC in situ hybridization in saline/NTX treated animals

Region	Saline not exposed [fmol/mg]	NTX not exposed [fmol/mg]	F-value	p-value	Saline exposed [fmol/mg]	NTX exposed [fmol/mg]	F-value	p-value
POMC	372.40 ± 14.61 n=7	540.69 ± 14.23 n=7	[1,12]=68.09	0.000***	482.48 ± 10.29 n=7	545.31 ± 11.52 n=7	[1,12]=16.56	0.002**

δ-opioid receptor

Suppl. Table 20: [^{3}H]-DPDPE binding in saline/NTX treated animals

Region	Saline **not exposed** [fmol/mg]	NTX **not exposed** [fmol/mg]	F-value	p-value	Saline **exposed** [fmol/mg]	NTX **exposed** [fmol/mg]	F-value	p-value
AcbS	23.64 ± 1.03 n=8	24.17 ± 0.86 n=8	[1,14]=0.16	0.70	25.59 ± 1.84 n=7	24.63 ± 0.96 n=7	[1,12]=0.21	0.65
AcbC	19.49 ± 1.56 n=8	17.85 ± 1.75 n=8	[1,14]=0.49	0.50	20.73 ± 1.57 n=7	20.65 ± 0.78 n=7	[1,12]=0.002	0.96
CPu	27.56 ± 0.69 n=8	31.71 ± 0.72 n=8	[1,14]=17.48	0.0009***	27.90 ± 1.37 n=8	32.08 ± 1.46 n=8	[1,14]=4.36	0.06
VTA	13.63 ± 1.08 n=8	22.22 ± 1.34 n=8	[1,14]=24.85	0.0002***	17.39 ± 2.22 n=8	18.83 ± 1.48 n=8	[1,14]=0.29	0.60

κ-opioid receptor

Suppl. Table 21: [^{3}H]-U69,593 binding in saline/NTX treated animals

Region	Saline **not exposed** [fmol/mg]	NTX **not exposed** [fmol/mg]	F-value	p-value	Saline **exposed** [fmol/mg]	NTX **exposed** [fmol/mg]	F-value	p-value
AcbC	50.21 ± 2.19 n=7	61.49 ± 3.29 n=8	[1,13]=7.66	0.02*	50.15 ± 2.13 n=7	71.99 ± 4.01 n=8	[1,13]=21.23	0.0005***
AcbS	56.62 ± 2.98 n=8	84.93 ± 3.46 n=8	[1,14]=38.39	0.00002****	61.88 ± 2.07 n=8	101.31 ± 5.91 n=8	[1,14]=39.59	0.00002****
CPu	22.72 ± 1.09 n=8	30.07 ± 1.46 n=8	[1,14]=16.24	0.001**	19.99 ± 0.71 n=6	25.81 ± 2.50 n=8	[1,12]=3.83	0.07
VTA	n.d.	n.d.	-	-	n.d.	n.d.	-	-

Study IV

Suppl. Table 22: Dopamine transporter (DAT), D1 and D2-like binding levels at different time points after air exposure of rats (controls) in the striatum. Data are expressed as fmol/mg (mean values ± SEM); n=number of animals/group. AcbC – nucleus accumbens core, AcbS – nucleus accumbens shell, CPu – caudate putamen.

	D1 (fmol/mg)	n	D2 (fmol/mg)	n	DAT (fmol/mg)	n
AcbS						
0d	4086,96 ± 78,69	7	328,19 ± 11,30	7	837,00 ± 58,73	5
1d	3593,05 ± 159,31	6	281,85 ± 24,06	6	755,06 ± 50,06	5
3d	3533,65 ± 118,09	6	292,87 ± 26,32	4	717,58 ± 59,57	6
7d	3211,08 ± 129,53	4	267,00 ± 25,44	5	706,73 ± 56,56	7
21d	3341,14 ± 122,21	6	271,99 ± 10,36	6	786,96 ± 61,20	6
AcbC						
0d	3375,96 ± 63,32	6	342,66 ± 9,57	6	990,85 ± 51,22	6
1d	2880,18 ± 250,63	7	301,64 ± 6,05	6	1151,70 ± 31,08	7
3d	3252,50 ± 79,18	6	318,10 ± 10,26	5	1074,52 ± 58,41	6
7d	2319,81 ± 101,37	8	280,41 ± 21,73	7	1026,81 ± 25,07	7
21d	2650,33 ± 117,17	7	277,88 ± 14,93	6	1208,06 ± 28,80	7
CPu						
0d	3709,91 ± 58,07	8	627,32 ± 7,98	7	1432,58 ± 53,24	4
1d	3297,98 ± 45,14	6	610,63 ± 11,03	8	1928,93 ± 73,43	7
3d	3313,21 ± 69,84	6	614,41 ± 13,30	4	2005,07 ± 34,54	6
7d	3154,57 ± 69,10	8	627,85 ± 9,01	7	1918,01 ± 32,15	7
21d	3013,47 ± 74,09	7	573,43 ± 10,56	7	2045,78 ± 32,36	7