Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
Diplombiologe Oliver Stählin
born in: Munich, Germany
Oral examination: 12th December 2013

Characterisation of transcriptome changes caused by alcohol addiction

Referees: Prof. Dr. Rainer Spanagel
Prof. Dr. Stefan Wiemann

Summary

Alcoholism is a psychiatric disorder whose main symptom is an uncontrollable desire to consume alcoholic beverages and which is often associated with a disturbed impulse control. The aim of this thesis was to improve the understanding of the molecular foundations of alcohol addiction and impulsive behaviour through four gene expression studies.

The first sub-project analysed if there is an interaction between alcohol addiction and the body's 'endogenous clock'. DNA microarrays were used to measure genome-wide transcription rates in the *nucleus accumbens* of alcohol-drinking rats and control animals at different times of the day. The experiment did not provide evidence that such a systematic interaction is taking place in the investigated brain region. Based on the vast number of genes that were found to be diurnally oscillating, the study could nonetheless show how important it is to consider the time of measurement as an important variable in gene expression experiments.

The second sub-project aimed at registering genome-wide transcription curves over a period of 24 hours in the blood of healthy human volunteers. Using also DNA microarrays, the main focus was again on genes with circadian oscillating expression levels. The obtained rhythmic expression profiles were mostly from immune genes and can now be used as a baseline for a follow-up study which will assess the impact of alcohol addiction on diurnal gene expression profiles in human blood.

In the third study we analysed genome-wide transcription levels in the *infralimbic cortex* and the *nucleus accumbens* of rats which had been selectively bred for extremes of high and low impulsivity. This microarray study discovered several genes which were closely linked to the degree of impulsive behaviour in the rats. Some of the most differentially expressed genes were *P2ry12*, *Frzb* and *Gprc5b*.

In the fourth sub-project we analysed the expression of four candidate genes, *AUTS2*, *GRIN3A*, *RASGRF2* and *TACR1* in brain tissue taken from deceased alcoholics and non-alcoholic control persons. The expression of *AUTS2* was linked to a single-nucleotide polymorphism (SNP) which was situated in an intronic region of the gene and which had been previously associated with increased alcohol consumption. No such link could be established for *RASGRF2* concerning a similarly situated SNP. The expression of *GRIN3A* was elevated in the *prefrontal cortex* and the expression of *TACR1* diminished in the *anterior cingulate cortex* of alcohol addicted subjects.

Zusammenfassung auf Deutsch

Alkoholismus ist ein Krankheitsbild der Psychiatrie, das durch einen unkontrollierbaren Drang zum Konsum alkoholhaltiger Getränke gekennzeichnet ist und häufig mit einer gestörten Impulskontrolle einhergeht. Ziel dieser Arbeit war es die molekularbiologischen Grundlagen der Alkoholsucht und der Impulsivität anhand von vier Genexpressionsstudien besser zu verstehen.

Die erste Teilstudie ging dabei der Frage nach, ob es eine Wechselwirkung zwischen Alkoholismus und eine Störung der körpereigenen „inneren Uhr“ gibt. Dazu wurde mittels DNA-Microarrays die genomweite Transkription im *Nucleus accumbens* von Alkoholtrinkenden Ratten sowie Kontrolltieren zu verschiedenen Tageszeitpunkten gemessen. Das Experiment hat keinen Hinweis auf eine derartige, systematische Wechselwirkung in der untersuchten Hirnregion geliefert. Aufgrund der Vielzahl an detektierten tageszeitabhängigen Genexpressionskurven konnte die Studie aber zeigen wie wichtig es ist bei Genexpressionsexperimenten den genauen Messzeitpunkt zu wählen und in allen Gruppen konstant zu halten.

Die zweite Teilstudie hatte zum Ziel über einen Zeitraum von 24 Stunden die genomweite Transkription mittels DNA-Microarray Technologie im Blut von gesunden Probanden zu messen. Der Fokus lag hier wiederum auf Genen mit tageszeitabhängig oszillierender Expression. Die detektierten rhythmischen Expressionskurven stammten zum größten Teil von Immungenen und können nun als Ausgangsbasis für eine Fortsetzungsstudie zur Störung der zirkadianen Genexpression im Blut von alkoholabhängigen Patienten dienen.

In der dritten Teilstudie wurde die Genexpression im *infralimbischen Kortex* und im *Nucleus accumbens* von Ratten untersucht, die bidirektional auf eine möglichst geringe oder eine möglichst hohe Neigung zu impulsivem Verhalten hin gezüchtet worden waren. Eine Reihe von Genen konnte ermittelt werden, die im Zusammenhang mit dem Hang zur Impulsivität standen, darunter *P2ry12*, *Frzb* und *Gprc5b*.

In der vierten Studie wurde die Expression von vier Kandidatengenen, *AUTS2*, *GRIN3A*, *RASGRF2* und *TACR1* im Hirngewebe von verstorbenen Alkoholikern und Kontrollpersonen untersucht. Während die Expression von *AUTS2* im Zusammenhang mit einem Einzelnukleotid-Polymorphismus (SNP) stand, der in einem Intron des Gens liegt und mit einem verstärkten Hang zum Alkoholkonsum assoziiert worden war, konnte für *RASGRF2* kein derartiger Zusammenhang bezüglich eines ähnlich gelegenen SNPs festgestellt werden. Im Gehirn von Alkoholkranken war die Expression von *GRIN3A* im präfrontalen Kortex verringert und die von *TACR1* im anterioren Teil des *Gyrus cinguli* erhöht.

To the taxpayer

Summary of Contents

Summary v

Zusammenfassung auf Deutsch vii

Abbreviations xvii

1. General Introduction 1

2. Influence of alcohol exposure and circadian rhythmicity on gene expression in the rat brain 9

3. Influence of circadian rhythmicity on gene expression in human blood 45

4. Gene expression profiling in high and low impulsive rats 63

5. Candidate gene expression studies in human post-mortem brain tissue 91

6. Overall conclusion 115

A. Appendix - Human brain tissue banks 119

Bibliography 125

Publications with content from this thesis 149

Acknowledgments 151

Contents

Summary v

Zusammenfassung auf Deutsch vii

Abbreviations xvii

1. General Introduction 1

2. Influence of alcohol exposure and circadian rhythmicity on gene expression in the rat brain 9

2.1. Introduction to study . . . 9
2.1.1. Circadian rhythms . . . 9
2.1.2. Alcohol addiction and circadian rhythmicity . . . 12
2.1.3. Transition to alcohol dependence and its molecular correlates . . . 14
2.1.4. Research goals . . . 14
2.2. Materials and Methods . . . 15
2.2.1. Alcohol exposure of rats . . . 15
2.2.2. Light-dark cycle . . . 15
2.2.3. Killing of rats . . . 16
2.2.4. Brain dissection . . . 16
2.2.5. RNA isolation and purification . . . 17
2.2.6. Illumina RatRef-12 Expression BeadChips . . . 17
2.2.7. Microarray data pre-processing . . . 18
2.2.8. Statistical analysis . . . 20
2.3. Results . . . 22
2.3.1. Principal component analysis . . . 22
2.3.2. Detection of daily oscillating genes by Fourier transformation . . . 22
2.3.3. Analysis of the core clock genes . . . 23
2.3.4. Analysis of selected E-box genes . . . 26
2.3.5. Screening for oscillating genes that change under the influence of alcohol . . . 28
2.3.6. Influence of exposure time: transition to addiction . . . 33
2.4. Discussion of study . . . 37

3. Influence of circadian rhythmicity on gene expression in human blood 45

3.1. Introduction to the study . . . 45

3.2. Materials and Methods . . . 47
3.2.1. Recruitment of test persons . . . 47
3.2.2. Blood taking procedure . . . 47
3.2.3. RNA isolation and purification . . . 47
3.2.4. Illumina HumanHT-12 v4 Expression BeadChip . . . 48
3.2.5. Microarray data pre-processing . . . 48
3.2.6. Microarray annotation . . . 48
3.2.7. Statistical analysis . . . 49
3.3. Results . . . 51
3.3.1. Principal component analysis . . . 51
3.3.2. Detection of oscillating genes by Fourier transformation . . . 52
3.3.3. Classification of the oscillating genes according to their time of peak expression levels . . . 55
3.3.4. Enriched pathways and gene ontology terms . . . 57
3.4. Discussion of the study . . . 58

4. Gene expression profiling in high and low impulsive rats 63
4.1. Introduction to the study . . . 63
4.1.1. Introduction to the concept of *impulsivity* . . . 63
4.1.2. Measurements of impulsivity . . . 63
4.1.3. Brain regions implicated in impulsivity . . . 65
4.1.4. Impulsivity and genetics . . . 66
4.1.5. Impulsivity and psychiatric disorders . . . 67
4.1.6. Research goals . . . 67
4.2. Materials and Methods . . . 69
4.2.1. Behavioural testing . . . 69
4.2.2. Brain dissection . . . 69
4.2.3. RNA isolation and purification . . . 69
4.2.4. Affymetrix Gene Chips . . . 70
4.2.5. Microarray data pre-processing . . . 70
4.2.6. Statistical analysis and data mining of microarrays . . . 71
4.2.7. Quantitative PCR validation . . . 72
4.3. Results . . . 75
4.3.1. Results of the 5-choice serial reaction time task (5CSRTT) . . . 75
4.3.2. Principal component analysis . . . 76
4.3.3. Volcano plots . . . 76
4.3.4. Filtering by t-test derived p-values and fold-changes . . . 77
4.3.5. KEGG pathways & gene ontology terms . . . 78
4.3.6. Correlation analysis . . . 78
4.3.7. Relation of gene expression findings to quantiative trait loci . . . 80
4.3.8. Validation of microarray results by quantitative PCR . . . 80
4.4. Discussion of the study . . . 85

5. Candidate gene expression studies in human post-mortem brain tissue 91
5.1. Introduction to candidate gene studies . . . 91
5.1.1. Measuring gene expression in human post-mortem brain tissue . . . 91
5.1.2. Investigated brain regions . . . 92
5.1.3. Candidate genes . . . 96
5.2. Materials and Methods . . . 100
5.2.1. Post-mortem samples from the Human Brain Tissue Bank . . . 100
5.2.2. Post-mortem samples from the New South Wales Tissue Resource . . . 100
5.2.3. DNA isolation and purification . . . 101
5.2.4. Single nucleotide-polymorphism genotyping . . . 101
5.2.5. RNA isolation and purification . . . 101
5.2.6. Quantitative PCRs . . . 102
5.2.7. Statistical analyses . . . 103
5.3. Results . . . 105
5.3.1. AUTS2 . . . 105
5.3.2. RASGRF2 . . . 107
5.3.3. TACR1 . . . 107
5.3.4. GRIN3A . . . 110
5.4. Discussion of candidate gene studies . . . 112
5.4.1. AUTS2 . . . 112
5.4.2. RASGRF2 . . . 112
5.4.3. TACR1 . . . 113
5.4.4. GRIN3A . . . 114

6. Overall conclusion 115
6.1. Study limitations . . . 115
6.1.1. Microarray studies . . . 116
6.1.2. Candidate gene studies in post-mortem tissue . . . 116
6.2. Conclusions for further studies . . . 117
6.3. Outlook . . . 117

A. Appendix - Human brain tissue banks 119

Bibliography 125

Publications with content from this thesis 149

Acknowledgments 151

Abbreviations

5CSRTT	5-choice serial reaction time task
AMPA	2-amino-3-hydroxy-5-methylisoxazol-4-propanoic acid
Ct	Cycle of threshold
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders - fourth edition - text revised
FDR	False discovery rate
GABA	γ-Aminobutyric acid ($H_2N - CH_2 - CH_2 - CH_2 - COOH$)
GWAS	Genome-wide association study
HBTB	Hungarian brain tissue bank
ILC	Infralimbic cortex
n.a.	[Data] not available
NacC	Nucleus accumbens core
NacS	Nucleus accumbens shell
NMDA	N-methyl-D-aspartate
NSW-TRC	New South Wales Tissue Resource Centre
PCR	Polymerase chain reaction
PMI	Post-mortem interval
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
VTA	Ventral tegmental area
ZT	Zeitgeber time

1. General Introduction

The ancient Greeks, having become masters in the art of wine making, soon realised the ambivalent nature of their liquid product. While they enjoyed its exhilarating and relaxing effects, they became wary of its inebriating and addictive properties and urged for moderation in consumption. In fact, wine was mostly served diluted with water. Nonetheless, drunkenness was commonplace and the Greek philosophers started speculating how wine could exert its magic effects on men. Among the most popular theories was the belief that there was a daemon, or spirit, of wine who would enter the human body with the ingestion of the fermented grape juice and take control over the drinkers thoughts and emotions causing madness (Thompson, 2010).

More than 2,500 years have passed since the Greeks made their first experience with alcohol but the problems caused by it are as prevailing today as they were then. Although the perception of what is the main problem with alcohol has shifted. Sure, drunkenness is still a problem, as accidents and emergency departments as well as law enforcement officers witness on a daily basis. But the biggest danger of alcohol does not lie in occasional surges of insobriety, as dangerous as they might be. It lies in the fact that alcohol is both addictive and, when applied in higher doses or over longer times, severely organotoxic. So the neutral daemon of wine of the Greek mythology turned out to be a demon, an evil spirit pushing men to loose control over their drinking habits and ruining their body and mind.

The majority of people consume alcohol occasionally and in moderate manner which is regarded safe from a medical point of view. But the toll of those whose consumption does not stay moderate is high. The federal government of Germany estimates that out of its current population of 82 million, 9.5 million (12%) consume alcoholic beverages so excessively that their health is at risk. An estimated 1.3 million of them (1.6% of the population) have a full-blown addiction to alcohol (Die Drogenbeauftragte der Bundesregierung, 2012). The definition of alcohol addiction is thereby based on the classification set out in the *Diagnostic and Statistical Manual of Mental Disorders* (Version IV) of the American Psychiatric Association (2000). It describes alcohol dependence as a psychiatric condition which is characterised by a 'maladaptive pattern of alcohol use, leading to clinically significant impairment or distress' which has to meet at least three of the following criteria within a period of twelve month:

- An abnormal tolerance towards the intoxicating effects of alcohol.
- Withdrawal symptoms when alcohol is not available.
- Alcohol is consumed in larger amounts or over a longer period than was intended.

- Unsuccessful attempts to reduce alcohol consumption despite having the desire to stop drinking.
- Obtaining, drinking and recovering from alcohol absorb a great deal of time.
- Neglect of other social and occupational activities for the sake of obtaining and drinking alcohol.
- Continued consumption of alcohol despite experiencing persistent or recurring physical or psychological negative consequences due to the consumption.

While the American Psychiatric Association used the term alcohol *dependence* in their manual, it has been proposed that this word should be reserved to describe the purely physiological dependence (Spanagel, 2009). Alcoholism as a whole, with its interwoven relationship of physiological as well as psychological and social aspects, should then be referred to as alcohol *addiction* as it will be done in this thesis.

Biological research on addiction tries to bridge the gap between these different aspects, especially when the focus of research is on the psychological phenomenon of craving, which describes the intense urge of an addicted person to re-administer the drug. Biomedical research tries to discover the modifications of cell receptor densities, gene expression rates and neuronal circuits which can be related to this phenomenon. Another example is the analysis of epigenetic modification, such as DNA methylation, which arise as a consequence of difficult childhood and which might modify gene expression and thereby brain responsiveness to stress and drugs throughout the whole life (McGowan and Szyf, 2010).

The first challenge in biological addiction research was to discover the targets of alcohol in the human body. Today we know that alcohol interacts primarily with five neuronal cell surface receptors and two ion channels (see table 1.1 for an overview). Alcohol slows down brain activity by increasing the signalling of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) via its activation of $GABA_A$ receptors and decreasing the signalling of the excitatory neurotransmitter glutamate via its inhibition of N-methyl-D-aspartate (NMDA)-sensitive glutamatergic receptors.

Alcohol also interacts with the glycine receptors but here the effect is more complex. It seems to act on at least two distinct locations of the receptor, one in the transmembrane domain and the other in the extracellular part, with opposing effects (Crawford et al., 2007). Further molecular interactions of alcohol are an activation of serotonergic and nicotine-responsive acetylcholine receptors. And alcohol also influences two ion channels without endogenous ligands as it inhibits the long-lasting voltage-dependent calcium channels and activates the G protein-coupled inwardly rectifying potassium channels (Spanagel, 2009).

In addition, there is evidence that the positive feelings triggered by alcohol are also mediated by receptors of the opioid and the endocannabinoid systems (Olive et al., 2001; Hungund and Basavarajappa, 2004), although alcohol seems to have no direct effect on the receptors of these neuromodulatory systems. Another important secondary effect is the increased release of dopamine in the nucleus accumbens shell by neurons that project from the ventral tegmental area (see below for anatomic details) (Spanagel and Weiss, 1999).

The identification of these alcohol-responsive neurotransmitter receptors has helped to

Table 1.1.: Receptors and channels targeted by alcohol

Receptor or Channel	Description	Type	Effect of alcohol
$GABA_A$-R	γ -aminobutyric acid receptor type A	inhibitory	activation
NMDA-R	NMDA*-responsive glutamate receptor	excitatory	inhibition
GlyR	glycine receptor	inhibitory	both
5-HT_3-R	serotonin (5-hydroxytryptamine-3) receptor	excitatory	activation
nAChR	nicotine-responsive acetylcholine receptor	excitatory	activation
L-type Ca^{2+}	long-lasting voltage-dependent calcium channel	-	inhibition
GIRK	G protein-coupled inwardly rectifying potassium channel	-	activation

* NMDA = N-methyl-D-aspartate

identify neuronal networks that play a crucial role in the development of alcohol addiction. The most salient finding is the mesolimbic pathway which consists of dopaminergic neurons of the medial forebrain bundle whose cell bodies are situated in a neuron cluster of the midbrain called the ventral tegmental area (VTA) and whose axons run to the nucleus accumbens, the amygdala and the hippocampus (Oades and Halliday, 1987) (figure 1.1). The nucleus accumbens is a part of the ventral striatum which belongs to the basal ganglia system. The latter structure is associated with the emotion processing limbic system, which includes the amygdala and the hippocampus among other structures. Another important neuronal connection is the mesocortical pathway which consists of dopaminergic neurons which lead from the ventral tegmental area (VTA) to the frontal lobe as well as other parts of the cerebral cortex. These connections have been labelled the *reward system* of the brain, as the experience of pleasure is associated with a surge in dopamine signalling in those neurons (Wise and Bozarth, 1984) although some authors use this term exclusively for the neuronal connections running from the VTA to the nucleus accumbens.

Different theories have been proposed to explain how mesolimbic dopamine exerts its role in reward. An increase in dopamine secretion might attribute a feeling of pleasure or 'liking' to rewarding stimuli or it might convey a prediction of future reward based on passed experience ('learning') or it might cause an *incentive salience* or 'wanting' of rewarding stimuli (Berridge, 2007). It was proposed that addiction follows a chronic repetition of a binge drinking intoxication phase, a withdrawal phase dominated by negative emotions and an anticipation phase dominated by craving for the drug. The reward system would be especially important during the binge drinking phase while the extended amygdala would dominate the withdrawal phase and the prefrontal cortex and the hippocampus would be pivotal for the anticipation phase (Koob and Volkow, 2010). The role of the prefrontal cortex in the latter phase would be based on its importance in decision making and top-down control of impulsive drives (Dalley et al., 2011).

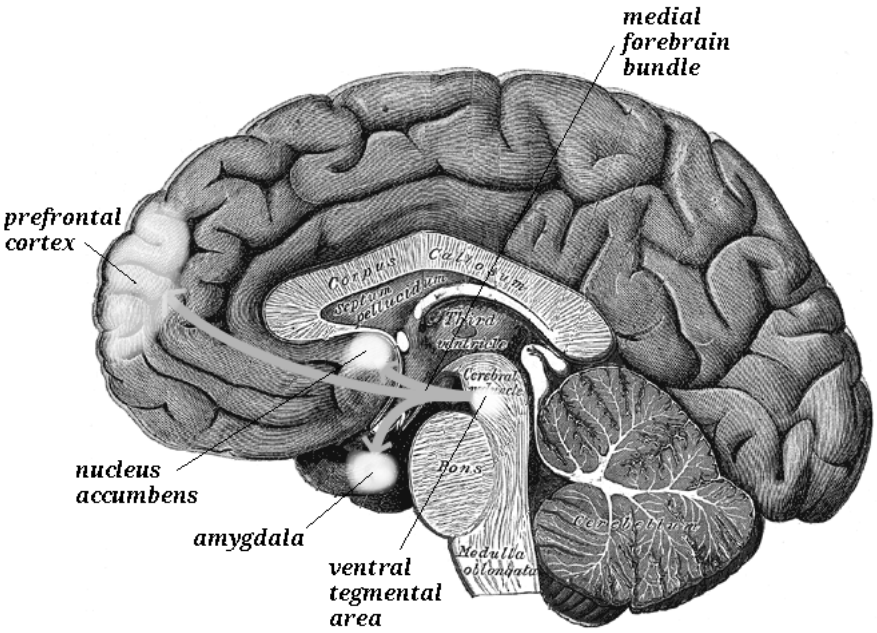


Figure 1.1.: Reward system of the human brain. The medial forebrain bundle, containing the axons of dopaminergic neurons, transmits signals from the ventral tegmental area to the nucleus accumbens and amygdala (mesolimbic pathway) and to the prefrontal cortex (mesocortical pathway). Image modified from Gray (1918) (book in public domain).

Based on the acquired information on the biological substrates of addiction, a number of drugs have been proposed for the treatment of alcohol addiction. On the one hand, there are drugs which are used to bring the patients safely through the phase of alcohol withdrawal which is the first step of nearly all treatment programs. Pharmacological management of withdrawal symptoms, which can range from mild agitation to seizures or full-blown delirium tremens, is based on benzodiazepines, anticonvulsants, betablockers and antihypertensives (Manasco et al., 2012). On the other hand are drugs specifically developed for relapse prevention in sober alcoholics. Table 1.2 gives an overview of the major drugs in use or in testing for this purpose and their putative biological targets.

The oldest of these drugs is *disulfiram* which interferes with the metabolism of alcohol. Under normal conditions, alcohol is converted to acetaldehyde by the enzyme alcohol dehydrogenase and then further processed to acetic acid by acetaldehyde dehydrogenase with the bulk of this metabolism happening in the liver. Disulfiram inhibits acetaldehyde dehydrogenase causing increased levels of acetaldehyde after alcohol consumption. High levels of acetaldehyde cause feelings of nausea and headache which are often associated with 'hangover' and which should deter the patient to drink further alcohol.

Another anti-craving drug used for relapse-prevention of sober alcoholics is *acamprosate*. Although it showed some efficacy in clinical trials (Mason et al., 2006) the drug's exact mechanism of action is still not completely understood. It has been proposed that acamprosate antagonises glutamatergic NMDA receptors, which are over-expressed in chronic alcoholics as a cellular reaction to alcohol's inhibiting effect on these receptors. This effect might be dose-dependent and lower concentrations of the drug could act stimulating on

Table 1.2.: Major drugs currently in use or in testing for the post-withdrawal treatment of alcohol addiction

Drug	Class	Status
Disulfiram (Antabuse)	acetaldehyde dehydrogenase inhibitor	FDA[1] approved
Acamprosate	putative NMDA receptor modulator	FDA[1] approved
Naltrexone	opioid receptor antagonist	FDA[1] approved
Nalmefene	opioid receptor antagonist	in testing
Baclofen	$GABA_B$ receptor agonist	in testing
Topiramate	$GABA_A$ receptor agonist / AMPA[2]- and Kainate[3] receptor antagonist	in testing

[1)] FDA = Food and Drug Administration of the United States Department of Health and Human Services; [2)] AMPA = 2-amino-3-hydroxy-5-methylisoxazol-4-propanoic acid, an agonist for a subtype of ionotropic glutamate receptor; [3)] Kainate = agonist for another subtype of ionotropic glutamate receptor

this receptor. Acamprosate would therefore act towards a normalisation of glutamatergic signalling. But there is also evidence that the drug has a subtly activating effect on $GABA_A$ receptors (Daoust et al., 1992; Zeise et al., 1994; Littleton, 1995; Mann et al., 2008).

There are also anti-craving drugs available that act on the opioid system. *Naltrexone* is a competitive antagonist of the μ- and κ-opioid and to a lesser degree of the σ-opioid receptors and probably exerts its action via the neuronal network of the mesolimbic pathway by interfering with the attribution of rewarding feelings to alcohol consumption (Herz, 1997). It is hypothesised that a polymorphism in the *OPRM1* gene for the μ-opioid receptor has a significant effect on treatment efficacy with carriers of the minor G allele showing higher naltrexone responsiveness (Anton et al., 2008). This constitutes an important example how genotyping of specific DNA locations could lead to alcohol addiction pharmacotherapy. The second opioid antagonist, *Nalmefene*, has a similar mechanism of action as naltrexone but has a longer pharmacokinetic half-life, is less liver toxic and acts as a partial agonist on the κ-opioid receptor (Swift, 2013). It has been proposed recently to give nalmefene to actively drinking alcoholics in an attempt to reduce their consumption levels without the goal of complete abstinence (Mann et al., 2012). Sinclair (2001) proposed a similar approach, consisting of giving naltrexone to non-abstinent alcoholics trying to provoke a pharmacological extinction of the association of rewarding effects with the consumption of alcohol.

Another drug proposed for the treatment of post-withdrawal alcoholisc is *baclofen* which was originally designed as a muscle relaxant for the treatment of spasticity. It acts as an agonist at the $GABA_B$ receptor. Ameisen (2005) suggested that a high-dose, long-term application of baclofen reduces alcohol craving.

Topiramate is a drug which was originally developed for the treatment of epilepsy. As in the case of acamprosate, its exact mechanism of action is unknown. Most likely it acts as an antagonist on the 2-amino-3-hydroxy-5-methylisoxazol-4-propanoic acid- (AMPA) and kainate-susceptible ionotropic glutamate receptors and as an agonist on the $GABA_A$ re-

ceptor. Additional mechanisms of action might include an inhibition of voltage-dependent sodium channels as well as an inhibition of carbonic anhydrase enzymes (Johnson, 2008). Topiramate significantly reduced alcohol consumption in both a rodent study (Zalewska-Kaszubska et al., 2013) and human trials (Johnson et al., 2007).

While these drugs might all work to a certain degree, none of them could claim a definite success in the treatment of alcohol addiction and relapse stays a prevailing problem for alcohol addicted patients. Based on a meta-analysis of studies from the United States, Miller et al. (2001) estimated that the percentage of patients who relapse after alcohol rehabilitation treatment is up to 75 %. Kiefer et al. (2003) obtained relapse rates of approximately 43 % during the first 12 weeks of anticraving therapy with either acamprosate or naltrexone. A meta-analysis of double-blind randomised controlled trials concluded that acamprosate reduced the risk of drinking after detoxification to 86 % of the risk under placebo treatment and one out of nine patients would benefit from acamprosate treatment (Rösner et al., 2010).

This dissertation aims to further the current understanding of the molecular underpinnings of psychiatric disorders. It will focus on a small subset of the cellular machinery, namely the rate at which genes are expressed in the central nervous system in the context of mental disorders. Four studies were conducted to investigate the correlation between gene transcription and mental problems:

In the first study, the focus is on alcohol addiction. Based on a rat model of chronic ethanol consumption, we analysed how gene expression patterns in the brain changed from non-alcohol exposed animals to rats with a history of short- and long-term drinking. As it has been proposed that alcoholism might impair the body's endogenous circadian rhythm which is responsible for our daily sleep-wake cycle, we investigated if this effect can be observed on the level of gene transcription as well.

The focus of the second study was also on circadian rhythmicity. The study aimed to discover genes with circadian oscillating expression levels in the blood of healthy human volunteers. As not only alcoholism but also other psychiatric conditions such as major depression seem to interact with endogenous circadian rhythms, the established expression profiles can be used as a baseline for future studies assessing the impact of these conditions on circadian gene expression in human blood.

The third study focused on impulsivity as a behavioural trait implicated in a variety of psychiatric disorders such as drug addiction, attention deficit hyperactivity disorder (ADHD), bipolar and borderline disorder. In a rat model of high and low impulsivity we screened for gene expression differences associated with this trait which has also been put forward as a factor of vulnerability for drug addiction.

And in the fourth part of this dissertation the focus was on the expression levels of selected candidate genes in post-mortem tissue of deceased mentally ill subjects as well as of control person who died of non-psychiatry related causes. The aim was to translate findings from animal models to the human situation.

Due to the molecular biological focus of these studies, they can only provide a partial view on the field of addictive disorders. There would be a lot to say about the social context of alcohol addicted persons and the way they grew up as adverse childhood conditions, including sexual abuse, as well as traumatising life events surely play a great role on the road to alcohol abuse and addiction. So, while focusing on the molecular aspects of addiction, the present thesis is not meant to curtail the role of psychotherapeutic interventions such as group therapies and individual behaviour therapies as well as the role of welfare workers who help alcohol addicted patients to regain control over their professional and private life.

2. Influence of alcohol exposure and circadian rhythmicity on gene expression in the rat brain

2.1. Introduction to study

Previous studies have suggested that drug addiction is often accompanied by a disruption of the sleep-wake cycle and perturbations of daily oscillating physiological parameters such as body temperature and blood pressure (Devaney et al., 2003; Kawano et al., 2002). This part of the thesis investigates the molecular mechanisms which form the underpinning of this interaction.

2.1.1. Circadian rhythms

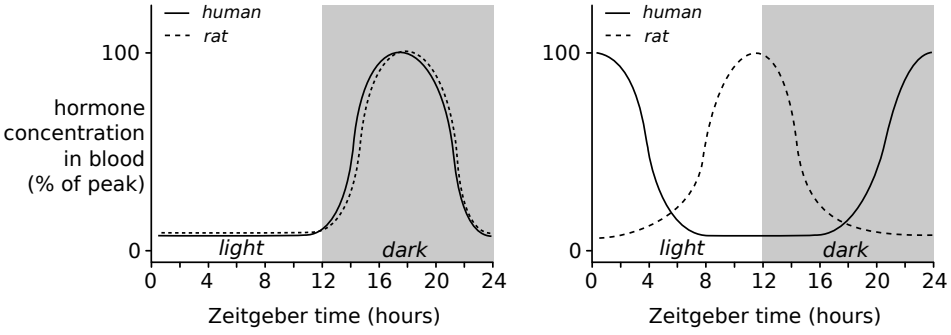
An endogenous clock to adapt to the day-night rhythm of the earth

Life on earth is subject to the presence of natural rhythms such as the seasonal changes in weather and daylight or the alternation between day and night due to the earth rotation around its own axis. It is essential for living creatures to adapt to these periodic changes in order to compete successfully in the struggle of natural selection.

Most living beings do not rely solely on external cues such as oscillations in daylight intensity or temperature to adapt to the 24-hour light-dark cycle of the earth. Instead they possess an internal - 'endogenous' - clock which sets an approximately diurnal rhythm. In mammals this function is fulfilled by the *suprachiasmatic nucleus* (SCN), a network of about 10 000 neurons which is part of the anterior hypothalamus and is situated just superior to the optic chiasm (hence the name *supra-chiasmatic*) (Hastings, 1997).

The SCN regulates numerous physiological functions such as the activity of the different parts of the endocrine system. For example, the SCN regulates the secretion of melatonin by the pineal gland whose plasma concentration peaks during the night and is almost not detectable during the day (Lincoln et al., 1985). The production of cortisol by the adrenal glands is also influenced by the SCN. It reaches a peak at the beginning of the active phase of an animal which would be in the early morning in - at least originally - diurnal humans and in the evening in nocturnal rodents such as most rat species (Bujis et al., 2003; Fries et al., 2009). As cortisol production is also part of the body's stress response this rhythm can be blurred by external events. Figure 2.1a and figure 2.1b depict the theoretical rhythms of the melatonin and the cortisol concentration in the blood as they

would occur in a 24 hour period with alternation of 12 hours light and 12 hours darkness. The time in a thus defined light-dark cycle is called *zeitgeber* time.



(a) Melatonin **(b)** Cortisol

Figure 2.1.: Schematic curves of the circadian oscillating concentrations of melatonin (a) and cortisol (b) in the blood of humans and rats. Melatonin always peaks in the night phase while cortisol peaks at the beginning of the active phase (human: day, rat: night). Time is presented as theoretical *zeitgeber* time with 12 h light followed by 12 h darkness. Data sources: see text.

The SCN is also implicated in the circadian regulation of blood pressure and body temperature, two physiological parameters which reach a minimum level during the inactive phase only to rise again before the beginning of the active phase (Janssen et al., 1994; Biaggioni, 2008; Scheer et al., 2005).

The diurnal rhythm of the SCN, with its quality of being entrainable by external cues, is called *circadian*, a compound of the Latin words for 'approximately' (*circa*) and 'day' (*dies*). But the rhythm of the SCN is only approximately diurnal and slightly deviates from the 24 hour scheme if an organism is kept under invariable light conditions without any external time cues (Czeisler et al., 1999). Under normal conditions, ganglion cells in the retina sense the external light intensity and communicate their daytime dependent excitation level to the suprachiasmatic nucleus, thus entraining its endogenous rhythm. Other factors, such as eating times and social activities also have an entraining influence (Klermann et al., 1998). These external cues, which are referred to as *zeitgebers* (German for 'time givers'), are thus responsible for adjusting the body's inner clock to the actual day-night pattern of the environment. They prompt, for instance, the re-adjustment of the body's wake-sleep cycle after a long-distance flight over several time zones.

The molecular machinery sustaining the circadian rhythms

Over the last decades, research has made substantial progress in deciphering the molecular mechanisms which are at the basis of circadian rhythms. Its foundation is a complex network of cross-talking regulatory transcriptional and post-transcriptional feedback loops of several proteins coded by the so-called clock genes (Ko and Takahashi, 2006; Lowrey and Takahashi, 2011).

A central role is played by the circadian clock genes *period 1*, *2* and *3* (*Per1*, *Per2*, *Per3*) as well as *cryptochrome 1* and *2* (*Cry1*, *Cry2*). All these genes contain specific sequences

in their promoter region called *enhancer boxes* (*E-boxes*). These regulatory sequences are the target of a heterodimer made of two further proteins, the *aryl hydrocarbon receptor nuclear translocator-like* (*Arntl* - sometimes also referred to as *Bmal1*) and the *circadian locomotor output cycles kaput* (*Clock*). As a feedback mechanism, the *Per* and *Cry* proteins also form heterodimers which get activated by phosphorylation by *casein kinase 1 epsilon* (*Csnk1e*) or a related protein. The *PER-CRY* dimers then enter the nucleus and inhibit the activity of the *ARNTL-CLOCK* dimer (figure 2.2) (Ko and Takahashi, 2006).

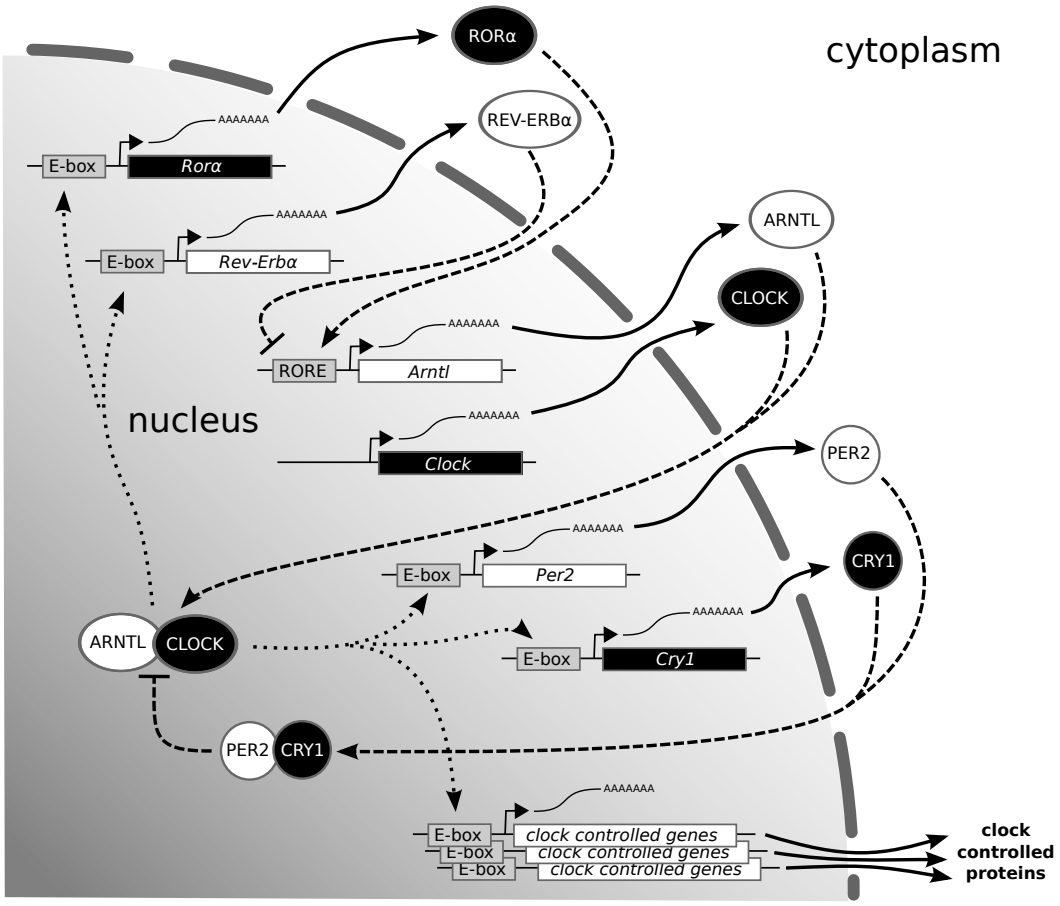


Figure 2.2.: Simplified scheme of the network of the feedback loops of the mammalian circadian clock. E-box and RORE are regulatory sequences in the promoter regions of the succeeding genes. Based on the network scheme proposed by Ko and Takahashi (2006).

In another regulatory loop, the *ARNTL-CLOCK* dimer also binds to the E-box elements of *retinoic acid related orphan receptor alpha* (*Rorα*) and *member 1 of group D of nuclear receptor subfamily 1* (*Nr1d1* also known as *Rev-ErbA α*) and increases the transcription of these genes. *RORα* and *REV-ERBA α* both bind to a regulatory sequence called *retinoic acid-related orphan receptor response element* (RORE) which is found in the promoter of *Arntl*. *RORα* increases and *REV-ERBA α* decreases *Arntl* expression. The proteins thus fine-tune the molecular clock (see also figure 2.2) (Ko and Takahashi, 2006).

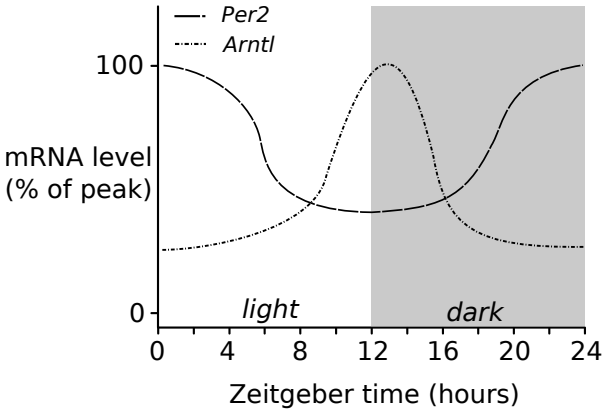


Figure 2.3.: Theoretical expression pattern of *Per2* and *Arntl* in the suprachiasmatic nucleus in rats. Based on data from Oishi et al. (1998).

E-box elements are not only found in the promoter regions of the core clock genes but also in a variety of other genes. It is via those genes that the rhythm of the molecular clock gets propagated to other parts of the cellular machinery. These additional E-box containing genes are often referred to as *clock controlled genes* (Zhang et al., 2004).

Concentration measurements of clock gene mRNAs in the suprachiasmatic nucleus have shown a distinctly circadian rhythm in the transcription rate of *Arntl* and *Per2* while the gene expression of *Clock* did not show signs of circadian oscillation (figure 2.3) (Oishi et al., 1998).

Lesion and transplantation studies have proven the role of the suprachiasmatic nucleus as the primary circadian rhythm pacemaker in mammals (Weaver, 1998). Nonetheless, molecular clock components are expressed in all transcriptionally active cells throughout the body and peripheral cells display signs of various periodic activities. Some of these activities, like the rhythmic contraction of the heart myocytes, have a comparatively short period, but numerous other cellular processes appear to have a circadian rhythm such as the expression of genes involved in metabolism and glucose homeostasis (Mohawk et al., 2012). Some of these oscillatory rhythms were shown to be self-sustaining in cell culture experiments and it was suggested that the SCN works as a master clock synchronising these peripheral or secondary oscillators (Kowalska and Brown, 2007).

2.1.2. Alcohol addiction and circadian rhythmicity

Clinical evidence

As mentioned in the beginning of the introduction, there is reasons to believe that alcohol addiction interferes with the proper functioning of the endogenous circadian clock. As an example, alcohol addicted patients often report disruptions in their sleep-wake cycle and insomnia is a common problem after withdrawal (Asheychik et al., 1989; Egbert, 1993; Jones et al., 2003). From the physiological point of view, alcohol is known to disturb the normal circadian cycle of body temperature (Wasielewski and Holloway, 2001; Devaney

et al., 2003) and blood pressure (Kawano et al., 2002). An analysis of emergency department records showed a significant daily pattern of drug-related admissions with a peak in the early evening. While a large portion of these admissions are due to opiate and cocaine consumption, alcohol intoxicated patients also tend to arrive in phases (Raymond et al., 1992). The variation in the number of hospital admissions depending on the time of day could of course also be caused by environmental factors such as shop and pub opening hours.

Evidence from animal studies

Animal studies have provided further evidence for a disturbance of the endogenous circadian clock by alcohol consumption. Similar to the situation in humans, Rosenwasser et al. (2005) have shown that alcohol consumption significantly alters the daytime-dependent occurrence of activity phases in rats. This finding was reproduced in two mice strains (Rosenwasser and Fixaris, 2013). Another study in rats reported that chronic alcohol consumption impairs the circadian rhythm of the transcription of the mRNA of *Per2* and *Per3* in the suprachiasmatic nucleus (Chen et al., 2004).

In addition, it was shown that a deletion mutation within the sequence of *Per2* significantly increased alcohol drinking in mice (Spanagel et al., 2005; Zheng et al., 1999) showing thus that there is also an effect of the circadian clock machinery on alcohol consumption.

Evidence from transcriptional regulation

There is also evidence from molecular biology that genes involved in alcohol addiction and core clock genes interfere with each other. It was suggested that the heterodimer of *ARNTL* and *CLOCK* does not only play a role as a transcription factor in the molecular clock machinery but might also influence the expression of addiction-related genes. Manev and Uz (2006) have mentioned a number of such addiction-related genes that contain E-boxes in their promoter regions and which could therefore be targets of *ARNTL*/*CLOCK*. Their list contained among others:

- Th: tyrosine hydroxylase
- Drd1: dopamine receptor D1
- Slc6a3: dopamine transporter (solute carrier family 6 member 3)
- Oprd1: opioid receptor delta 1
- Slc6a2: norepinephrine transporter (solute carrier family 6 member 2)
- Slc1a3: glial glutamate transporter (solute carrier family 1 member 3)
- Grm4: metabotropic glutamate receptor 4

Often the E-boxes are in the proximity of other regulatory sequences such as cAMP response elements (CRE) which are the target of the *cAMP response element-binding protein* (*CREB*) and TPA responsive elements (TRE) that are bound by the *activator protein 1* (*AP-1*) transcription factor which is a heterodimer of *Fos* and *Jun*. Alcohol might influence the efficiency with which the *ARNTL*/*CLOCK* dimer binds to the E-boxes of addiction-related genes by modulating CREB and AP-1 binding which alternates the availability of the surrounding promoter region (Manev and Uz, 2006).

2.1.3. Transition to alcohol dependence and its molecular correlates

A fully developed alcohol addiction is the result of a long history of high alcohol consumption in the course of which a transition takes place from controlled recreational alcohol drinking to compulsive consumption. In a rodent model of cocaine addiction, Kasanetz et al. (2010) showed how synaptic plasticity, measured by the ability of neurons to induce long-term depression at their synapsis, is permanently impaired in animals which undergo a transition to addiction but not in rats which showed no signs of compulsive consumption. It is conceivable that changes in gene expression underlie this observation and that a similar mechanism takes place during the development of alcohol addiction. Epigenetic modifications of the promoter regions of implicated genes could be a possible mechanism behind this differential regulation of gene expression (Spanagel, 2009).

Spanagel and Heilig (2005) proposed that transition to alcohol addiction might be correlated to a 'molecular switch' which could involve an up-regulation of *Delta FosB*, a truncated form of *FosB*, in the mesolimbic dopamine pathway. But while the up-regulation of *FosB* after drug consumption lasts only a few weeks another gene with a longer-lasting up-regulation has been brought forward: *Per2*. This suggests an involvement of the circadian clock - addiction interaction in the transition to addiction.

Another mechanism which might act as a 'molecular switch' to addiction is an imbalance in the concentration ratio between synaptic and non-synaptic glutamate in the prefrontal cortex which might cause an impairment in top-down behavioural control on drug consumption (Kalivas, 2009; Meinhardt et al., 2013).

2.1.4. Research goals

The present study is based on the hypothesis that alcohol addiction and circadian rhythm systems interact with each other on the transcriptional level. The aim of the study was therefore to identify genes in the brain's reward system which show diurnally oscillating expression levels and whose transcription rhythmicity would be altered by a history of chronic alcohol consumption.

We therefore simulated alcohol addiction in a rat model of voluntary self-administration with repeated withdrawal phases. Rats were divided in a short-term and long-term alcohol-exposed group which should make it possible to assess gene expression before and after the transition to addiction. By sacrificing the animals at different time points throughout the day we were able to identify diurnally oscillating genes and the modulation of their expression patterns by the short-term and long-term alcohol exposure.

We isolated the RNA of the nucleus accumbens of the animals and measured the gene expression using microarrays. The focus was thereby on a brain region which plays a pivotal role in drug addiction and was large enough for the extraction of microarray-grade RNA.

2.2. Materials and Methods

The drinking paradigm of this study was implemented by my co-workers Stephanie Perreau-Lenz and Valentina Vengeliene. I assisted in sacrificing the animals and continued with the RNA preparation and the microarray experiment.

2.2.1. Alcohol exposure of rats

In order to study the molecular mechanisms of the interaction between circadian rhythmicity and alcohol dependence, we used a well-established animal model of addictive behaviour to alcohol (Spanagel and Hölter, 1999). The model is based on rats and consists of the voluntary self-administration of alcohol which is interrupted by repeated alcohol deprivation phases. In brief, rats were kept solitary with free access to standard rat food and water as well as three solutions of different ethanol concentration: 5%, 10%, and 20%. The continuous presence of the water bottle guaranteed that the consumption of the ethanol solutions was always voluntary and never driven by thirst. The access to ethanol was interrupted by deprivation phases where the three ethanol solutions were removed. This paradigm with its alteration between access and deprivation reliably causes an escalating, uncontrolled and dependent-like alcohol consumption in rats (Spanagel and Hölter, 1999).

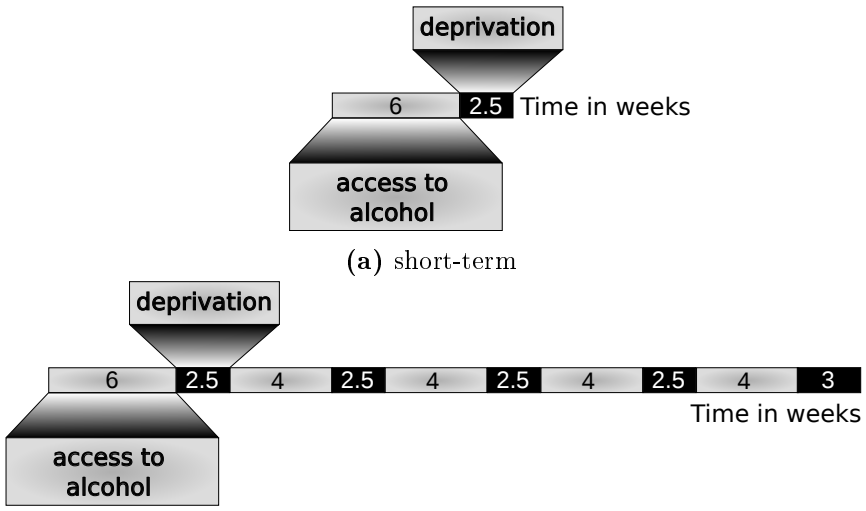
In our study we used a total of 96 male Wistar rats which were divided into four equally large experimental groups:

- short-term ethanol exposed animals
- short-term control animals
- long-term ethanol exposed animals
- long-term control animals

All animals were eight weeks old at the beginning of the experiment. The short-term ethanol exposed animals had six weeks of access to the ethanol concentrations followed by a deprivation period of two and a half weeks after which they were sacrificed (figure 2.4a). The long-term ethanol exposed animals underwent the same six week access, two week deprivation scheme but this was followed by another four units of four weeks access to alcohol which were alternated by two and a half weeks deprivation phases (figure 2.4b). After the last four weeks of ethanol access animals underwent a three week deprivation phase before they were sacrificed. The prolongation of the last deprivation phase is necessary to produce a high level of craving in the animals as the rats tend to get used to the fact that alcohol becomes available again after two and a half weeks. The rats of the short-term and long-term control group underwent exactly the same time schedule as the respective alcohol exposed animals, except that they always only had access to the water.

2.2.2. Light-dark cycle

To obtain an environment with controlled time cues, rats were kept in a windowless room with alternating periods of 12 hours light and 12 hours darkness. Time was reported



(a) short-term

(b) long-term

Figure 2.4.: Schedule of alcohol access and deprivation phases for the ethanol exposed rats

according to a standardised notation, the so-called *zeitgeber time* where ZT00 is the beginning of the light phase and ZT12 the beginning of the dark phase (see for example Gerstner and Yin, 2010).

2.2.3. Killing of rats

Animals were killed at four different time points throughout the day to obtain time-dependent gene expression profiles: ZT05, ZT11, ZT17, and ZT23 (see also figure 2.5). These time points were selected to present the mid of the light phase, the end of the light phase, the mid of the dark phase, and the end of the dark phase. All time points were one hour before the actual mid time points (ZT06, ZT12, ZT18, and ZT24) to allow for the consecutive killing of six animals before the start of the next quarter of the day. Killing at time points ZT17 and ZT23 were performed under red light to maintain the circadian rhythm. Always six animals of each experimental condition were killed at one time point with the exception of long-term control rats at killing time point ZT23, where only five animals were sacrificed due to a premature death in this group. Animals were briefly anaesthetised with CO_2 and beheaded with a hand-held guillotine. Brains were immediately taken out from the sculls, shock-frozen in $-50\,^{\circ}\mathrm{C}$ cold isopentane and stored at $-80\,^{\circ}\mathrm{C}$ until further processing.

2.2.4. Brain dissection

The frozen rat brains were cut into 120 µm thick coronal slices at an ambient temperature of $-20\,^{\circ}\mathrm{C}$ in a Leica CM 3000 Cryostat (Leica, Wetzlar, Germany). The nucleus accumbens was identified according to images from a rat brain atlas ((Paxinos and Watson, 1998) and extracted with 0.75 to 1.5 mm diameter tissue punches (Stoelting, Wood Dale, IL, USA).

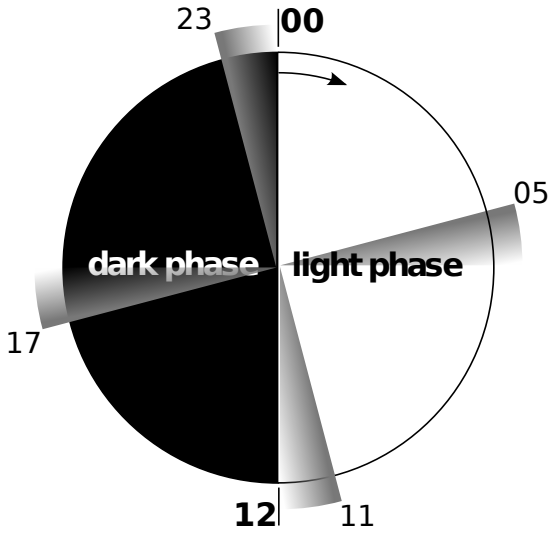


Figure 2.5.: Time-points of sacrifice of the rats throughout the circadian rhythm in zeitgeber notation with lights on from 00 hours till 12 hours and lights out from 12 hours till 24 hours. The grey shaded areas correspond to the four time slots for sacrificing the animals starting at 05, 11, 17 and 23 hours.

2.2.5. RNA isolation and purification

RNA was isolated by phenol-chloroform extraction (Chomczynski and Sacchi, 1987). 1 ml of TRIzol® Reagent (Life Technologies, Darmstadt, Germany), a monophasic solution of phenol and the chaotropic agent guanidinium thiocyanate, was added to the punched tissue samples and the suspensions were homogenised by multiple passages through a 22 gauge needle. Samples were filled up with 200 µl of chloroform, mixed and centrifuged to obtain a separation of the aqueous upper and the organic lower phase. The RNA containing upper phases were carefully collected and purified with an RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the RNAs were analysed with a Nanodrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany). All samples had a ratio of absorption at 260 nm versus 280 nm in the range of 1.8 to 2.2, signifying low contamination with leftover proteins. RNA integrity was further analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples had *RNA integrity number* (RIN) values above eight.

2.2.6. Illumina RatRef-12 Expression BeadChips

RNA samples were prepared for microarray analysis at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) using the Illumina TotalPrep RNA Amplification Kit (Life Technologies, Darmstadt, Germany) following the manufacturer's protocol. In brief, 500 ng RNA were reverse transcribed to first strand complementary DNA (cDNA) using Array Script™ reverse transcriptase with oligo-thymidine primers connected to the promoter region of the T7 bacteriophage. The cDNA was purified and processed using RNAse H to clean the first strand DNA from remaining RNA. DNA polymerase was

used for second strand synthesis after which the DNA was purified once again. T7 RNA polymerase was used for in vitro transcription. As a part of the required uridine triphosphate (UTP) was tagged with biotin this process yielded biotinylated complementary RNA (cRNA). The cRNA was purified, quantified with the Nanodrop 1000 Spectrophotometer and quality checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All cRNA samples had an acceptable RNA size distribution.

The cRNA was hybridised on the microarrays using the RatRef-12 Expression BeadChip Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol. In brief, 750 ng cRNA per sample were hybridised on RatRef-12 Expression BeadChips and incubated for 14 hours on a rocker mixer. BeadChips were subjected to two washing steps according to manufacturer's protocol to remove unbound cRNA. BeadChips were then stained with Cyanine 3 coupled to streptavidin which binds to the biotinylated UTPs of the cRNA. This was followed by another washing step.

BeadChips were scanned on an Illumina BeadArray Reader using Bead Scan Software (Illumina) and read out as text files.

2.2.7. Microarray data pre-processing

The acquired fluorescence values from the hybridised microarrays were pre-processed in the DKFZ Core Facility's R pipeline. The pre-processing in the pipeline included the removal of outliers, quality assessment, between array normalisation, and averaging of the bead-level data. As a last step I updated the array annotation as described below.

Removal of outliers

The first step was to exclude all beads with fluorescence values below 20, as lower values can no longer be distinguished from the array's background signal. Outliers were defined as having a median deviation of more than 2.5 times greater than a consistent estimation of the standard deviation:

If X was the set of the fluorescent values $x_1, x_2, ..., x_i$ from the multiple replicates of one probe on the array and k = 1.4826 the scaling factor for a consistent, robust estimation of the standard deviation assuming a normal distribution of X (Sachs and Hedderich, 2006) then the outliers were calculated according to the following equations:

Median absolute deviation:	$MAD = median(\|x_i - median(X)\|)$
Estimator of standard deviation:	$\sigma^* = 1.4826 \cdot MAD$
Definition of outliers:	$\|x_i - median(X)\| > 2.5 \cdot \sigma^*$

Quality control

Microarray data were quality assessed using the quality control features present on the Illumina arrays. These consisted of the following items and their complementary probes:

- Hybridisation control: Detection of six different Cy3-labelled oligonucleotides which were spiked in the hybridisation buffer.
- Negative control: Detection of the system background noise with random sequence probes which are not complementary to any known rat RNA.
- Hybridisation stringency control: Detection of the Cy3-labelled oligonucleotides form the hybridisation control with complementary probes that contain two mismatches.
- Gene intensity control: Detection of selected housekeeping genes.
- Biotin control: Detection of two heavily biotin-tagged oligonucleotides which were spiked in the hybridisation buffer.
- High stringency control: Detection of a spiked-in oligonucleotide with a very high GC content that would hybridise and yield a signal even if hybridisation stringency was too high.

All samples but one passed the quality control. The failed sample belonged to one of the long-term control animals and was excluded from subsequent steps. Given the one incident of premature death which also affected this group, the long-term control group had only four array data available. All other groups had six array data sets.

Normalisation and averaging of bead level values

All bead values were log2 transformed to reduce the influence of absolute intensity magnitude on variation and to approximate the bead intensity values to a Gaussian distribution. Arrays were normalised over all samples and beads using the quantile normalisation algorithm (Bolstad et al., 2003) of Bioconductor's affy package (Gautier et al., 2004). Intensity values of one bead type were averaged using arithmetic mean.

Annotation

The most up-to-date Illumina annotation for the RatRef-12 Bead Array was '*RatRef-12 V1 0 R5 11222119 A*'. Excluding the quality control probes, each BeadChip contained 22 517 probes. But only 6 366 (28,3%) of those probes had a biologically interpretable annotation, while the remaining probes consisted of locus identities, predicted proteins, or non-specified rat genome database and mammalian gene collection entries.

We therefore made use of the *Re-annotation and Mapping for Oligonucleotide Array Technologies* (ReMOAT) project, which provides re-computed probe annotations for Illumina BeadArrays (Barbosa-Morais et al., 2010). We compared ReMoat's '*Rat-RS-V1 rn4 V1.0.0 Aug09*' annotation to Illumina's '*RatRef-12 V1 0 R5 11222119 A*' and kept all congruent probes (3 433). Probes which had only a locus identity, mammalian genome corporation or rat genome database number were eliminated. To this we added the remaining probes which had a ReMoat annotation labelled "good" or "perfect" (893) despite having a purely numerical Illumina probe annotation. And finally we added those probes that were well-annotated in the Illumina version (no locus ID, etc.) despite having a bad ReMoat annotation. This led to a final number of 7 656 probes per array.

2.2.8. Statistical analysis

All statistical analyses were performed using LibreOffice 3 and R statistical programming language version 2.15.0.

Principal component analysis

Principal component analysis was performed on the quantile normalised data using the *prcomp*-function of the *stats*-package of R. For graphical presentation, the data points representing the microarray data sets from the individual rats were plotted against the first two principal components. The legend was added and the plot was coloured in *inkscape 0.48*.

Detection of daily oscillating genes

For each treatment group, the four time points were concatenated and then z-score transformed so that every gene had a mean expression of zero and a standard deviation of one (Cheadle et al., 2003) according to the following formula:

$$x' = \frac{x - \mu}{\sigma}$$

In the formula, x corresponded to a given log2-transformed expression value of a gene, μ and σ to the arithmetic mean and standard deviation of all expression values of this gene and x' to the z-score transformed value.

Genes with circadian oscillating expression levels were then identified using the *fdrfourier*-function of the *cycle*-package of R (Futschik and Herzel, 2008) based on a period of 24 h. The function calculated a *Fourier score* for each gene which became larger if a gene was closer to a cosine curve of the given period length of 24 hours:

$$Fourier\ score = \sqrt{\left(\sum_{i=1}^{n} cos(2\pi \cdot \frac{t_i}{T}) \cdot x'_i\right)^2 + \left(\sum_{i=1}^{n} sin(2\pi \cdot \frac{t_i}{T}) \cdot x'_i\right)^2}$$

In the formula, n corresponded to the number of measurement points (16 for the long-term control group, 24 for all other groups), T to the period length (24 hours) and x'_i to the z-score transformed expression value at the time t_i. The *fdrfourier*-function also computed an empirical false discovery rate (FDR) (see Futschik and Herzel (2008) for details) which was calculated on the basis of 100 permutations within the rows in our case.

The thus calculated oscillation scores and false discovery rates were used as a basis for the analysis of the daily expression profiles of the core clock genes, the E-box containing genes and the hypothesis-free screening of the microarray data. In the latter - exploratory - analysis, we considered all genes with a FDR $< 5\,\%$ as oscillating while all genes with a FDR $> 50\,\%$ were considered non-oscillating.

Sorting of the genes according to their expression profiles and display in heatmaps

Pairwise Pearson correlation distances were computed between the expression patterns of the oscillating genes using the *cor.dist*-function of the *bioDist*-package of R. The *hclust*-function of the *stats*-package was then used to hierarchically cluster the genes based on these pairwise distances using *complete linkage* as agglomeration method.

For graphical presentation, heatmaps with the results were drawn using the *heatmap.2*-function of the *gplots* R package. The heatmaps of the short-term and long-term rats were each arranged into a composite plot using *inkscape 0.48* and fitted with labels and a legend.

T-tests and fold change calculations

Genes that were differentially regulated under the influence of alcohol exposure were detected based on *Student's t-tests* using the Welch adaptation for possibly unequal variances. The tests were performed on the log2-transformed but not Z-score transformed data. In addition, the fold change, corresponding to the ratio of the average alcohol-exposed mRNA concentration versus the average control mRNA concentration, was calculated for each gene and time point and each treatment-duration group according to the following formula:

$$fold\ change = \frac{\frac{1}{n}\sum_{i=1}^{n} x_{alcohol,i}}{\frac{1}{m}\sum_{i=1}^{m} x_{control,i}}$$

In the formula, the variables $x_{control}$ and $x_{alcohol}$ corresponded to the non-logarithmic, non-Z-score transformed expression values, n to the number of replicates in the alcohol-exposed groups (always six) and m to the number of replicates in the control groups (six, except for ZT17 of long-term rats were it was four).

Genes were considered significantly differentially expressed if the t-test p-value was below 0.05 and the absolute fold change (= |fold change|) above 1.2.

Volcano plots were created in R based on the calculated p-values and fold changes. For purpose of presentation, the values of the x-axis were transformed to $log_2(fold\ change)$ and the values of the y-axis were converted to $log_{10}(p\ values)$. The graphs were arranged into a composite plot using *inkscape 0.48*.

2.3. Results

Messenger RNA extracted from the nucleus accumbens of short-term and long-term alcohol drinking rats and from age-matched control animals was quantified by DNA microarray technology. After quality control and exclusion of probes missing a proper gene annotation we obtained a data set consisting of expression values of 7656 genes in 94 samples, each sample corresponding to one rat. Based on the time of day when the rats where sacrificed, the access to alcohol or only water and the duration of the experiment, the samples could be divided into 16 groups (see table 2.1).

Table 2.1.: Overview of the 16 different treatment groups and their number of samples. ZT 5 and 11 lay in the light phase while ZT 17 and 23 lay in the dark phase.

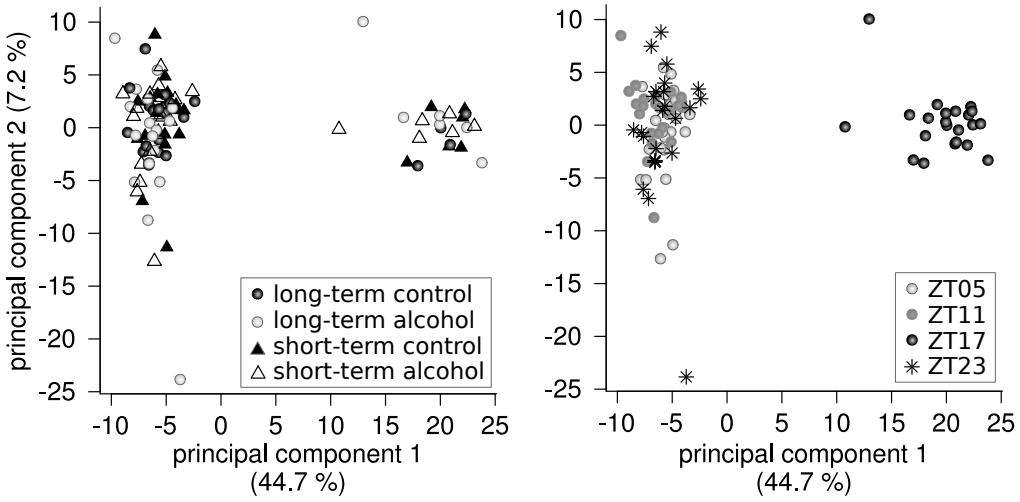
zeitgeber time	short-term		long-term	
(in hours)	control	alcohol	control	alcohol
05:00	6	6	6	6
11:00	6	6	6	6
17:00	6	6	4	6
23:00	6	6	6	6

2.3.1. Principal component analysis

To get an impression of the main sources of variability in our data set we conducted a sample-focused principal component analysis on the quantile normalised and log-transformed values of all arrays combined. The first two principal components were responsible for 44.7 % and 7.2 %, respectively, of the variability in the data. The samples were plotted on a coordinate system whose axis corresponded to the first two principal components and labelled according to the rat's treatment (figure 2.6a) or the time when the animal was sacrificed (figure 2.6b). Animals did neither cluster according to their access to alcohol or only water nor did they group according to the length of the experiment. The time point of sacrifice, on the other hand, led to a visible differentiation with zeitgeber time 17, corresponding to the midst of the rodents' active dark phase, clearly apart from all other time points. No further subdivision for zeitgeber times 05, 11 or 23 was visible.

2.3.2. Detection of daily oscillating genes by Fourier transformation

We then proceeded to identify genes which showed a daily oscillating expression pattern using a fast Fourier transform algorithm which calculated *Fourier scores* as a measurement of how far a gene expression profile consistently resembled a 24-hour cosine curve. In addition, the algorithm calculated a false discovery rates for each gene until which it would be considered daily oscillating. As the rats had been kept under a cycle of 12 hours light followed by 12 hours darkness (LD 12:12) the Fourier transform algorithm detected daily oscillating genes regardless whether the oscillation of their gene expression was driven by underlying molecular circadian rhythms or by external factors.



(a) Treatment group **(b)** Time of day

Figure 2.6.: Scatter plot of the nucleus accumbens RNA samples projected on the first two axis of variation as calculated by principal component analysis (PCA). Samples were labelled according to treatment group (a) and time of day (b).

Table 2.2 shows the numbers of genes which were detected as daily oscillating in the four subgroups according to a false discovery rate of 10%. Up to 50 % of the genes were identified as showing regular daily oscillations in their mRNA levels. The relatively low number of such genes in the long-term control rats was probably due to the fact that this group contained only four replicates which could be concatenated while all other groups had six.

Table 2.2.: Number of daily oscillating genes in the four treatment groups (out of 7 656 genes in total).

	control	alcohol
short-term	4 096	3 748
long-term	2 308	3 830

2.3.3. Analysis of the core clock genes

Having identified the oscillating genes, we first concentrated our attention on the core clock genes which had been presented in figure 2.2 of the introduction.

Table 2.3 presents an overview of the Fourier scores and associated false discovery rates (FDR) of these genes. *Rora* and *Csnk1e* were not present in the quality filtered microarray data set but *Csnk1a1*, which is closely related to *Csnk1e*, was present in the data. While *Arntl*, *Cry1* and *Csnk1a1* displayed an oscillating profile in all four treatment groups according to a FDR $< 10\,\%$, *Cry2* was oscillating in all groups but the long-term control rats. *Nr1d1*, *Per1*, *Per2* and *Per3* gene expression oscillated each in only one treatment group.

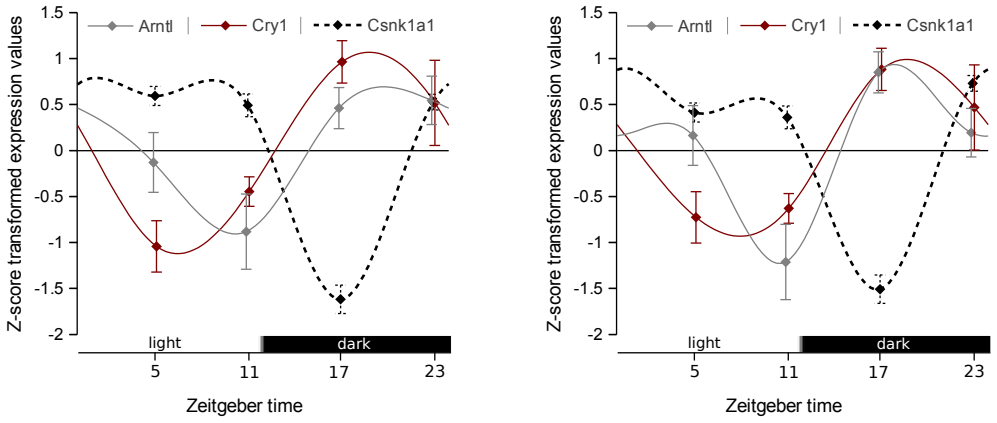
Table 2.3.: Fourier scores and false discovery rates of core clock genes. Scores with an associated FDR $< 10\,\%$ **in bold**.

	short-term				long-term			
	control		alcohol		control		alcohol	
Gene	score	FDR	score	FDR	score	FDR	score	FDR
Arntl	**9.29**	**0.046**	**9.41**	**0.043**	**8.75**	**0.029**	**11.19**	**0.008**
Clock	5.74	0.349	2.67	0.815	2.71	0.751	4.31	0.568
Cry1	**13.39**	**0.001**	**11.70**	**0.004**	**7.29**	**0.084**	**11.27**	**0.008**
Cry2	**9.43**	**0.041**	**11.14**	**0.009**	6.09	0.194	**9.79**	**0.032**
Nr1d1	**11.95**	**0.004**	6.12	0.306	5.66	0.248	6.52	0.260
Per1	6.19	0.291	**8.46**	**0.088**	2.24	0.825	7.30	0.176
Per2	3.33	0.716	6.59	0.249	**7.27**	**0.085**	7.47	0.161
Per3	7.72	0.134	**10.62**	**0.014**	6.99	0.105	7.90	0.127
Csnk1a1	**13.30**	**0.001**	**11.76**	**0.004**	**8.39**	**0.036**	**12.35**	**0.002**

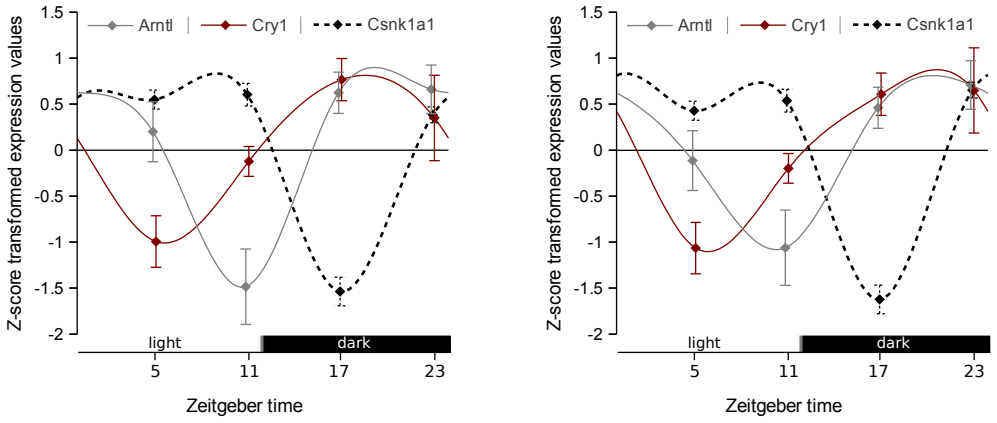
Next, we wanted to know if the expression profiles of *Arntl*, *Cry1* and *Csnk1a1* which had shown oscillating mRNA levels in all treatment groups had always the same appearance. The other option would be that either alcohol consumption or treatment duration or both factors combined alter the expression profile while maintaining its oscillating appearance. We plotted the expression profiles in the four treatment groups (figure 2.7) and visually compared the daily curves. Besides of slight variations in the profile of *Arntl* at ZT05, ZT17 and ZT23, the three genes showed roughly the same expression profiles in all four conditions.

We also plotted the expression profiles of *Per1* and *Per2* because of these genes' pivotal role in both circadian rhythm generation and transition to addiction (figure 2.8). *Per1* showed a phase reversal with a peak of mRNA concentration during the light phase in short-term rats and during the dark phase in the long-term animals. There was no consistent effect of alcohol in the short-term and long-term treatment group and the low mRNA level amplitudes combined with the big error bars explain why the algorithm did not consider these profiles circadian oscillating with the exception of the one of the short-term alcohol drinking rats.

In the case of *Per2*, all treatment groups showed the highest mRNA concentration at zeitgeber time 11, corresponding to the end of the light phase with the peak approximately twice as big in the rats of the long-term paradigm. This difference in peak height was not significant according to a t-test comparing the Z-score transformed expression values at 11 hours of all short-term to the corresponding values of all long-term rats (p-value: 0.16). The long-term control animals had their lowest *Per2* expression at zeitgeber time 23 and showed a bigger amplitude than all other groups which explains why this group profile was rated as oscillating by the Fourier algorithm. The short-term control animals had the lowest amplitude and the short- and long-term alcohol groups were in between.

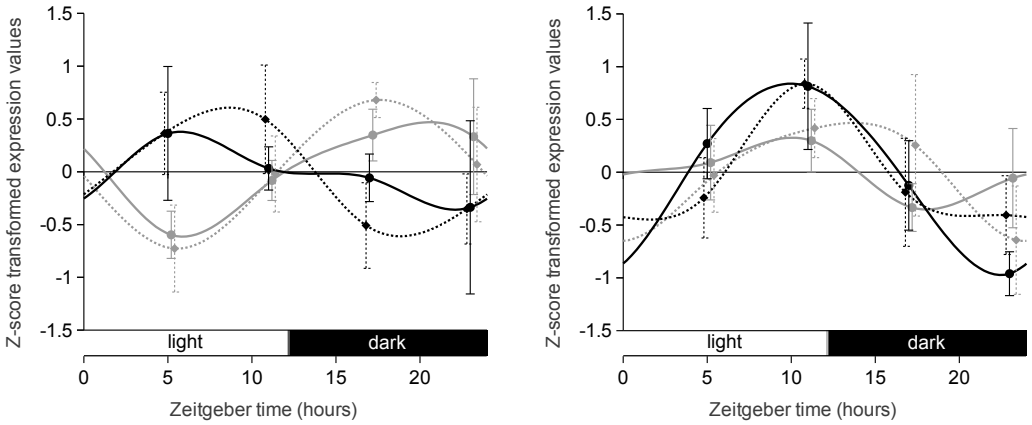


(a) short-term control **(b)** short-term alcohol



(c) long-term control **(d)** long-term alcohol

Figure 2.7.: Daily expression profiles of *Arntl*, *Cry1* and *Csnk1a1* in the four treatment groups. Shown are mean values ± standard error of the mean.



(a) Per1 **(b)** Per2

Figure 2.8.: Daily expression profiles of *Per1* and *Per2* in the four treatment groups. Shown are mean values ± standard error of the mean.

Legend: = short-term control, = short-term alcohol
= long-term control, = long-term alcohol

2.3.4. Analysis of selected E-box genes

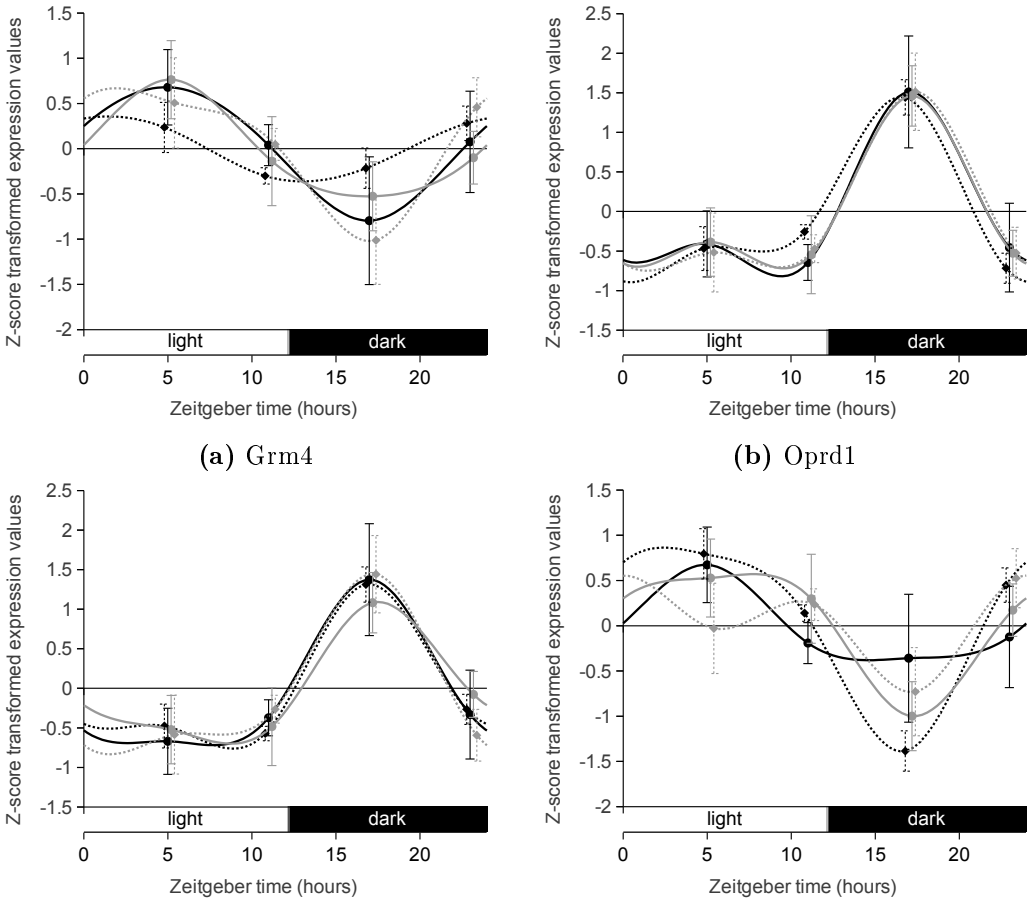
In the next step, we wanted to assess the degree of diurnal oscillation in the expression of E-box genes. We concentrated the analysis on the expression profiles of the addiction-related E-box containing genes listed in the introduction (see section 2.1.2).

Table 2.4 presents the Fourier scores and false discovery rates of these genes. *Oprd1* and *Slc6a2* showed significant oscillation in their expression pattern in all four treatment groups based on a false discovery rate $< 10\,\%$. *Th* and *Grm4* showed oscillating expression levels in two or only one group, respectively. *Drd1* was not present in the quality filtered microarray data.

Table 2.4.: Fourier scores and false discovery rates of selected E-box genes. Scores with an associated FDR $< 10\,\%$ **in bold**.

	short-term				long-term			
	control		alcohol		control		alcohol	
Gene	score	FDR	score	FDR	score	FDR	score	FDR
Grm4	7.74	0.133	**9.44**	**0.042**	5.90	0.217	4.14	0.553
Oprd1	**11.07**	**0.009**	**12.19**	**0.003**	**7.72**	**0.061**	**11.79**	**0.004**
Slc1a3	3.63	0.669	8.04	0.114	2.54	0.782	4.72	0.506
Slc6a2	**9.93**	**0.027**	**12.33**	**0.002**	**8.17**	**0.043**	**10.88**	**0.011**
Slc6a3	2.08	0.881	2.02	0.892	0.72	0.981	4.43	0.551
Th	**9.19**	**0.050**	4.55	0.532	4.14	0.495	**13.21**	**0.001**

As in the case of the core clock genes, we plotted the expression profiles of the oscillating genes to assess if their appearance was different between the treatment groups. *Grm4* had about the same profile in all four treatment groups with the biggest difference in the short-term alcohol-exposed rats. *Oprd1* and *Slc6a2* had each approximately identical expression profiles in all groups. *Th* showed the biggest deviations in the profiles of short-term alcohol-drinking and long-term control rats. The expression difference appeared most pronounced at ZT17 but did not reach statistical significance with an uncorrected p-value of a Student's t-test of 0.247 (see figure 2.9).



(a) Grm4 **(b)** Oprd1

(c) Slc6a2 **(d)** Th

Figure 2.9.: Daily expression patterns of E-box containing genes: *Metabotropic glutamate receptor 4* (*Grm4*), *opioid receptor δ1* (*Oprd1*), *norepinephrine transporter* (*Slc6a2*) and *tyrosine hydroxylase* (*Th*) in the four rat groups. Shown are mean values ± standard error of the mean.

Legend: = short-term control, = short-term alcohol
= long-term control, = long-term alcohol

2.3.5. Screening for oscillating genes that change under the influence of alcohol

Having analysed the expression profiles of core clock genes and selected E-box containing genes, we wanted to screen our data set for genes whose circadian expression rhythm was disturbed by the rats' alcohol consumption. In this exploratory approach, we defined genes showing daily oscillating mRNA levels according to a false discovery rate below 5 % in the Fourier analysis as truly oscillating and genes that oscillated with a false discovery rate above 50 % as not oscillating. Table 2.5 shows the number of genes which were identified according to these conditions.

Table 2.5.: Number of oscillating genes according to different false discovery conditions (out of a total of 7656 genes).

		FDR $<$ 05 %	FDR $>$ 50 %
short-term	control	2 864	1 099
	alcohol	2 613	1 203
long-term	control	874	1 677
	alcohol	2 691	1 211

We then started with the short-term paradigm rats and determined the overlap between the truly oscillating genes of the control and alcohol samples. In addition, we identified those genes which oscillated in the control but not in the alcohol-drinking rats and those genes which did not oscillate in the control but showed rhythmicity in the alcohol-exposed rats.

In order to get a visual expression of the oscillation pattern we sorted the genes according to a hierarchical clustering based on a Pearson correlation of the control samples and displayed the results in form of a heatmap (figure 2.10). The topmost heatmap contains the genes which were oscillating in both control and alcohol-exposed rats. It visually depicts that the vast majority of genes maintained the same oscillation pattern in both conditions. The mid-level heatmap shows the genes which oscillated in control but not in alcohol-exposed rats. It becomes evident that the expression pattern in the alcohol group resembled a pale copy of the pattern in the control group indicating that the genes in the former group had a similar oscillation tendency but a lower amplitude. The bottommost heatmap depicts the genes which oscillate in alcohol-exposed rats but not in controls. As in the mid-level heatmap, the non-oscillating genes seem to mirror the expression pattern of their oscillating counterparts albeit with a smaller amplitude.

We then performed the same analysis in the long-term paradigm animals and obtained roughly the same result (figure 2.11). Overall, the long-term animals showed less overlap between the different oscillation condition of control and alcohol rat genes which was probably due to the fact that the long-term control group contained only four replicates while all other groups had six.

Regarding those genes which had a significantly oscillating expression profile in both control and alcohol-exposed animals, there was an overlap of 576 genes between the short-term (n = 1 995 genes) and long-term (n = 704 genes) treatment group. There was not a

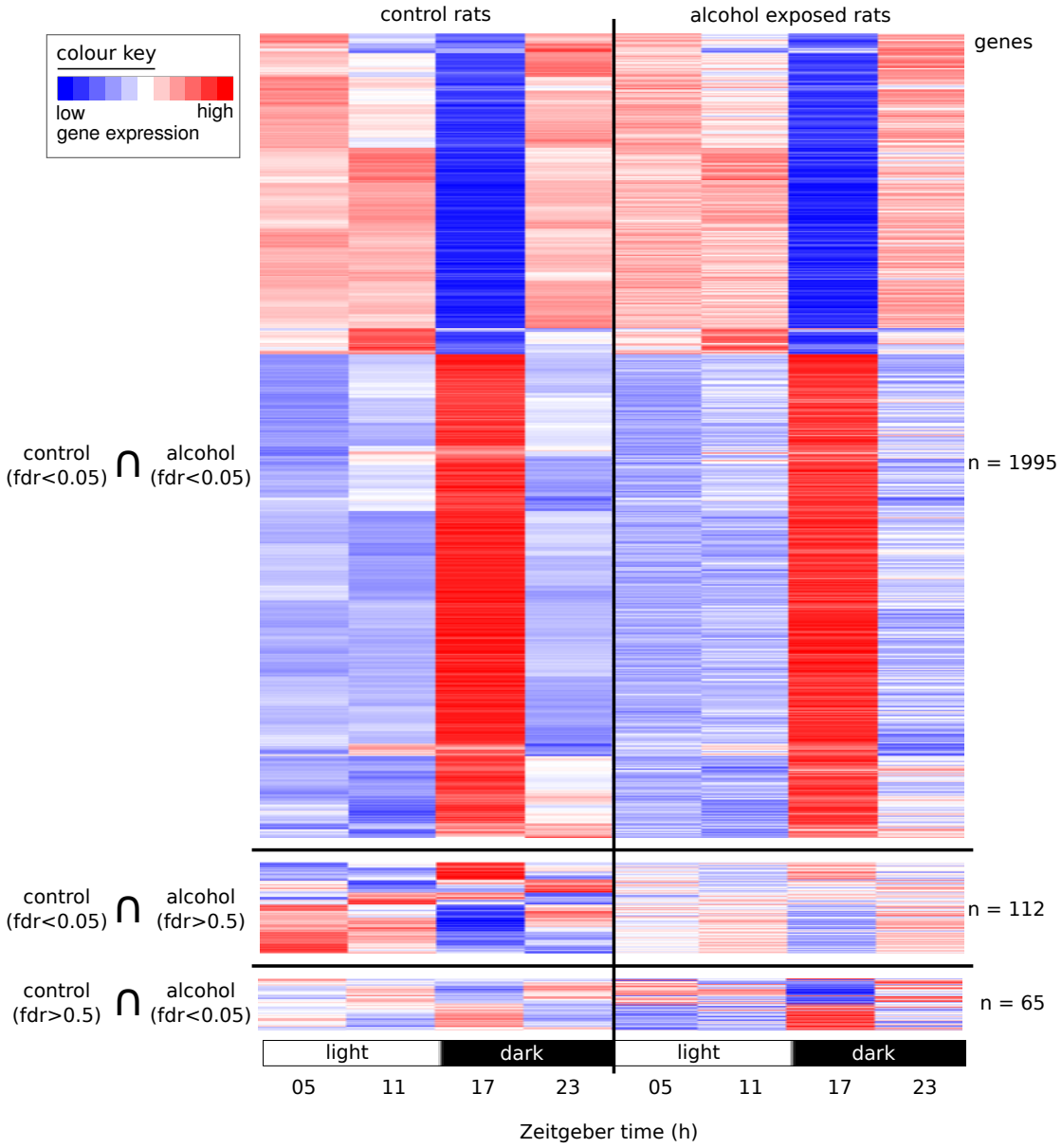


Figure 2.10.: Heatmaps of genes with oscillating mRNA levels in rats of the ***short-term*** paradigm. The uppermost heatmap shows genes which oscillated in both control and alcohol rats, the map in the middle depicts those genes which oscillated only in control rats and the heatmap at the bottom shows the genes which oscillated only in alcohol-drinking rats. The height of the uppermost heatmap is half the proportion of the other two maps for purpose of presentation.

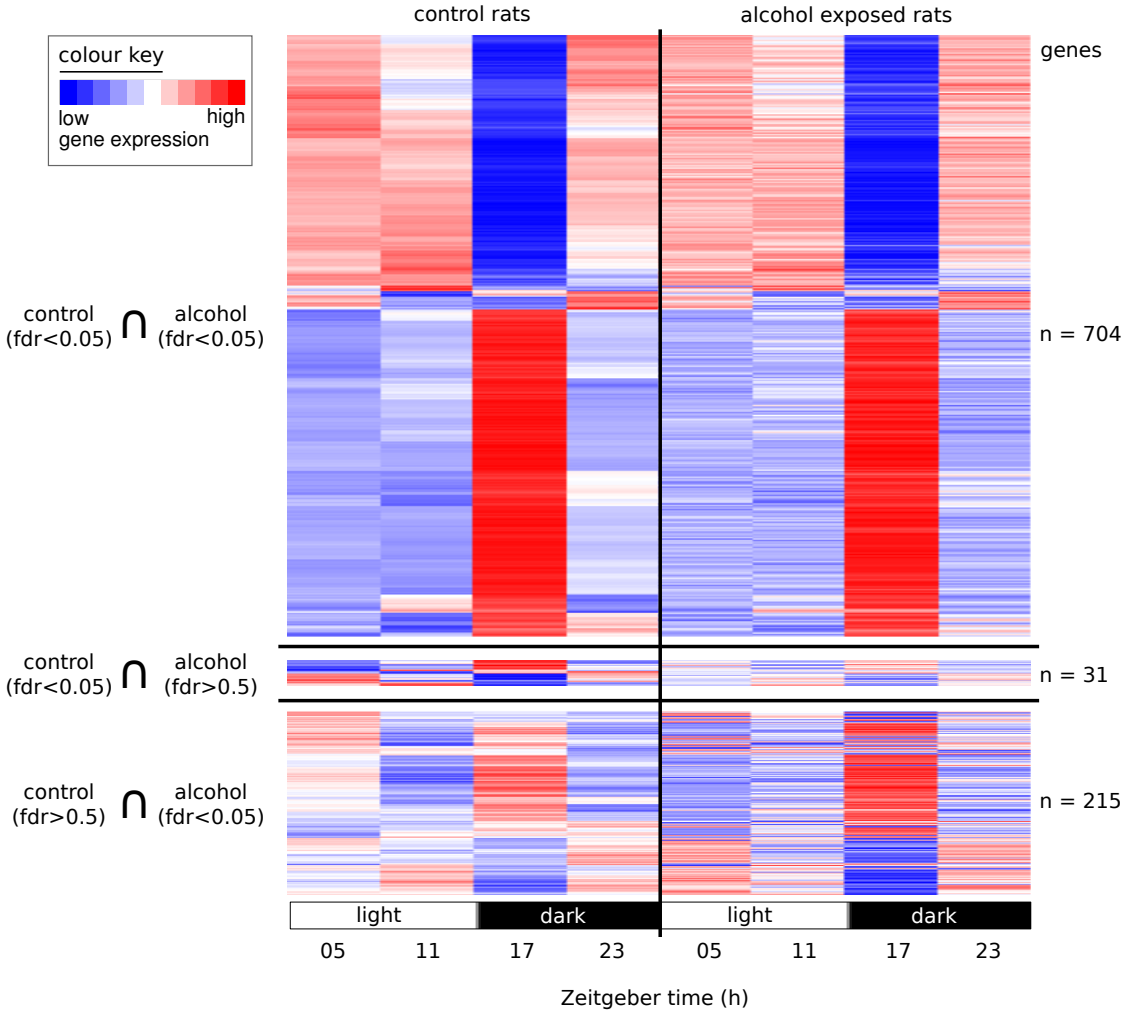


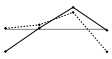
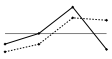
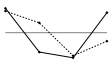
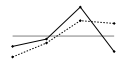
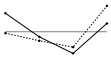
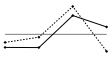
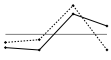
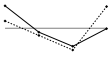
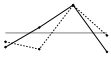
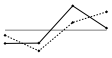
Figure 2.11.: Heatmaps of genes with oscillating mRNA levels in rats of the ***long-term*** paradigm. The uppermost heatmap shows genes which oscillated in both control and alcohol rats, the map in the middle depicts those genes which oscillated only in control rats and the heatmap at the bottom shows the genes which oscillated only in alcohol-drinking rats.

single common finding between the two treatment-length cohorts in the lists of those genes which had oscillating expression profiles in control but not in alcohol-exposed rats. And there was one gene in common between the two treatment-length cohorts in the lists of those genes which had oscillating expression profiles in alcohol-exposed but not in control animals: *neuropeptide B* (*Npb*).

In the long-term exposed rats, there were much more genes (n = 215) which showed a diurnally oscillating pattern only in the alcohol-exposed animals than genes (n = 31) which showed such an expression pattern only in the control animals. This is in contrast to the short-term animals where there had been more gene oscillating only in control rats (n = 112) than genes oscillating only in alcohol rats (n = 65).

Besides the visual analysis of oscillation pattern with the heatmaps we also calculated the Pearson correlation coefficients (Pearson's ρ) of the averaged gene expression profiles of control and alcohol rats. Table 2.6 contains the genes with the lowest coefficients. There was no overlap between those genes in short-term and long-term animals and the lowest Pearson correlation coefficients were still above 0.4 in both paradigms.

Table 2.6.: Top findings of oscillating genes that change under the influence of alcohol: Genes with a FDR < 0.05 in both control and alcohol-exposed rats.

short-term			long-term		
gene	Pearson's ρ	profile	gene	Pearson's ρ	profile
Ddx47	0.425		Rps3	0.412	
Ugt2b17	0.488		Tas2r114	0.476	
Prpf4b	0.601		Pomgnt1	0.583	
Jph2	0.632		Plunc	0.612	
Lmcd1	0.693		Prkab2	0.628	

Profile: —•— = control, ···•··· = alcohol exposed

Ddx47	= DEAD (Asp-Glu-Ala-Asp) box polypeptide 47
Ugt2b17	= UDP glucuronosyltransferase 2 family, polypeptide B17
Prpf4b	= PRP4 pre-mRNA processing factor 4 homolog B (yeast) (serine/threonine kinase)
Jph2	= junctophilin 2 (component of junctional complexes in excitatory cells)
Lmcd1	= LIM and cysteine-rich domains 1 (putative transcription factor)
Rps3	= ribosomal protein S3
Tas2r114	= taste receptor, type 2, member 114
Pomgnt1	= protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-)
Plunc	= BPI fold containing family A, member 1 (Bpifa1) (injury response in nasal epithelium)
Prkab2	= protein kinase, AMP-activated, beta 2 non-catalytic subunit

(gene information taken from: www.ncbi.nlm.nih.gov/gene/)

2. Influence of alcohol exposure and circadian rhythmicity on gene expression in the rat brain

We also extracted the most prominent cases of genes which showed oscillating mRNA levels in either the control or the alcohol paradigm but not the other. Table 2.7 shows their Fourier scores and false discovery rates in the control and alcohol groups for the short-term exposed rats and table 2.8 lists the corresponding values for the long-term consuming rats. The genes were selected from the two heatmaps at the bottom of the heatmap chart for the short-term and long-term animals, respectively. For each scenario, we selected the ten genes with the lowest false discovery rate in one drinking-condition and a false discovery rate above 50 % in other drinking-condition. There were no common findings among the top ten genes of any of these conditions.

Table 2.7.: Top 10 genes which showed rhythmic expression in one treatment condition but not the other. Table for the ***short-term exposed animals***.

control but not alcohol					alcohol but not control				
	control		alcohol			control		alcohol	
gene	score	FDR	score	FDR	gene	score	FDR	score	FDR
Angptl4	13.87	0.0003	3.88	0.64	Csmd1	3.13	0.74	11.17	0.008
Arl3	12.40	0.0023	4.25	0.58	Esr2	3.92	0.62	11.62	0.005
Cxcl13	12.36	0.0024	4.25	0.58	Ggnbp1	3.26	0.73	11.90	0.003
Dcir2	11.84	0.0042	2.99	0.77	Gimap6	4.65	0.51	11.29	0.007
Enth	12.36	0.0024	4.74	0.50	Mafk	2.80	0.79	11.33	0.007
Esm1	12.95	0.0013	3.07	0.76	Psbpc1	4.09	0.60	12.35	0.002
Itgbl1	13.70	0.0003	4.65	0.52	Rasl11a	2.73	0.80	10.86	0.011
Mboat5	12.13	0.0032	2.88	0.79	S100a3	1.66	0.92	12.50	0.002
Tpp2	12.95	0.0012	4.57	0.53	Trat1	2.93	0.77	10.80	0.012
V1rc20	11.94	0.0038	4.03	0.61	Ugt1a1	3.93	0.62	11.43	0.006

Table 2.8.: Top 10 genes which showed rhythmic expression in one treatment condition but not the other. Table for the ***long-term exposed animals***.

control but not alcohol					alcohol but not control				
	control		alcohol			control		alcohol	
gene	score	FDR	score	FDR	gene	score	FDR	score	FDR
Accn1	8.80	0.029	4.12	0.600	Arpc5	3.73	0.57	12.27	0.0025
Dmrt1	9.08	0.027	4.32	0.567	Casp3	4.03	0.52	12.60	0.0017
F2rl1	8.73	0.029	4.36	0.561	Crisp2	3.98	0.52	12.87	0.0013
Galnt3	8.79	0.029	1.90	0.899	Cryge	3.76	0.56	12.99	0.0010
Orai1	8.86	0.027	4.58	0.528	Limk1	1.87	0.87	12.74	0.0016
Prkx	8.99	0.026	3.84	0.641	March7	3.14	0.68	12.25	0.0026
Psgb1	8.82	0.028	2.97	0.770	Mtfmt	2.87	0.72	14.00	0.0003
Ptpn21	8.70	0.029	2.68	0.808	Pcsk9	3.87	0.54	12.40	0.0022
Taar5	9.70	0.012	4.06	0.608	Pgk1	3.67	0.58	13.00	0.0010
Taz	9.31	0.024	4.35	0.563	St3gal1	2.14	0.84	12.38	0.0022

2.3.6. Influence of exposure time: transition to addiction

Finally, we used the created microarray data set to screen for genes with significantly differential expression levels in alcohol-exposed compared to control rats. To get a first impression of the amount of gene expression differences in the nucleus accumbens of the alcohol versus the control rats at each time point and in both age groups, we plotted our data in the form of Volcano plots (figure 2.12). These plots are a special case of scatter plots in which each data point corresponds to one gene, its x-value indicating the logarithm of the ratio of average alcohol versus average control mRNA concentration and its y-value corresponding to the negative logarithm of the p-value of a t-test between the alcohol and control group. Genes with a significant p-value (in our case defined as below 0.05) and a big difference in the average expression levels (in our case: absolute fold change > 1.2) are of particular interest. They can be found in the upper right and left corners of our plots and were marked by asterisks. Our plots showed that the impact of alcohol on overall gene expression was approximately the same at all time points. There was also no clear trend for bigger gene expression differences in the long-term paradigm rats although there were more significantly differentially regulated genes in the long-term rats at zeitgeber time 17.

Table 2.9 lists the genes in the short-term paradigm animals which we considered significantly differentially expressed according to the above mentioned critera and which were marked by asterisks in the plot. Table 2.10 does the same for the long-term rats. Only two genes appeared in more than one condition (marked in bold in the tables). The *Cell division cycle-associated protein 7* (*Cdca7*) was up-regulated in short-term alcohol drinking rats at five and eleven hours zeitgeber time. *Cytoglobin* (*Cygb*) was down-regulated in short-term alcohol-drinking rats at time point five and up-regulated at time point eleven in long-term alcohol-drinking rats.

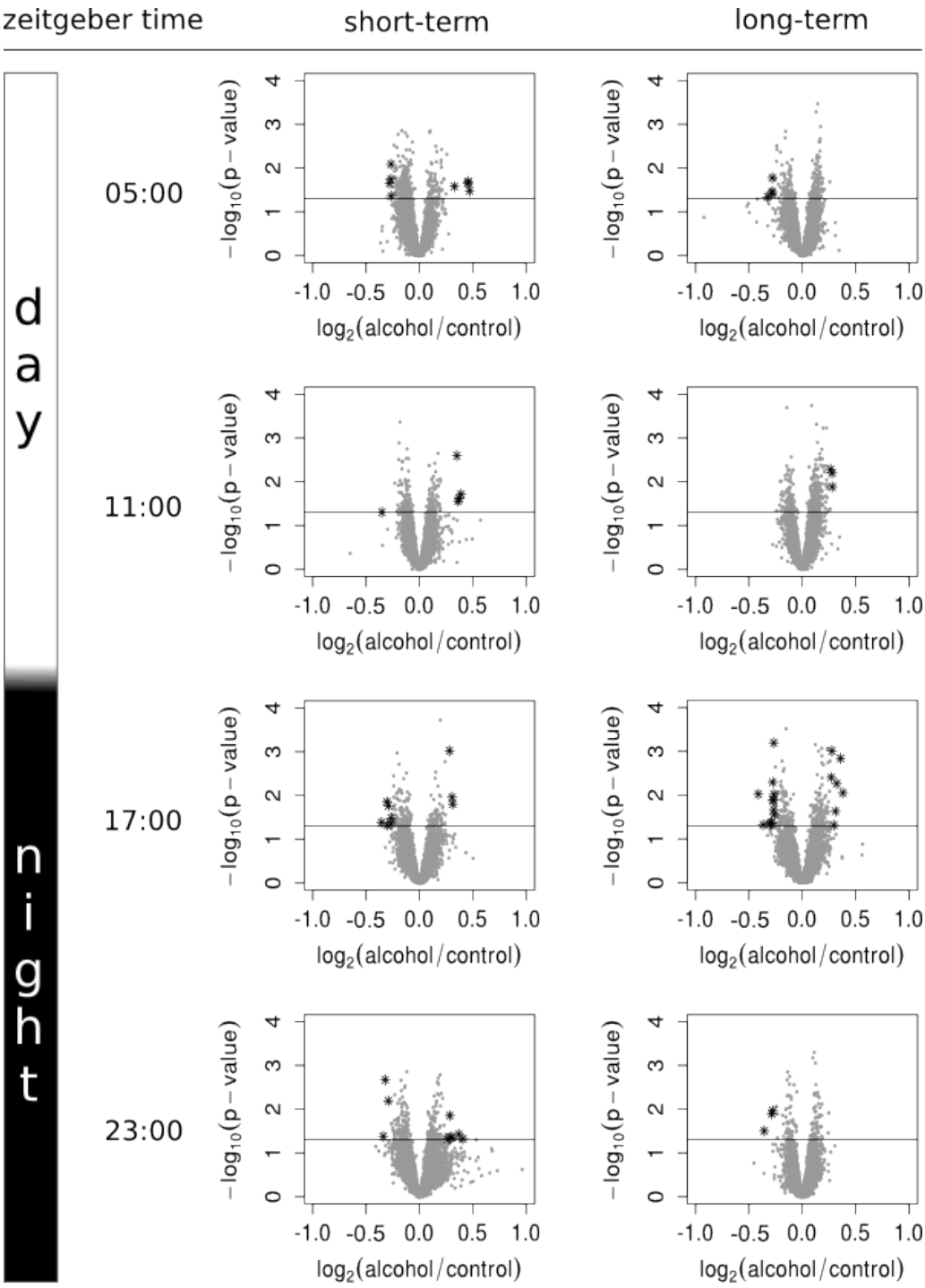


Figure 2.12.: Volcano plots of the comparison of alcohol versus control rats. Data points correspond to individual genes. The horizontal black lines correspond to a p-value level of 0.05 and significantly differentially regulated genes (based on a t-test p-value < 0.05 and an absolute fold change > 1.2) are represented by asterisks.

Table 2.9.: Top findings of differentially regulated genes* in alcohol exposed versus control rats of the ***short-term*** paradigm. Genes that appeared in several groups in this or the next table were marked in **bold**. Genes with a known relation to addiction or circadian rhythmicity were underlined and their role was addressed in the discussion section.

Light phase

ZT05			ZT11		
gene	t-test p-value	ratio alcohol/control	gene	t-test p-value	ratio alcohol/control
B4galt6	0.022	1.37	**Cdca7**	0.003	1.28
Cdca7	0.020	1.37	Ctgf	0.024	1.29
Cygb	0.022	0.82	Nr4a3	0.019	1.31
Fxc1	0.018	0.83	Sla	0.028	1.28
Nxf	0.026	1.25	Slc16a3	0.050	0.78
Pacsin2	0.043	0.83			
Rnf138	0.033	1.38			
Trappc1	0.008	0.83			

Dark phase

ZT17			ZT23		
gene	t-test p-value	ratio alcohol/control	gene	t-test p-value	ratio alcohol/control
Arg1	0.011	1.24	Dlgap1	0.038	1.29
Fip1l1	0.043	0.83	Glg1	0.044	1.23
Hba-a1	0.041	0.78	Grip2	0.042	0.79
Nefh	0.048	0.81	Hapln2	0.006	0.82
Pde1b	0.033	0.83	Hpcal4	0.047	1.33
Pscd3	0.014	0.81	RT1-A2	0.045	1.22
Rarres2	0.017	0.82	Sirt2	0.002	0.80
Slc17a6	0.016	1.24	Tnfsf12	0.050	1.20
Znf672	0.001	1.22	Tuba1	0.014	1.22

*) selected on the basis of a t-test p-value < 0.05 and an absolute ratio > 1.2

Table 2.10.: Top findings of differentially regulated genes* in alcohol exposed versus control rats of the ***long-term*** paradigm. Genes that appeared in several groups in this or the previous table were marked in **bold**. Genes with a known relation to addiction or circadian rhythmicity were underlined and their role was addressed in the discussion section.

Light phase

ZT05			ZT11		
gene	t-test p-value	ratio alcohol/control	gene	t-test p-value	ratio alcohol/control
Bdnf	0.035	0.82	**Cygb**	0.013	1.22
Prph1	0.046	0.80	Slc6a9	0.006	1.21
Reln	0.017	0.82	Znrd1	0.005	1.20
Unc5a	0.038	0.82			

Dark phase

ZT17			ZT23		
gene	t-test p-value	ratio alcohol/control	gene	t-test p-value	ratio alcohol/control
Amz2	0.005	1.25	Col1a2	0.031	0.78
Angptl4	0.013	0.82	Igf2	0.011	0.83
Cap1	0.013	0.83	Tpbg	0.013	0.82
Cfdp1	0.045	0.81			
Chn2	0.042	0.82			
Cugbp1	0.022	0.83			
Dhrs4	0.044	0.82			
Gabbr1	0.009	0.75			
Gria4	0.001	1.21			
Heph	0.004	1.21			
Hspa8	0.009	1.30			
Lpl	0.001	0.83			
Mtrf1l	0.023	1.24			
Nudt3	0.010	0.83			
Phactr1	0.028	0.83			
Pmch	0.001	1.28			
Ppp3r1	0.048	0.78			
Rbm34	0.005	0.83			
Tnfrsf11b	0.049	1.23			

*) selected on the basis of a t-test p-value < 0.05 and an absolute ratio > 1.2

2.4. Discussion of study

In the present study we wanted to analyse the influence of alcohol consumption on the transcription profiles of diurnally oscillating genes. Using microarrays, we had measured mRNA concentrations in the nucleus accumbens of voluntary alcohol-consuming and control rats because of the prominent role of this brain region in addiction.

Principal component analysis

In order to get a first impression of the main sources of variance on global gene expression in the nucleus accumbens we had performed a principal component analysis on our data. The fact that alcohol consumption had no visible effect on overall gene expression is probably due to moderate transcription alterations caused by the drug and to the limited number of genes which are affected. This is in line with a previous study by Matthäus et al. (2009) which had also reported that alcohol induced gene expression differences had little impact on the overall variability in gene expression data.

Nonetheless, we did not expect such a low influence of the duration of alcohol consumption on overall gene expression. Of course, the difference in the drinking history wasn't likely to play a major role given alcohol's limited effect on gene expression. But the long-term drinking rats were 185 days older than their short-term counterparts which is a substantial time span for a laboratory rat with its average life expectancy of three years. McCutcheon and Marinelli (2009) have argued in a systematic review of neuroscience articles that the age of an experimental animal can have a profound influence on the experimental outcome. We attribute the lack of this effect on the fact that our animals were 115 and 300 days of age at time of death, both ages being far beyond puberty. Neuronal maturation had been completed long before in both age cohorts and no substantial brain development happened during this period (Sengupta, 2011).

The big influence of the daytime on overall gene expression was almost exclusively due to zeitgeber time 17 which corresponded to the middle of the active night phase of the rats. Given the setup of our study we could not distinguish gene expression changes due to an endogenous circadian rhythm from changes due to differences in the animals activity levels. We suspect a greater influence of the latter as only zeitgeber time 17 stands apart while the other time points had formed a non-divisible cluster. We are not aware of any study specifically assessing the effect of behavioural activity levels on gene expression in the mammalian brain but it is conceivable that the working brain of an awake animal has a higher protein turnover and subsequently higher gene expression. Two studies, conducted in rat fibroblasts (Duffield et al., 2002) and rat pineal gland (Fukuhara and Tosini, 2008) also reported that gene expression showed the biggest alterations during the active night phase.

Detection of daily oscillating genes

It seems probable that the ability of the Fourier algorithm to discern genes with truly daily oscillating mRNA levels from those with aleatory fluctuations was diminished by the fact

that we had only four time points of measurement per day.

As shown in the heatmaps, the gene expression profiles were almost completely dominated by the amplitude at zeitgeber time 17 - the middle of the rats active phase. As a consequence, approximately 50 % of the genes were detected as oscillating at a false discovery rate of 10 %. We had nonetheless kept this FDR for the analysis of the core clock and the E-box genes as we considered sensitivity to be more important than specificity in the analyses.

Influence of alcohol consumption on core clock genes

Based on our hypothesis of an interplay between core clock and addiction-related genes, we had hoped to find core clock genes whose mRNA levels showed oscillation in control but no such oscillation or an altered rhythmicity in alcohol-drinking rats. In the best case such a loss or modification of oscillation should have been visible in both the short-term and the long-term treated animals.

While none of the core clock genes in our rats had fulfilled the best case criteria, two genes, *Rev-ErbA* α (*Nr1d1*) and *period 2* (*Per2*), had lost their diurnal expression rhythm in either the short-term or the long-term alcohol-drinking rats. Those genes, which had shown daily oscillations in all treatment conditions (*Arntl*, *Cry1* and *Csnk1a1*), had not displayed any noteworthy differences in their rhythmic profiles.

The importance of the *Per2* finding was reduced by the fact that the expression profile of this gene had reached almost significant oscillation in the long-term alcohol-drinking rats as well. *Nr1d1* had therefore been the only gene which unambiguously lost its rhythm under the influence of alcohol. This is particularly interesting as this gene was reported to have a strong impact on post-transcriptional mechanisms such as the expression of the thyroid hormone receptor (Hastings et al., 2000). *Nr1d1* had previously turned up as a finding in a gene expression study on ethanol vapour-exposed mice (Melendez et al., 2012) but an association study on methamphetamine dependent patients had failed to find a link between this nuclear receptor and addiction (Kishi et al., 2011).

Previous studies reported inconsistent results regarding the expression profiles of *Per1* and *Per2* (Masubuchi et al., 2000; Iijima et al., 2002; Uz et al., 2003; Li et al., 2009, 2010). The peak of *Per1* gene expression was reported either at the beginning, in the middle or at the end of the light phase or at the beginning of the dark phase. The expression maximum of *Per2* was reported either at the beginning or the end of the the dark phase or in the middle of the light phase (Iijima et al., 2002; Li et al., 2009, 2010). In our experiment, the expression maxima of *Per2* had been at the end of the light phase in all treatment groups. The lowest expression level of *Per2* had been at the end of the dark phase in the long-term paradigm rats. In the short-term rats, the lowest expression point had been in the middle of the dark phase for the control animals but had shifted to the end of this phase for the alcohol-exposed animals. Only the *Per2* expression profile of the long-term control group had been considered oscillating according to the Fourier analysis. Overall the expression oscillation in *Per2* but also *Per1* of the rats of the long-term treatment group had been more pronounced. As the different duration of the short- and long-term treatment also implied an age difference of several month between the animals, we suggest that the age

of an experimental animal might have a substantial influence on the circadian expression of *Per1* and *Per2* in the nucleus accumbens.

Previous studies have also reported that the expression profiles of the *period* genes can be markedly different in the nucleus accumbens core and shell region with *Per2* even showing opposing rhythms in these subregions (Li et al., 2009, 2010). This could explain why we failed to detect a significant circadian oscillation in these genes as we might have obliterated their region specific rhythms by extracting the mRNA of the nucleus accumbens as a whole.

Taken together, the analysis of the core clock genes did not substantiate the hypothesis that alcohol-drinking substantially modifies the circadian expression profiles of the core clock genes in the nucleus accumbens. A possible interpretation of our results is that the centre of integration between circadian rhythms and addiction does not lie in the nucleus accumbens. Several studies had reported alterations of the diurnal core clock gene expression in the hypothalamus of rats after chronic alcohol consumption or in the hypothalamus, suprachiasmatic nucleus and cerebellum of rats after prenatal exposure to alcohol (Chen et al., 2004, 2006; Farnell et al., 2008). Alterations of core clock gene oscillation had also been reported in the case of other drugs of abuse: in the mouse striatum after methamphetamine consumption (Iijima et al., 2002) and in the nucleus accumbens of rats following opiate withdrawal (Li et al., 2009, 2010). In a study on human post-mortem brain tissue, Li et al. (2013) had shown that major depressive disorder leads to significant phase interruptions of core clock gene expression in several brain regions. Interestingly, the greatest impact was in the dorsolateral prefrontal cortex while the expression profiles in the nucleus accumbens were more conserved between depressive patients and control subjects. Their findings suggest that the prefrontal cortex might therefore play a greater role than the nucleus accumbens in the connection between psychiatric disorder and circadian rhythm disturbance.

While we did not find changes in the expression rhythm of the core clock genes, there was still a chance that alcohol increased or decreased the expression of these genes at all time points. This eventuality was discussed below in the discussion of the t-test results.

Influence of alcohol consumption on selected E-box containing genes

Given the presumed role of E-box regulatory DNA sequences in the interference between alcohol addiction and the circadian rhythm machinery, we had also analysed a selection of addiction related genes which contained such E-box elements in their promoter region. As in the case of the core clock genes, we had speculated to find a neutralisation or marked alteration of diurnal oscillating gene expression under the influence of alcohol.

In the case of the *metabotropic glutamate receptor 4* (*Grm4*) we had found instead a gain in rhythmicity in short-term alcohol rats although the importance of this result is lessened by the fact that the gene was almost rhythmic in the corresponding control group as well. While *Grm4* had been linked to schizophrenia and epilepsy (Shibata et al., 2009; Muhle et al., 2010), there are no reports of a direct relation between this gene and addiction.

The *delta opioid receptor 1* (*Oprd1*) has been linked both to addiction in general as well as specifically to chronic alcohol abuse (Zhang et al., 2008; Hansell et al., 2009) but its

strong rhythmic profile had not been altered by alcohol-drinking in the present experiment.

Slc6a2, coding for the norepinephrine transporter which empties the synaptic clefts of norepinephrine and dopamine, had also shown strong but basically unaltered circadian rhythmicity in all groups. Its function is related to the action of the *tyrosine hydroxylase* (*Th*) which converts tyrosine to L-dopa thus paving the way for the synthesis of the catecholamine neurotransmitters dopamine and norepinephrine (Nagatsu, 1995). *Th* had been the only E-box containing gene which lost its diurnal mRNA oscillation in alcohol-drinking rats, albeit this happened only in the short-term treated rats. Lemmer et al. (2003) reported circadian expression oscillations of both *Slc6a2* and *Th* in the adrenal glands of rats. While their *Th* result was consistent with our findings their *Slc6a2* profile in the peripheral tissue was phase-reversed compared to our data from the central nervous system.

Taken together, our analysis of this small sample of E-box containing genes did not provide substantial evidence for an alteration of rhythmic expression profiles of these genes. The substantial diurnal oscillation of *Oprd1* and *Slc6a2* expression in the central nervous system has not been reported previously to the best of our knowledge and constitutes therefore an interesting accessory finding.

General screening for oscillating genes that change under the influence of alcohol

We had also screened our data in a more general approach for genes whose diurnally oscillating expression was markedly altered by alcohol, hoping to find clusters of genes which showed a characteristic circadian-oscillating expression pattern in control rats which would get disturbed in alcohol drinking rats. The visual comparison of gene expression profiles in the heatmaps had not provided any noteworthy examples of such clusters in short-term or long-term treated rats. The Pearson correlation coefficients of the genes with the least congruent expression profiles between control and alcohol-drinking rats had still been above 0.4 and would therefore conventionally be considered to indicate a modest to moderate correlation (Taylor, 1990). In addition, the least congruent genes were *DEAD box polypeptide 47* (*Ddx47*), *ribosomal protein S3* (*Rbs3*), *polypeptide B17 of family 2 of glucuronosyltransferases* (*Ugt2b17*) and *member 114 of type 2 taste receptor* (*Tas2r114*). We assume that these findings are due to statistical noise as none of these genes had a reasonable connection to alcohol consumption or circadian rhythm and the findings of short-term and long-term animals did not overlap.

As in the previous steps, this part of the analysis did not provide substantial evidence for an alteration of circadian gene expression profiles by alcohol consumption.

Comparison of mRNA levels in control and alcohol drinking rats of the short- and long-term paradigm

In the last part of our analysis we had compared the gene expression levels in control versus alcohol drinking rats at all the different time points and for both treatment duration groups.

This analysis, based on very moderate cutoff citeria of an uncorrected t-tests below 0.05 and an absolute fold change above 1.2, had produced surprisingly small lists of genes.

The limited number of significantly differentially expressed genes had made any reasonable analysis for enriched pathways or gene ontology terms impossible. The lack of substantial overlap between the different time points and the short- and long-term treatment groups again suggested a large number of biologically insignificant chance findings. *Cell division cycle-associated protein 7* (*Cdca7*) and *cytoglobin* (*Cygb*) had been the only genes that were significantly differentially regulated in more than one subgroup. To the best of our knowledge, none of the two proteins had been previously related to alcohol addiction or circadian rhythm.

Overall, the lists of differentially expressed genes contained many transcripts with no plausible connection to addiction or even neuronal activity. But there was also a number of genes whose predicted function could play a role in alcohol addiction:

In the ***short-term*** paradigm, the mRNA level of *neuron-derived orphan receptor 3* (*Nr4a3*) had been increased in alcohol drinking rats at zeitgeber time 11. In line with our result this gene had also been up-regulated in the striatum of rats following nicotine and methamphetamine consumption in a study by Saint-Preux et al. (2013) while it was shown to be down-regulated in the infralimbic cortex of alcohol-vapour exposed rats (Meinhardt et al., 2013).

The *phosphodiesterase 1B* (*Pde1b*) had been down-regulated in alcohol-drinking rats at ZT17. This gene has a high expression level in the striatum and has been associated with dopamine turnover (Siuciak et al., 2007) as well as DARPP32-dependent signalling in striatal medium spiny neurons (Ehrman et al., 2006; Spanagel, 2009).

Another gene of interest was the *excitatory amino-acid transporter* (*Slc17a6*), also known as *vesicular glutamate transporter 2* (*Vglut2*), which had been down-regulated in the alcohol-exposed animals at ZT11. The gene codes for a glutamate transporter which transfers glutamate into the synaptic vesicles (Shigeri et al., 2004) and which is co-expressed in dopaminergic neurons of the ventral tegmental area which project to the nucleus accumbens (Birgner et al., 2010). Alsiö et al. (2011) reported that mice in which the expression of this transporter had been suppressed in the midbrain dopaminergic neurons showed a higher affinity to high-sucrose food and cocaine in an operant self-administration paradigm. Together with our findings, this suggests that *Slc17a6* is related to addictive-like behaviours.

The *glutamate receptor interacting protein 2* (*Grip2*) had been down-regulated in alcohol-drinking rats at ZT23. This protein might play a role in the targeting of AMPA-sensitive glutamate receptors to the synapses (Dong et al., 1999). *Grip2* has not been linked to alcoholism or any other addiction so far to the best of our knowledge.

Also at ZT23, *sirtuin 2* (*Sirt2*) had been down-regulated in the alcohol drinking rats. The gene codes for a member of the *sirtuin* family of proteins and it was suggested that *Sirt2* acts as a deacetylase on different substrates such as α-tubulin (Maxwell et al., 2011) and histone H4 (Donmez and Outeiro, 2013). The protein family had been linked to circadian rhythmicity in previous studies although this was mainly due to studies on *Sirt1* (Wijnen, 2009). Renthal et al. (2009) reported that both *Sirt1* and *Sirt2* were up-regulated in the nucleus accumbens of cocaine-challenged mice. The authors could show in addition that the sirtuins were responsible for an altered electrical excitability of the nucleus accum-

bens neurons and that *sirtinol*, an inhibitor of the sirtuins, reduced the self-administration of cocaine. Our finding is thus in line with this previous study that sirtains might play a role in the establishment of drug addiction. But while the gene expression of *Sirt2* had been significantly oscillating in the short-term paradigm rats in our study, we did not find a modulation of this oscillation by alcohol.

In the ***long-term*** paradigm, the *brain-derived neurotrophic factor* (*Bdnf*) was down-regulated in alcohol-drinking rats at ZT05. *Bdnf* had been previously linked to alcohol abuse although there are conflicting reports about its expression levels upon chronic alcohol consumption (for an overview see Davis, 2008). Wolstenholme et al. (2011) reported an inverse correlation between *Bdnf* expression in the nucleus accumbens and ethanol consumption in mice. A similar finding had been reported for rats (Yan et al., 2005). But both studies were based on a comparison of preferring to non-preferring animals and the authors argued that low *Bdnf* levels might have existed before the alcohol consumption thus constituting a protective effect against alcohol addiction. Melendez et al. (2012) showed a down-regulation of *Bdnf* in the prefrontal cortex of alcohol vapour-exposed mice and a study in humans found lower *Bdnf* levels in the blood of alcohol dependent patients (Joe et al., 2007) which would be in line with our finding from the nucleus accumbens.

The *glycine transporter 1* (*Slc6a9*) was up-regulated in alcohol drinking rats at ZT11. This transporter protein removes the inhibitory neurotransmitter glycine from synapses. Previous studies have shown that glycine is involved in the dopamine surge in the nucleus accumbens after ethanol consumption and that inhibition of *Slc6a9* decreases the alcohol intake in rats and reduces their relapse rate (Molander et al., 2007; Vengeliene et al., 2010). Our results would concord to these findings although none of these studies had reported significant differential expression of *Slc6a9*.

The *GABA B1 receptor1* (*Gabbr1*) was down-regulated in alcohol drinking rats at ZT17. This finding is interesting as alcohol acts primarily on the $GABA_A$ receptor (Spanagel, 2009). In addition, baclofen, a $GABA_B$ receptor agonist, was proposed as a possible anti-craving drug for recovering alcoholics (Ameisen, 2005). Our finding of a down-regulation would make sense in this context but it is at odds with an earlier report that *Gabbr1* is up-regulated in the striatum of alcohol addicted mice (Ribeiro et al., 2012). A study conducted on human post-mortem hippocampus tissue showed that *Gabbr1* was significantly down-regulated in alcoholics (Enoch et al., 2012) which would be more in line with our finding although *Gabbr1* might well show contrasting expression levels in different brain regions of post-dependent individuals.

The *AMPA-sensitive ionotropic glutamate receptor 4* (*Gria4*) was up-regulated in alcohol drinking rats at ZT17. We found only one previous study which had measured *Gria4* gene expression under the influence of chronic alcohol consumption: Acosta et al. (2011) reported, in line with our findings, that *Gria4* was up-regulated in prefrontal cortex and anterior cingulate cortex of voluntarily alcohol-consuming cynomolgus monkeys (*Macaca fascicularis*).

Noteworthy is the absence of core clock genes on the lists of differentially regulated

genes. Huang et al. (2010) had reported that the baselines of the core clock genes were significantly lower in the blood of alcohol addicts as compared to control subjects. We could not corroborate these results with our data from the rat nucleus accumbens.

Outlook

All results taken together, our data indicate that the nucleus accumbens does not seem to be the brain area where the interaction between circadian rhythms and the development of alcohol dependence takes place. Nonetheless, our study demonstrated decisively how important it is to consider the influence of the circadian phase when taking biopsies for gene expression analysis. On the one hand, it is essential to choose which time of day produces the most meaningful results, while on the other hand, it is crucial that all samples are taken at approximately the same time.

As in the case of all negative research findings, the outcome of this study could be due to a real absence of the investigated phenomenon or to a lack of sensitivity in our experimental setup. Especially the inability to distinguish activity-level related gene expression differences from circadian rhythm oscillations proved to be a big limitation to our study.

Based on the results of this study several future experiments would be of interest. An option would be to repeat the measurements in the suprachiasmatic nucleus of long-term drinking rats to see if alcohol effects the rhythm generating clockwork at its core. Given the negative results in the nucleus accumbens, the caudate putamen and the ventral tegmental area would be further regions of interest where the interaction between circadian rhythmicity and development of alcohol addiction could take place. They could therefore be investigated in a similar study setup. More measurement times would thereby increase the accuracy of the Fourier analysis. The integration of a rat group kept in constant darkness could help to distinguish activity-related from truly circadian oscillations of gene expression. In addition, this would allow to assess the impact of alcohol on animal behaviour under a free-running circadian rhythm without external zeitgebers.

The final aim of these proposed studies would be to improve the treatment options for alcohol addicted patients. It makes therefore sense to switch to human subjects in follow-up experiments. Large questionnaire studies on signs of circadian disturbance in drinking and recovered alcoholics could improve our knowledge of the epidemiological aspects of the relationship between addiction and circadian rhythm alterations. Another option would be to screen for genes with circadian expression profiles in an easily available biological material, such as blood, and compare the profiles of addicted patients to those of healthy volunteers. In our next study we tried to lay the foundations for such an approach.

3. Influence of circadian rhythmicity on gene expression in human blood

3.1. Introduction to the study

As discussed in the previous project, alcohol addiction interferes with the proper functioning of the body's intrinsic circadian rhythm. The disturbances of the day-night cycle and the respective activity pattern often continue or even become worse after withdrawal from alcohol (Asheychik et al., 1989; Egbert, 1993; Jones et al., 2003). This might be partially the result of depressive mood states which are typical for the immediate days after discontinuation of alcohol (Laine et al., 1999; Pang et al., 2013) as major depression is well-known to be accompanied by insomnia (Jindal, 2009).

Given the lack of sufficiently effective psychological treatments and pharmaceutical drugs to control the high relapse risk of recovering alcoholics, the investigation of the alcoholism-related circadian distortions might lead to additional treatment approaches. Especially the degree of the disturbance of the body's endogenous clock might be an important clinical parameter as there is evidence that alcohol consumption and relapse risk are increasing with the severity of insomnia (Allen and Wagman, 1975; Brower et al., 2001). High risk patients with gravely deranged circadian rhythmicity could thus receive extra medical attention. Such patients might also respond to chronotherapeutic interventions such as *bright light therapy* or optimisation of the environmental factors of their sleep (*sleep hygiene*) (Schmitz et al., 1997; Landolt and Gillin, 2001; Hasler et al., 2012). In addition, the analysis of cellular circadian systems and their perturbation by alcohol might provide new molecular targets for the pharmacotherapy of alcoholism, as already mentioned in the previous study.

Assessment of the severity of the circadian rhythm perturbation could be made on the basis of questionnaires, sleep laboratory recordings and behavioural monitoring but all these techniques would either yield inexact results or be too elaborate for a routine clinical diagnosis. Measurements of melatonin or other oscillating hormone concentrations are another option but would not yield new pharmaceutical targets. It is therefore of high interest to look at the molecular level and assess how far the transcription patterns of genes with circadian oscillating mRNA levels are disturbed.

The effect of alcohol consumption and post-withdrawal states on circadian gene expression in the suprachiasmatic nucleus is well documented (Chen et al., 2004) but sampling patient tissue of this region or any other brain structure is practically impossible. Blood, on the other hand, is readily available and routinely taken in a hospital setting. In addition, Achiron and Gurevich (2006) as well as Sullivan et al. (2006) have shown that blood gene expression mirrors the transcription profile of the central nervous system. As many

other tissues in the human body, blood also shows clear signs of circadian rhythmicity. O'Neill and Reddy (2011) showed that red blood cells, which lack a cell nucleus in their mature state, show nonetheless circadian rhythmicity in the redox state of peroxiredoxins. And leucocytes have genes with circadian transcription levels (Watanabe et al., 2012).

The first step in the identification of genes whose circadian oscillating mRNA levels are altered by alcohol consumption is the identification of consistently diurnally fluctuating mRNAs in the blood of healthy subjects.

The aim of the present study was therefore to take blood samples from healthy test persons throughout a period of 24 hours and to identify genes with circadian oscillating transcription rates by microarray analysis. The result is a baseline curve of the candidate genes which can then be compared in a follow-up study to blood gene expression patterns in post-dependent individuals.

3.2. Materials and Methods

The clinical part of this study was performed by our collaborators at the Clinic of Psychiatry and Psychotherapy of the University Hospital of Göttingen (Göttingen, Germany).

3.2.1. Recruitment of test persons

Healthy male volunteers aged between 35 and 50 years of age were recruited for this study. All subjects were examined with standard psychiatric diagnostic tools for physical, psychiatric and neurological disorders and only subjects without any positive findings were invited for the subsequent study.

The original number of test subjects was set at ten but due to experimental difficulties with the nocturnal blood taking procedure, five of the first ten participants did not have a physiological sleep. Seven more participants were recruited to counter this fact.

3.2.2. Blood taking procedure

The participants spent one night of adaptation in the sleep laboratory of the Clinic of Psychiatry and Psychotherapy of the University Hospital of Göttingen. Blood taking started the next morning at 8:00 hours. A peripheral venous catheter was placed in one arm and two times 2.5 ml blood were taken every two hours till 8:00 hours in the next morning. The blood was collected directly into PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland). Throughout the experiment, the participants stayed in the Hospital. In the evening, the subjects went to the sleep laboratory at 22:00 hours and lights were switched off at 23:00 hours.

In the case of the first five subjects, staff would walk into the room every two hours during the night to withdraw blood samples from the catheter. With the exception of subject 4, the sleep of all other participants had been markedly disturbed by this procedure. Subject 6 dropped out of the study at 22:00 hours the second night.

For the following participants, the blood taking procedure during the night was modified: A plastic tube was used to connect the peripheral venous catheter to a valve which was situated behind a private screen. This allowed blood collection without disturbing the sleep of the test subjects.

The sleep quality of all participants was monitored by electroencephalography (EEG). Medical staff at the sleep laboratory of the University Hospital of Göttingen assessed the EEG recordings and categorised the patients in three groups with physiological, almost physiological and not physiological sleep.

3.2.3. RNA isolation and purification

The blood-filled PAXgene Blood RNA Tubes had been stored and shipped at −80 °C. They were thawed overnight at 4 °C and RNA was isolated using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's protocol. The RNA was subsequently purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. In the final step, RNA was eluted in

14 µl of RNase-free water. The concentration and purity of the RNAs were analysed with a Nanodrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany) and the degree of RNA degradation was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with a ratio of absorption at 260 nm versus 280 nm in the range of 1.8 to 2.2 as well as an *RNA integrity number* (RIN) above seven were considered for microarray analysis. Not all samples from subject 04, 09 and 12 met those criteria. As we did not want incomplete time series data we excluded these subjects and continued with the samples from subject 08, 11, 13, 15, and 17 for the microarray experiment.

Total RNA samples isolated from blood contain a high amount of globin mRNA which can impair the sensitivity of the microarray analysis. Hence, a variety of globin mRNA reduction methods have been invented to treat the RNA before gene expression analysis. We did not subject our RNA to globin mRNA reduction following the reasoning of Liu et al. (2006) that such a treatment can induce bias as these techniques can also affect non-globin messenger RNAs.

3.2.4. Illumina HumanHT-12 v4 Expression BeadChip

As in the study on circadian rhythm and alcohol consumption in rats, RNA samples were prepared for microarray analysis at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) in Heidelberg, Germany. A description of the protocol can be found in the corresponding material and method section of the previous project (see page 17). Hybridisation was done on HumanHT-12 v4 Expression BeadChips using the accompanying kit (Illumina, San Diego, CA, USA).

3.2.5. Microarray data pre-processing

The acquired data from the BeadChips were processed in the DKFZ Core Facility's R pipeline. A description of the pipeline and its steps can be found in the material and method section of the previous project (see page 18). All samples scored acceptable on Illumina's BeadChip quality control features showing that the abundant presence of globin mRNA did not cause a problem with the hybridisation. As in the previous project, the microarray data were normalised based on the *robust multi-array averaging* (RMA) procedure which includes a quantile normalisation and a log2 transformation (Irizarry et al., 2003).

3.2.6. Microarray annotation

In contrast to the previous microarray project, we kept the original Illumina annotation provided by the DKFZ Core Facility as the *Re-annotation and Mapping for Oligonucleotide Array Technologies* (ReMOAT) project did not offer a recomputed annotation for the HumanHT-12 v4 Expression BeadChip (Barbosa-Morais et al., 2010). In addition, the standard annotation of the HumanHT-12 v4 Expression BeadChip was much better than the one for the RatRef-12 Bead Array: The Human BeadChip contained 48 107 probes, 883 of which were control probes, 3 270 were labelled as UniGene cluster identities, 11 650 as locus identities, and 1 755 probes were labelled as predicted genes. The remaining 30 549 probes were well annotated.

3.2.7. Statistical analysis

All statistical analyses were performed using LibreOffice 3 and R statistical programming language version 2.15.0.

Principal component analysis

Principal component analysis was performed on the quantile normalised data using the *prcomp*-function of the *stats*-package of R. For graphical presentation, the data points representing the individual blood samples were plotted against the first three principal components using the *cloud*-function of the *lattice*-package. The labels were added and the plot was coloured in *inkscape 0.48*.

Identification of circadian oscillating genes

As in the previous project, the time-series gene expression data of all test-persons were concatenated and then z-score transformed so that every gene had a mean expression of zero and a standard deviation of one (Cheadle et al., 2003).

Genes with circadian oscillating expression levels were then identified using the *fdr-fourier*-function of the *cycle*-package of R (Futschik and Herzel, 2008) based on a period of 24 hours. The function calculated a *Fourier score* which became larger if a gene was closer to a cosine curve of the given period length as well as a false discovery rate (FDR) which was calculated on the basis of 100 permutations within the rows.

A more detailed description of the z-score transformation and the Fourier function can be found in the corresponding section of the previous project (see chapter 2.2.8 on page 20).

Classification of the oscillating genes according to their time of peak expression levels

Pairwise Pearson correlation distances were computed between the expression patterns of the oscillating genes using the *cor.dist*-function of the *bioDist*-package of R. The *hclust*-function of the *stats*-package was then used to hierarchically cluster the genes based on these pairwise distances using *complete linkage* as the method of agglomeration.

For graphical presentation, a heatmap with the results was drawn using the *heatmap.2*-function of the R package *gplots*. Image labels were arranged in *inkscape 0.48* and a dendrogram of the hierarchical clustering was added to the side.

Analysis of enriched pathways and gene ontology terms

The lists with the oscillating genes were screened for over-represented enzymatic pathways and gene ontology terms using the *Database for Annotation, Visualization and Integrated Discovery* (DAVID) version 6.7 (`http://david.abcc.ncifcrf.gov/`) of the National Institute of Allergy and Infectious Diseases (NIAID) in Bethesda (MD, USA) which has been introduced in two publications by Huang da et al. (2009a,b). Gene lists were screened for pathways of the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) and *biological pathway*, *molecular function* and *cellular component* gene ontology terms of level 5 using the

whole Illumina HumanHT-12 array as a background. Results were considered significant if the Bonferroni corrected p-value was below 0.05 or the enriched structure had a false discovery rate below 20 %.

3.3. Results

In order to search for oscillating gene expression pattern in non-diseased individuals, blood was taken from seventeen healthy volunteers (mean age = 40.25 years, standard deviation = 5.69 years) during a period of 24 hours. As the experiment included an overnight stay in a hospital and our focus was on analysing the mRNA concentrations during a normal daily routine, the test persons' sleep quality was monitored by EEG. The age of subjects, as well as the EEG-based categorisation of their sleep quality and the quality of the extracted RNA can be found in table 3.1.

Table 3.1.: Overview of the sleep quality of the study participants.

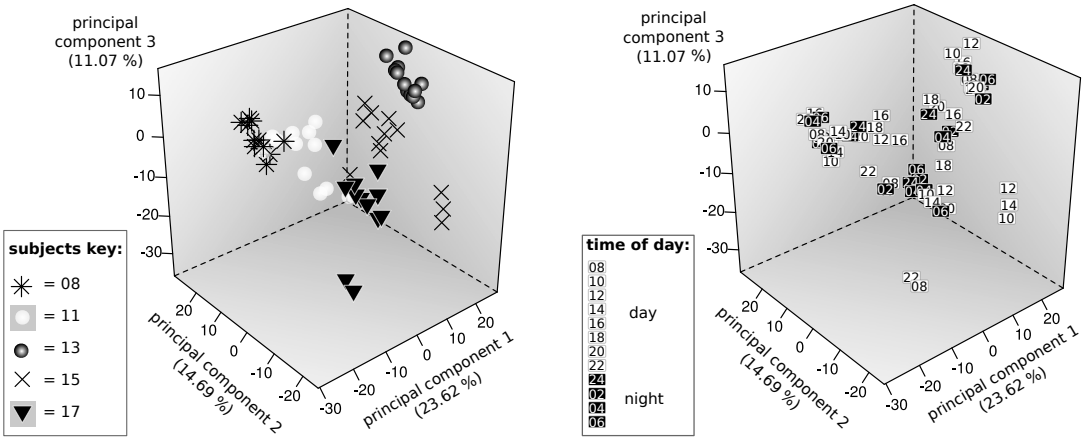
subject number **- selected for microarray**	age in years	sleep profile according to EEG	RNA of all time points in good quality available
1	45	not physiological	(not extracted)
2	48	not physiological	(not extracted)
3	43	not physiological	(not extracted)
4	41	physiological	✗
5	47	not physiological	(not extracted)
6	n.a.*	n.a.*	(not extracted)
7	47	not physiological	✔
8	37	almost physiological but patient woke up to early	✔
9	44	physiological	(not extracted)
10	45	not physiological	✗
11	35	physiological	✔
12	41	almost physiological	✗
13	37	physiological	✔
14	37	not physiological	(not extracted)
15	29	almost physiological	✔
16	33	not physiological	(not extracted)
17	35	almost physiological	✔

*) not available, as participant aborted the study in the evening.

Only the test persons number 8, 11, 13, 15 and 17 showed both acceptable sleep quality and acceptable RNA quality for each time point and were thus used for global gene expression measurement with microarrays.

3.3.1. Principal component analysis

After normalisation of the microarray data we first applied a principal component analysis to see if the identity of the blood donor or the time of blood sampling had a higher overall



(a) Study participant **(b)** Time of day

Figure 3.1.: Scatter plot of the blood RNA samples projected on the first three principal components detected by principal component analysis (PCA).

effect on the similarity of the global gene expression. The first three principal components, which identified the mathematically abstract directions along which the variation in the data set was maximal, were plotted as the axes of a three dimensional coordinate system. The microarray chips, representing each a blood sample, were then positioned in the coordinate system and labelled according to the blood donor (figure 3.1a) or according to the time of blood sampling (figure 3.1b). The graphs show that samples were clearly clustered based on the blood donor but not based on the time point.

3.3.2. Detection of oscillating genes by Fourier transformation

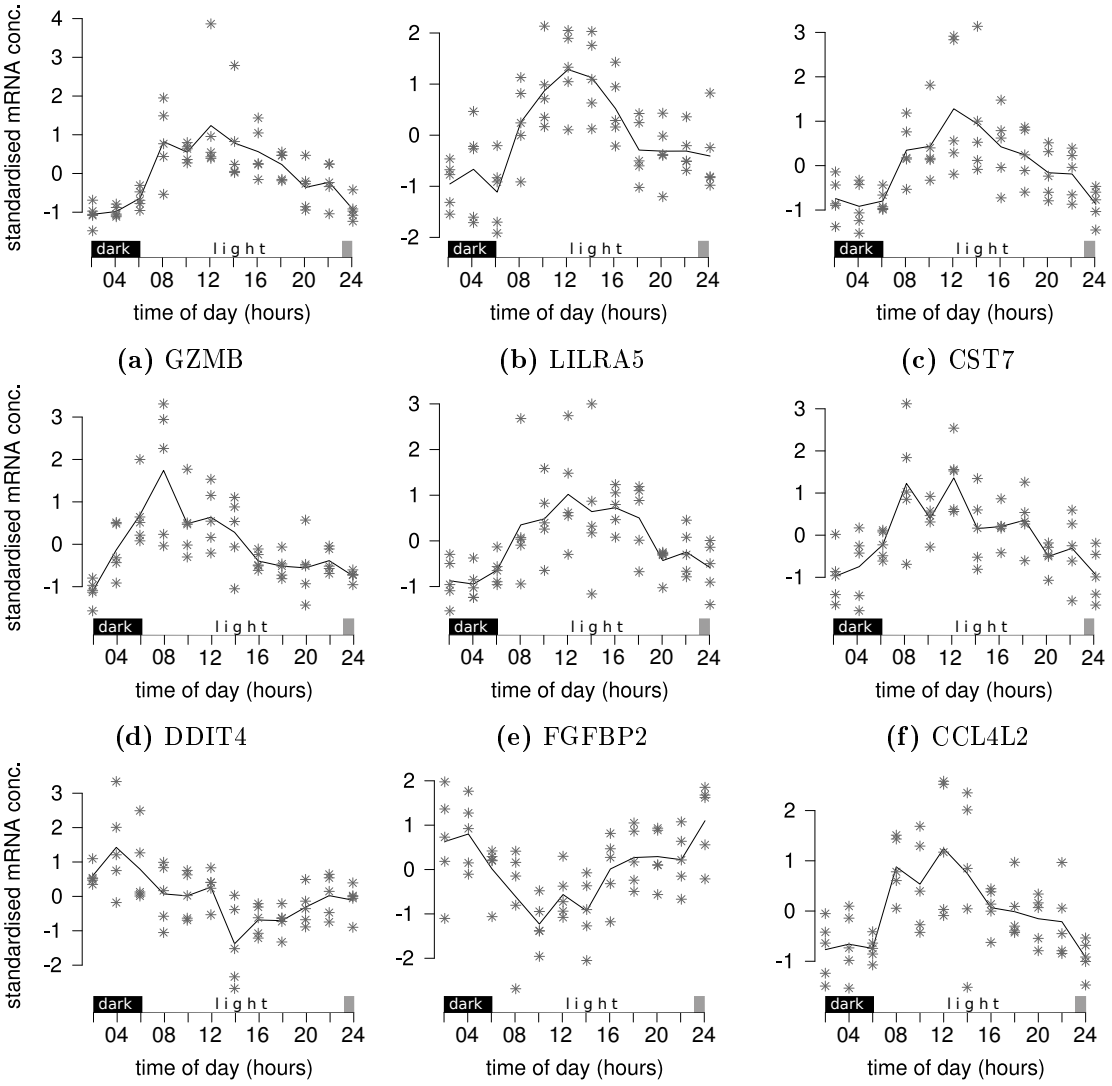
As a next step, we wanted to identify those genes which showed a truly diurnally oscillating mRNA concentration. We used a fast Fourier transform algorithm which calculated abstract *Fourier scores*, with higher scores indicating that a gene expression time-series resembles more closely a cosine curve of a period of 24 hours. This yielded a list of 169 circadian oscillating genes based on a false discovery rate of 10 %. The top 20 genes are displayed in table 3.2. Besides the circadian clock gene *PER2*, which had its peak mRNA concentration at 04:00 hours in the morning, the list contained a number of genes which were representative for leukocytes. The mRNA concentration measurements of the first nine genes were plotted in figure 3.2.

To obtain an impression of the consistency of the gene expression pattern throughout the different subjects, *PER2* gene expression was also represented in another graph with individual time curves for each subject (figure 3.3). While the test persons all showed the same tendency of higher *PER2* gene expression in the hours after midnight, differences existed between individual persons. Thus, subject 13 showed a dip in *PER2* gene expression at 04:00 hours and subject 17 showed a less pronounced circadian oscillation than the other subjects.

Table 3.2.: List of top 20 oscillating genes according to Fourier score.

Gene symbol	Description	Fourier score	FDR*	Peak time
GZMB	granzyme B	30.21	0.0000	12:00
LILRA5(1)	leukocyte immunoglobuline-like receptor A5	28.46	0.0000	14:00
CST7	cystatin F	27.78	0.0067	12:00
DDIT4	DNA damage inducible transcript 4	26.92	0.0050	08:00
FGFBP2	fibroblast growth factor binding protein 2	26.51	0.0040	12:00
CCL4L2	C-C motif chemokine ligand 4-like 2	26.18	0.0067	12:00
PER2	period circadian clock 2	25.68	0.0100	04:00
ZNF512	zinc finger protein 512	25.64	0.0088	24:00
TTC38(1)	tetratricopeptide repeat domain 38	25.39	0.0089	12:00
PRF1	perforin 1	25.35	0.0080	14:00
TTC38(2)	tetratricopeptide repeat domain 38	25.15	0.0082	12:00
FKBP5	FK506 binding protein 5	25.06	0.0075	10:00
NELL2	neural epidermal growth factor-like 2	24.96	0.0092	02:00
NKG7	natural killer cell group 7 sequence	24.90	0.0100	14:00
PINK1	PTEN induced putative kinase 1	24.80	0.0100	10:00
LILRA5(2)	leukocyte immunoglobuline-like receptor A5	24.78	0.0100	16:00
BAG5	BCL2-associated athanogene 5	24.75	0.0106	04:00
TMEM91	transmembrane protein 91	24.72	0.0111	10:00
KIR2DL4	killer cell immunoglobulin-like receptor 2DL4	24.70	0.0105	12:00
CLIC3	chloride intracellular channel 3	24.47	0.0125	12:00

*) FDR = false discovery rate; LILRA5 and TTC38 were targeted by two different probes on the array.



(a) GZMB (b) LILRA5 (c) CST7

(d) DDIT4 (e) FGFBP2 (f) CCL4L2

(g) PER2 (h) ZNF512 (i) TTC38

Figure 3.2.: Daily mRNA concentration curves of selected genes. The grey asterisks indicate the individual values while the black line shows the averaged curve. The black-white bar just above the x-axis indicates the light-dark periods during the blood sampling.

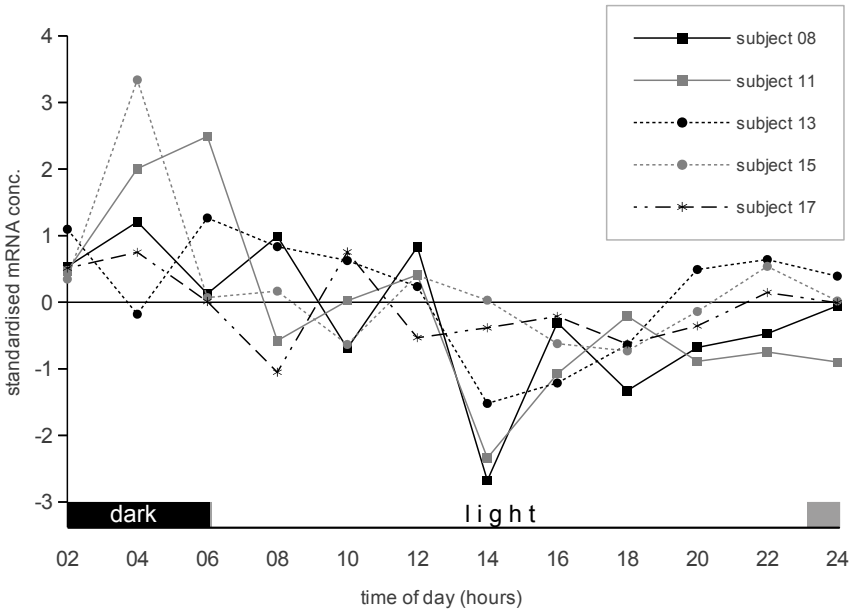


Figure 3.3.: *PER2* gene expression curves of the five test persons.

3.3.3. Classification of the oscillating genes according to their time of peak expression levels

In a next step, we wanted to group the 169 oscillating genes into classes with similar circadian expression patterns. We therefore calculated the pairwise Pearson correlation coefficients between the oscillating genes which had been selected in the previous step and then used hierarchical clustering to split the list of genes into subgroups based on the closeness of their Pearson correlation. The results were displayed in a heatmap (see figure 3.4).

Based on the heatmap image, we visually defined four groups which had each a characteristic circadian oscillating expression pattern. Group 1 contained 68 genes which had their peak expression at noon and their lowest expression levels around midnight. *GZMB*, *LILRA5* and *CST7* were the most intensively oscillating members of this group, based on their Fourier score. Group 2 contained 13 genes whose expression levels peaked in the morning around 8:00 hours and whose lowest expression level was around 22:00 hours. *DDIT4* was part of this group. Group 3 contained 81 genes with a peak expression level around midnight and the lowest expression at around 14:00 hours. *ZNF512* and *NELL2* were the most significantly oscillating members of this group. Group 4 contained five genes whose peak expression was also during the night but later as in the genes of group 3 and whose low expression periods expanded well in the late afternoon. *PER2* was part of this group.

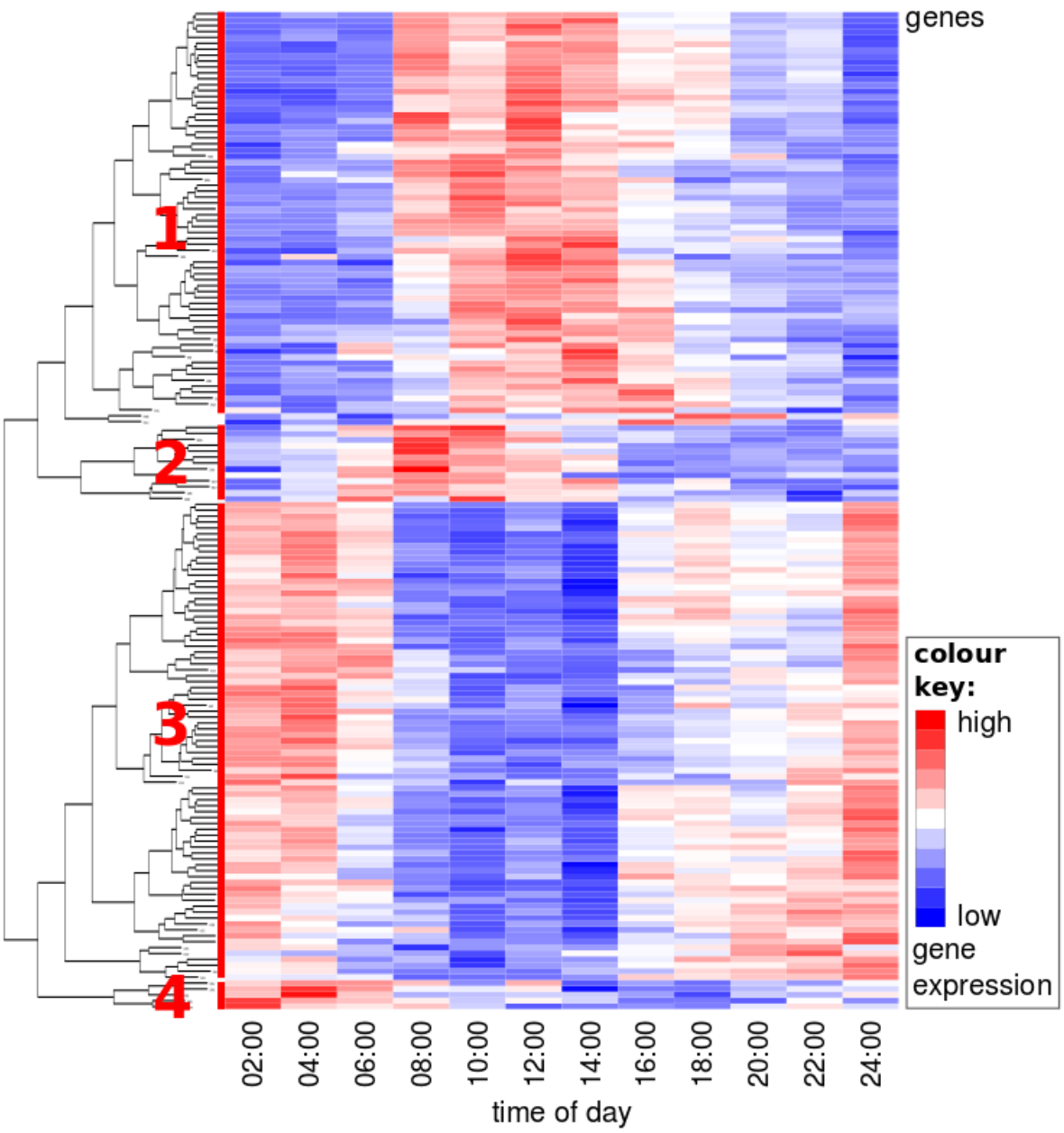


Figure 3.4.: Heatmap of oscillating genes clustered according to their Pearson correlation coefficients. Each row stands for one gene and each column for one time point. Higher gene expression is indicated by a more intensive red colouring while blue signifies lower gene expression. The vertical red bars on the left side indicate the four main - visually identified - subgroups of genes which had their peak expression levels around noon (1), in the morning (2), around midnight (3) and between 2 and 4 o'clock in the morning (4).

3.3.4. Enriched pathways and gene ontology terms

We tried to characterise our lists of oscillating genes more closely by analysing if the genes from our list belonged to specific enzymatic pathways or gene ontology terms. The analysis was based on the web-based resources of the *Database for Annotation, Visualization and Integrated Discovery* (DAVID).

The list of the 169 genes with daily oscillating mRNA concentrations did not show any signs of enriched gene ontology terms or over-representation of genes of specific pathways. When repeating the analysis for the four subgroups, only group 1, containing the genes with a peak expression around noon, showed two over-represented pathways which remained significant after Bonferroni correction but not according to a false discovery rate calculation (see table 3.3). No gene ontology terms were enriched in this group and none of the other groups showed signs of enrichment of either gene ontology terms or enzymatic pathways.

Table 3.3.: Enriched KEGG pathways in the list of genes with a peak expression at noon.

KEGG Pathway	number of genes*	p-value	fold enrichment	Bonferroni	FDR
Graft-versus-host disease	4	0.00045	24.6	0.013	0.38
Natural killer cell mediated cytotoxicity	5	0.00160	9.1	0.046	1.0

*) Number of genes from the candidate list of oscillating genes implicated in the KEGG pathway.

3.4. Discussion of the study

The aim of this study was to identify genes with circadian oscillating expression levels in the blood of healthy volunteers by microarray screening. The expression pattern of these genes can then serve as a baseline for the comparison of transcription rates in alcohol dependent individuals.

Principal component analysis

In order to obtain a first impression of the generated data set, we had applied a principal component analysis to look for the main sources of variance. We found that the blood donor had a much higher impact on the overall gene expression profile than the time of day at which the blood sample was taken. This result corresponded to our expectations, as we had assumed that only a fraction of the transcriptional activity in the blood cells would show circadian rhythmicity. We therefore speculate that the vast majority of transcriptional processes differed only slightly between the time points leading to a higher impact of inter-individual differences. Previous studies have shown that gene expression varies significantly in the same tissue of different organisms of the same species and that the cellular composition of leukocytes fluctuates in healthy individuals depending on their mental and physical state on the day of measurement (Kaplow, 1975; Fei et al., 1993; van Rossum et al., 2008).

It also lies in the nature of oscillating processes that the same states - gene expression levels in our case - are periodically recurring. The overall impact of this phenomenon on the variation in the data set will therefore be smaller than the time-consistent differences in gene expression between individuals.

Role of core circadian genes

In line with the aim of this study we identified a number of circadian oscillating genes in the blood of the healthy test persons. It is noteworthy that the core clock genes, like cryptochrome 1 and 2, *ARNTL*, *CLOCK*, *RORα* and *REV-ERBA α* were not present in the list of circadian oscillating genes leaving *PER2* as the only oscillating member of the group of period genes. Blood cells are known to possess a self-sustaining circadian rhythm whose generation does most likely also involve the core clock genes that build the molecular basis for the rhythm creation in the suprachiasmatic nucleus. The detection of a circadian expression pattern of *PER2* fits into this hypothesis and has been previously reported by others (Teboul et al., 2005; Watanabe et al., 2012). Unlike in our study, these authors also reported circadian modulation of other core clock genes such as *BMAL1*. A possible reason for this diverging results is the fact that we used whole blood mRNA for our analysis while Teboul et al. (2005) used peripheral blood mononuclear cells (lymphocytes, monocytes). Existing circadian expression rhythms might therefore have been blurred in our approach as different types of immune cells could have different daily expression patterns of the core clock genes. Teboul et al. (2005) also reported that *PER2* and *BMAL1* gene expression varied considerably between the test persons. Watanabe et al. (2012) used the same blood

extraction system as we did but also reported that circadian rhythmicity of the core clock genes differed markedly between individuals. Unlike in our experiment their subjects were forced to stay awake during the whole 24 hour measurement period.

Role of immune system genes

The screening for enriched pathways and gene ontology terms had only yielded two immune system-related pathways which were detected in the group of genes with a peak expression level at noon. Although the screening algorithms for common biological functions did not find further pathways or ontology terms, our top findings included a number of genes related to the function of the immune system such as *GZMB*, *PRF1* and *LILRA5*. The large portion of immune genes on the list of circadian oscillating genes is plausible as gene expression measurements in the whole blood will be dominated by transcription in leukocytes as neither red blood cells nor platelets have a nucleus and therefore no gene expression is taking place in these cells. It is also known that the number of circulating haematopoietic stem cells, which are the progenitors of all other blood cells, oscillates with a peak during the inactive phase in mammals (Scheiermann et al., 2013).

Granzyme B (*GZMB*) is a serine protease which is principally expressed in natural killer cells and cytotoxic T lymphocytes and which is involved in the lymphocyte-mediated initiation of apoptosis in virus-infected cells (Bots and Medema, 2006). *Perforin* (*PRF1*) is also a protein product of cytotoxic T lymphocytes and natural killer cells and plays a crucial role in the cytolysis of infected cells (Kawasaki et al., 1992; Trapani, 1995). A study performed in rats had previously reported that both granzyme B and perforin showed circadian oscillating mRNA levels in lymphocytes (Arjona and Sarkar, 2005). In another study, the same authors also reported that these circadian expression patterns were disturbed after chronic ethanol consumption (Arjona et al., 2004) suggesting granzyme B and perforing as two top candidate genes to serve as biomarkers for post-dependent circadian rhythm perturbations.

Another gene which was found to have circadian oscillating expression levels was the *leukocyte immunoglobulin-like receptor A5* (*LILRA5*), which codes for a receptor that is located on the surface of monocytes and that is implicated in the secretion of proinflammatory cytokines as part of the unspecific innate immune response. Its gene expression maximum was shortly after noon and could lead to a peak alertness of the innate immune system shortly afterwards when the receptor is fitted into the outer cell membrane. Gibbs et al. (2012) reported that cytokine secretion is much more pronounced at the beginning of the active phase in mice (i.e. at night) than at the start of the resting phase. The fact that the authors only used two measuring time points makes it difficult to deduce a circadian pattern but their finding is consistent with our report that innate immune response is more alert during the active phase of a mammal.

The list of immune system related genes with strongly oscillating expression levels also included *cystatin F* (*CST7*), a protease inhibitor which is expressed primarily in leukocytes and whose role in immune function remains yet to be elucidated (Hamilton et al., 2008; Colbert et al., 2009). The *fibroblast growth factor binding protein 2* (*FGFBP2*), which is selectively secreted in cytotoxic T lymphocytes, was also part of this list, giving further

support to our hypothesis that a large variety of immune system genes show daily oscillating mRNA levels.

Further circadian oscillating genes

Nonetheless, our list of oscillating genes also contained candidates which were neither associated with the transcriptional-translational network of the endogenous clock nor directly associated with the immune system. An example is the transcription factor *FOSB*. The gene, a transcription factor of the Fos family, was previously associated with the processing of photic stimuli in the suprachiasmatic nucleus during the night phase in rats (Schwartz et al., 2000). It is implicated in a great variety of cellular processes which makes it difficult to pin down its function to a single process. We therefore assume that the expression peak of *FOSB* at noon indicated a general climax of leukocyte activity at this time of day.

Another gene, whose expression peaked shortly after noon, was the *RAS oncogene family member 24* (*RAB24*), which codes for a small G protein. Its expression has been reported in macrophages where it plays a role in vesicular transport (Jacobsen et al., 2005). As in the case of *FOSB*, the peak expression of this protein could indicate a more general peak of immune system activity at that time of day.

Use of the combined expression pattern of several genes

It appears unlikely that the daily expression pattern of a single gene will be sufficient to predict the degree of circadian disturbance in recovering alcoholics as we observed high inter-individual differences in the oscillating genes. An assessment of circadian perturbation based on blood gene expression will therefore need to rely on the comparison of a more complex pattern based on the expression profiles of a combination of several genes. A better picture of which genes should be included in this approach will be available once the daily gene expression data have been collected from a group of alcohol addicted patients in the follow-up study. Given the profound impact of chronic alcohol consumption on the immune system (Cook, 1998; Molina et al., 2010) it is likely that immune system-related genes will dominate the list of genes which will be used for the expression pattern comparison.

Suggestions for future studies

A possible modification in the protocol of the follow-up study, which will sample the blood of recovering alcoholics, is to extract the mRNA of a limited spectrum of blood cell types such as peripheral blood mononuclear cells (PBMC). In the present study we used the PAXgene Blood RNA Kit which extracts the mRNA of all blood cells leading to a high amount of globin mRNA which is still present in matured red blood cells although these cells have no longer any transcriptional activity as they lack a nucleus. Focusing on peripheral blood mononuclear cells would also allow to eliminate bias caused by varying numbers of polymorphonuclear leukocytes (i.e. granulocytes) such as basophils, eosinophils, and neutrophils.

The participants of our study had also been free to choose among a variety of available dishes from the hospital kitchen for lunch and dinner during the blood taking phase. As it cannot be ruled out that blood gene expression was influenced by this dietary variety, future studies might consider offering only one standardised dish for all participants.

The test persons had been recruited on the preconditions that they have no sleep problems and are not shift workers. Independent of this, the participants had their individual chronotypes ranging somewhere from early morning-active (lark-type) to evening-active (owl-type). It is perceivable that this individual preferences could be correlated with specific circadian gene expression pattern in the blood. Future studies might therefore consider assessing the chronotypes of the participants using questionnaires already available for this purpose (Zavada et al., 2005; Dosseville et al., 2013).

Study limitation

The approach to use blood mRNA as a biomarker for circadian disruption in post-treatment alcoholics has a crucial limitation which has to be taken into account when interpreting the results. Human beings do not only differ from each other by morphological features but also have significant differences in cellular features and gene expression characteristics. As an alcohol addicted patient is analysed, there is no information how the specific circadian gene expression pattern in his blood looked like when he was still healthy. It will therefore be a demanding challenge to tell the inter-individual differences from the alterations due to the disease status. The use of a combination of gene expression profiles will hopefully mitigate this fact.

Outlook

The design of this study, requiring blood sampling at several time points throughout a 24 hour period, is too elaborate for a routine diagnostic tool. Nonetheless, a follow-up study in recovering alcoholics will be interesting from a scientific point of view, as it will show which circadian oscillating genes in blood will be most affected by alcohol addiction. This could also increase our understanding how alcoholism interacts with the immune system. If the follow-up study reveals that a limited number of genes shows striking differences at two or three time points during the day, a diagnostic application will become more likely. Taking blood samples in the morning and the evening from an inpatient and analysing the gene expression of a small number of genes by quantitative PCR could be feasible in many psychiatric hospital settings.

4. Gene expression profiling in high and low impulsive rats

4.1. Introduction to the study

4.1.1. Introduction to the concept of *impulsivity*

Human beings differ from each other not only in outward appearance but also in their psychological attributes. Attempts to understand and classify such personality differences have a long history dating back even before the times of Hippocrates and his humoral theory. An important step was made by Allport and Allport (1921) with the definition of personality traits as defined, characteristic sub-features of the human personality which can be assessed by questionnaires. While the Allport brothers proposed a vast number of possible traits, the use of factor analysis allowed to reduce the number of personality traits which do not correlate with each other. Today, the *Five Factor Model* which defines *openness*, *conscientiousness*, *extraversion*, *agreeableness*, and *neuroticism* as the five core factors is widely accepted (McCrae and Costa, 1987). Nonetheless, further traits have been suggested, such as *impulsivity* which shall be the subject of this study.

Impulsivity can be defined in simple words as '*a tendency to act prematurely without foresight*' (Dalley et al., 2011). A more classical definition describes impulsivity as a personality trait characterised by a tendency for '*actions which are poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and that often result in undesirable consequences*' (Durana and Barnes, 1996). While impulsivity is considered a personality trait, it can be seen as a multifactorial psychological construct: multifactorial because it is an umbrella term which encompasses a variety of closely related but independent psychological factors such as a failure to adequately process and interpret sensory information before acting, a lack of motor inhibition, and a lack of patience (Evenden, 1999). And impulsivity can be considered a psychological construct as it is an explanatory variable which is not directly observable. Nonetheless, a person's degree of impulsivity is a behavioural pattern which is relatively stable over time, thus justifying the classification as a personality trait.

4.1.2. Measurements of impulsivity

In order to scientifically analyse impulsivity it is important to find ways to measure this personality trait. This can be done by using either self-report questionnaires or neuropsychological tests.

A widely used self-report questionnaire is the ***Barratt Impulsiveness Scale*** (BIS)

(Patton et al., 1995). Its current version, the BIS-11, consists of 30 questions with four possible answers: (1) *rarely/never*, (2) *occasionally*, (3) *often*, and (4) *almost always/always*. Factor analysis allowed to derive six first-order factors from the questions and these can be even further summarised to three second-order factors (see table 4.1). These factors can be considered as subdivisions of the trait of impulsivity.

Table 4.1.: Factors of the Barratt Impulsiveness Scale (BIS-11).

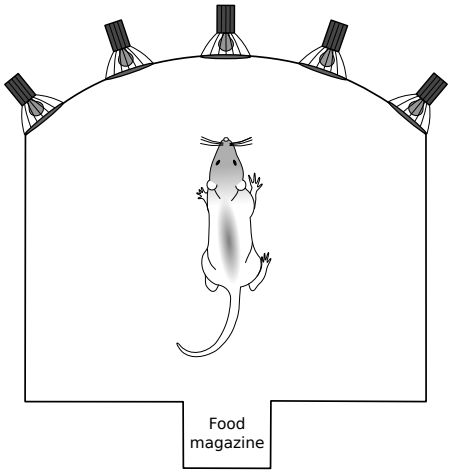
Number of Questions	First-order factors	Second-order factors
5	attention	attentional
3	cognitive instability	
7	motor	motor
4	perseverance	
6	self-control	non-planning
5	cognitive complexity	

source: Patton et al. (1995)

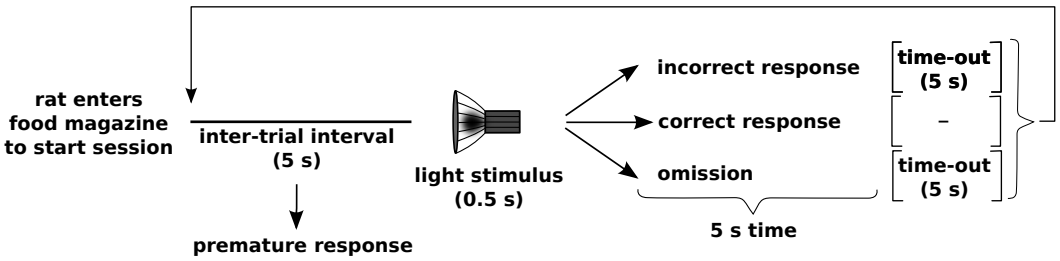
A measure of impulsive behaviour is provided by the ***Delay discounting task*** which demands subjects to choose between an immediate small reward or a delayed larger reward. Impulsive individuals will go for the small award. This test can be adapted for animal models (Ainslie, 1974; Evenden and Ryan, 1996). Another version of this test is based on *probability discounting*. This time subjects have to choose between small but likely or larger but less likely rewards.

There are also neurophysiological tests like the ***Go/No-Go test***. In this paradigm subjects are asked to perform as fast as possible a certain action such as pressing a button upon appearance of a specific visual stimulus such as a circle on a screen. But from time to time the original stimulus will be replaced by a different input, for example a rectangle instead of the circle. In this case the test subject should not respond (Rubia et al., 2001). A variation of this paradigm is the ***Stop Signal Reaction Time task***. Again subjects are requested to react as fast as possible to a stimulus but in this test from time to time a second, different stimulus will appear shortly after the first one. In this case participants are asked to not respond to the first signal. So participants are for example asked to press a button if a light appears on a screen in front of them. But sometimes the light appearance is followed after a short delay by a sound and in this case the subjects are required to do nothing (Nichols and Waschbusch, 2004). Both of these tests measure the attentional and the motor-control aspect of impulsivity. They can be applied to humans as well as other animals (Eagle et al., 2008).

The present work is based on another neurophysiological impulsivity test which was developed specifically for rodents: the ***5 Choice serial reaction time task*** (5CSRTT) (Robbins, 2002). In this test, a rat or mouse is placed in a box with a set of five lights on one side and a food magazine on the opposite side (figure 4.1a). The session is initiated when the animal accesses the food magazine. Five seconds later one of the five lights will light up for 0.5 s after which the animal has five seconds to poke against the corresponding light with its nose. A correct response will be rewarded with a food pellet. A premature



(a) 5CSRTT apparatus



(b) 5CSRTT paradigm

Figure 4.1.: The 5-choice serial reaction time task (5CSRTT). (a) Sketch of apparatus adapted from Bari et al. (2008). (b) Test procedure adapted from Robbins (2002).

response during the five second inter-trial interval before any light has been lit, a wrong choice (incorrect response) or a failure to respond (omission) will lead to a time-out of five second during which the main box light will go out. After this, the trial resumes with a new inter-trial interval of five seconds until the session is over (figure 4.1b). A detailed description of the test can be found in Bari et al. (2008). The test allows to collect a multitude of data such as the number of correct and incorrect responses or the number of omissions. For our study we were interested in the number of premature responses which is considered to be a measurement of impulsivity (Evenden, 1999).

4.1.3. Brain regions implicated in impulsivity

Anatomical information about human brain structures implicated in impulsive behaviour derive almost exclusively from *functional magnetic resonance imaging* (fMRI) studies. A study by Costa Dias et al. (2012) linked the brain's reward system and especially the *nucleus accumbens* to impulsivity as measured by a delay discounting task. This is no surprise as the behavioural test is based on making a decision between a small but immediately available or a bigger but later-arriving reward. It is therefore likely that high impulsive individuals show differences in their reward processing. But impulsivity is also correlated to

the prefrontal cortex which plays a dominant role in executive control and decision making. Castellanos and Tannock (2002) showed how a disturbance of these prefrontal processes can lead to the maladaptive impulsive behaviour which can be observed in attention deficit hyperactivity disorder (ADHD).

Animal models offer the possibility to use brain lesions in order to identify those regions which play a vital role in impulsivity. Comparable to the human fMRI studies, rodent lesion studies indicate an important role for the nucleus accumbens. The core region of this brain structure seems to play an even greater role in rats. But there is also evidence for a link between prefrontal cortex structures such as the infralimbic cortex and impulsivity (Dalley et al., 2011). Winstanley et al. (2006) summarised the roles played by different rat brain regions in regard to impulsive behaviour (see adopted table 4.2).

Table 4.2.: Summary of the influence of different rat brain regions on impulsivity. Table adapted from (Winstanley et al., 2006).

Brain region	Stop-signal reaction time task	Delay discounting Task	5 Choice serial reaction time task
Prelimbic cortex	✗	✗	✗
Infralimbic cortex	?	✗	✔
Anterior cingulate cortex	?	✗	✔
Orbitofrontal cortex	?	✔	✔
Nucleus accumbens	✗	✔	✔
Subthalamic nucleus	?	✔	✔

✔ = influence, ✗ = no effect, ? = unknown

4.1.4. Impulsivity and genetics

As in every trait which plays a role in psychiatric disorders, the question has been brought forward in how far a person's degree of impulsivity is the result of a genetic disposition and how far it is a result of the environment. Most information in this regard stems from studies which were analysing a potential genetic influence on attention deficit hyperactivity disorder (Hinshaw, 2003; Heiser et al., 2006). A meta-analysis of twin and adoption studies conducted by Bezdjian et al. (2011) found that approximately half of the variance in peoples level of impulsivity is due to genetic disposition. The genetic link seemed to be more important in males, which are also reported to score higher on several subsets of impulsivity than females (Cross et al., 2011). Candidate gene studies have led to inconsistent results but suggested an involvement of the dopaminergic and serotonergic system (Varga et al., 2012). In line with this hypothesis, Varga et al. (2012) reported an association between polymorphisms in the *D_4 dopamine receptor* (*DRD4*) and the *5-hyrodxytryptamine receptor 1B* (*HTR1B*) and impulsivity. A similar result was obtained in a study on *DRD4* and *tyrosine hydroxylase* (*TH*) polymorphisms in dogs (Wan et al., 2013). In the frame of a large scale genome-wide association study, Heinrich et al. (2013) reported that an intronic variation in the *AMBRA1* gene, coding for the *autophagy/beclin-1 regulator 1*, was linked to the degree of impulsivity in the test subjects. The level of *D_2 dopamine receptors*

in the striatum has also been linked to impulsivity (Trifilieff and Martinez, 2013).

4.1.5. Impulsivity and psychiatric disorders

A high level of impulsivity obviously has a negative overall affect on people's lives as it incites them to make decisions which they will regret later on. But being impulsive itself is not a pathological condition. In fact, Dickman and Meyer (1988) reportet that it can be of an advantage in situations which demand rapid information processing. Nonetheless, high impulsivity plays a role in several psychiatric disorders.

Attention deficit hyperactivity disorder (**ADHD**) is characterized by severe deficits in attention and hyperactivity. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV-TR) distinguishes three subtypes of ADHD (American Psychiatric Association, 2000):

1. predominantly inattentive type
2. predominantly hyperactive-impulsive type
3. combined type

Type II and III are closely linked to excessive impulsivity and symptoms of this trait are part of their diagnostic criteria as outlined by the DSM-IV-TR: '1. Often blurts out answers before questions have been finished.', '2. Often has trouble waiting one's turn' and '3. Often interrupts or intrudes on others (e.g., butts into conversations or games)' (American Psychiatric Association, 2000).

Impulsivity has also been brought in connection with **bipolar disorder** where it is especially important during manic phases (Moeller et al., 2001; Najt et al., 2007). The often reported excessive and impulse-driven purchases during manic episodes are a good example of this. Impulsivity is also present in the DSM-IV-TR definition of another psychiatric condition, the **borderline disorder**: 'Impulsivity in at least two areas that are potentially self-damaging (e.g., spending, sex, substance abuse, reckless driving, binge eating)' (American Psychiatric Association, 2000).

Of particular interest is the link between impulsivity and **drug addiction**, the latter being the overall theme of the present thesis. A role of impulsivity in the initiation of drug consumption is easily conceivable as it leads individuals to choose the immediate rewarding effects of a drug despite well-known long term negative consequences. It is a well established fact that high impulsive people have a higher risk of becoming drug addicted (Ersche et al., 2010; Dalley et al., 2011). This relationship becomes more complicated as the addiction progresses as it has been shown that drug consumption itself makes people more impulsive (Perry and Carroll, 2008). In addition, higher levels of impulsivity have been shown to correlate with a higher relapse rate in treated drug addicts (Doran et al., 2004; Bankston et al., 2009).

4.1.6. Research goals

The aim of the present study was to find a link between the degree of impulsivity in a rodent model of this trait and underlying gene expression differences in relevant brain

areas of the animals. We therefore analysed global gene expression in the brains of rats bred for extremes in high and low impulsive behaviour. The measurements were done using microarrays and quantitative PCR. The analysed brain regions were the infralimbic cortex and the nucleus accumbens core and shell. All three regions had been previously connected to impulsivity (Dalley et al., 2011). We speculated to find expression differences of genes involved in dopaminergic and serotonergic signalling as well as genes which are part of other neurotransmitter systems and subsequent intra-cellular signalling cascades.

4.2. Materials and Methods

4.2.1. Behavioural testing

The behavioural part of this experiment was performed by our collaborators from the *Cambridge Neuroscience* network in Cambridge, United Kingdom. Starting with an outbred strain of Lister hooded rats, the group of Jeff Dalley had set up a breeding scheme to generate animals showing extremes of either high or low impulsive behaviour.

Rats were selected according to their number of premature responses in the 5-choice serial reaction time task (5CSRTT). The animals received 60 to 70 training sessions before the actual three weeks behavioural assessment followed. During this testing phase the rats were tested Monday through Friday with always one 30 min session during the animals active dark phase. On Wednesday, the sessions lasted 60 minutes and the number of premature responses was counted. Rats having less than 30 premature responses were considered to be low impulsive while rats with more than 50 such responses were considered high impulsive.

After behavioural characterisation, rats were sacrificed in the middle of their active night phase and the brains were extracted. We received the brains from the sixth generation of the bidirectional breeding program for whole gene expression profiling of impulsivity-related brain areas.

4.2.2. Brain dissection

Frozen rat brains were sectioned into 120 µm thick coronal slices at an ambient temperature of $-20\,^{\circ}\mathrm{C}$ in a Leica CM 3000 Cryostat (Leica, Wetzlar, Germany). The infralimbic cortex and the nucleus accumbens core and shell region were identified based on images from a Rat Brain Atlas (Paxinos and Watson, 1998) and extracted with 0.75 to 1.5 mm diameter tissue punches (Stoelting, Wood Dale, IL, USA).

4.2.3. RNA isolation and purification

RNA was isolated by phenol-chloroform extraction (Chomczynski and Sacchi, 1987). 1 ml of TRIzol® Reagent (Life Technologies, Darmstadt, Germany), a monophasic solution of phenol and the chaotropic agent guanidinium thiocyanate, was added to the punched tissue samples and the suspensions were homogenised by multiple passages through a 22 gauge needle. Samples were filled up with 200 µl of chloroform, mixed and centrifuged to obtain a separation of the aqueous upper and the organic lower phase. The RNA containing upper phases were carefully collected and purified with an RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the RNAs were analysed with a Nanodrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany) and the integrity of the RNAs was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with a ratio of absorption at 260 nm versus 280 nm in the range of 1.8 to 2.2 as well as an *RNA integrity number* (RIN) above eight were considered for microarray analysis.

4.2.4. Affymetrix Gene Chips

RNA samples from six high and six low impulsive animals were prepared for microarray analysis using the GeneChip® 3´IVT Express Kit (Affymetrix, High Wycombe, UK) following the manufacturer's protocol. In brief, 100 ng RNA were reverse transcribed to first-strand complementary DNA (cDNA) using oligo-thymidine primers connected to the promoter region of the T7 bacteriophage. After second-strand cDNA synthesis, an in-vitro transcription was performed for 16 hours using T7 RNA polymerase with part of the uridine triphosphate (UTP) labelled with biotin. The thus produced biotinylated complementary RNA (called aRNA in the Affymetrix protocol) was purified and quantified with a Nanodrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany). 15 µg of each sample were subsequently fragmented to increase assay sensitivity. The fragmented aRNA was handed over to the Core Laboratory for Microarray-Analysis of the University Medical Centre Mannheim for the hybridisation and scanning process. 12.5 µg of fragmented aRNA were hybridised to GeneChip Rat Genome 230 2.0 Arrays (Affymetrix, High Wycombe, UK) and incubated for 16 hours using Affymetrix's GeneChip Hybridization, Wash, and Stain Kit and the corresponding protocol. After washing, the GeneChips were scanned in an Affymetrix GeneChip Scanner 3000 run with Affymetrix GeneChip Command Console Software and red out as cell intensity (CEL) files.

4.2.5. Microarray data pre-processing

Quality control

Affymetrix scan software produced low-resolution JPEG images of the scanned arrays. These images were visually controlled for stains. While several arrays had little hybridisation-free spots, none of the arrays had more than one tenth of its surface blurred which is a common limit above which assays should no longer be analysed.

The obtained cell intensity (CEL) files were quality assessed using a web-based Affymetrix quality control pipeline (www.arrayanalysis.org) from the Department of Bioinformatics of Maastricht University (Maastricht, Netherlands). The pipeline's R scripts were downloaded and run in R 2.14.2 software environment. The pipeline consisted of the following items and their complementary probes:

- Detection of spiked-in sample preparation probes: Lysine (Lys), Phenylalanine (Phe), Threonine (Thr), and Diaminopimelic acid (Dap) should be detected in the following intensity order: Lys < Phe < Thr < Dap.
- Detectability of the lowest concentrated spiked-in sample preparation probe lysine.
- Detection of the 3´ and 5´ end of beta-actin: The ratio 3´/ 5´ should not exceed three.
- Detection of the 3´and 5´end of GAPDH: The ratio 3´/ 5´ should not exceed 1.25.
- Detection of spiked-in hybridisation probes: The enzymes BioB, BioC, and BioD from the biotin synthesis pathway of *Escherichia coli* as well as the creX gene from bacteriophage P1 should be detected in the following intensity order: BioB < BioC < BioD < creX.

- Detectability of the lowest concentrated spiked-in hybridisation probe BioB.
- Less than 10 % variation in the percentage of probes called present between the different arrays.
- Less than 20 % variation in the background spread between the arrays. Background spread is calculated on the basis of the mismatch probes between the arrays.
- Limited spread of the log-transformed scaling factors: The different arrays are scaled with constants so that all arrays have the same mean intensity. The decadic logarithms of the scaling factors should never exceed three.

All samples passed the quality control features.

Annotation

Following the reasoning of Dai et al. (2005) and Sandberg and Larsson (2007) that Affymetrix's original annotation lacks accuracy and has no stringent probe set to gene relationship, we used updated probe set definitions from the Brainarray project (`http://brainarray.mbni.med.umich.edu`), which is a web-based re-annotation resource maintained by the University of Michigan. We summarised the probe intensities according to Brainarray's custom-made *chip definition file version 14.1.0* (CDF) which was based on Entrez Gene genomic locations.

Normalisation and averaging of bead level values

Data were subsequently quantile normalised using the *robust multiarray average*-function of the *affy* package from the Bioconductor project (Gautier et al., 2004). Genes with expression values below 80 were excluded to avoid confounding array background noise and data were subsequently log2 transformed for the statistical analysis.

As a result, we obtained a final number of 7 961 genes for the infralimbic cortex, 7 928 genes for the nucleus accumbens core and 7 926 genes for the nucleus accumbens shell.

4.2.6. Statistical analysis and data mining of microarrays

All statistical analysis were performed using LibreOffice 3 and R statistical programming language version 2.14.2.

Principal component analysis

As in the circadian microarray studies of the previous chapters, principal component analysis was performed using the *prcomp*-function of the *stats* package of R. The calculation was based on the data of the four high and the four low impulsive rats with the most pronounced phenotypes. Data points representing the microarray data sets obtained from the individual animals were plotted against the first three principal components. An image legend was added and the plot was coloured in *inkscape 0.48*.

T-tests and fold change calculations

Candidate genes were selected on the basis of uncorrected *Student's t-tests* using the Welch adaptation as we assumed unequal variances in the different groups. We compared the expression data of the four high and four low impulsive rats with the most pronounced phenotypes. The fold change, corresponding to the ratio of average gene expression in high-impulsivity versus average gene expression in low impulsivity rats, was calculated for each gene according to the following formula:

$$\text{fold change} = \frac{\frac{1}{n}\sum_{i=1}^{n} x_{high\ impulsivity,\ i}}{\frac{1}{n}\sum_{i=1}^{n} x_{low\ impulsivity,\ i}}$$

The variables $x_{high\ impulsivity}$ and $x_{low\ impulsivity}$ corresponded to the non-logarithmic expression values for the examined gene and n corresponded to the number of replicates which was four in both group as we had limited this analysis to the animals with the most pronounced high and low impulsive phenotypes.

Volcano plots were created in R based on the calculated p-values and fold changes with the x-axis representing the $log_2(fold\ change)$ values and the y-axis the $log_{10}(p\ values)$.

Candidate genes for true differential expression between the two impulsivity groups were selected on the basis of an uncorrected t-test p-value below 0.05 and an absolute fold change above 1.2.

Analysis of enriched pathways and gene ontology terms

As in the previous subproject, the lists with candidate genes were screened for over-represented enzymatic pathways and gene ontology terms using the *Database for Annotation, Visualization and Integrated Discovery* (DAVID) version 6.7 (`http://david.abcc.ncifcrf.gov/`) (Huang da et al., 2009a,b). The lists were screened for pathways of the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) and *biological pathway*, *molecular function* and *cellular component* gene ontology terms of level 5. As a background we used all genes of our array which had passed the microarray quality control and were above the background cut-off.

Correlation analysis

Gene expression data were also correlated with the number of premature responses in the 5CSRTT by *Spearman's rank correlation coefficient*. The values were calculated by using the *cor.test*-function of the *stats* package of R. In contrast to the above mentioned t-test, we used the data of all six high and six low impulsive animals for the correlation analysis.

4.2.7. Quantitative PCR validation

To confirm the core microarray findings, we assessed gene expression levels of selected candidate genes with quantitative PCR. 100 ng total RNA were reverse transcribed using the High Capacity RNA-to-cDNA Master Mix (Life Technologies, Darmstadt, Germany)

according to the manufacturer's protocol. The resulting cDNA was diluted 1:10. Candidate genes were quantified on an Applied Biosystems 7900 HT Real-time PCR system (Life Technologies) using the Power SYBR® Green PCR Master Mix (Life Technologies). Reactions were performed in a total volume of 10 µl with 3 µl diluted cDNA and a final concentration of 400 nmol/l of both the forward and the reverse primer. *Actb*, *Rpl6* and *Ubb* were used as housekeeping genes and all samples were run in triplicates with the following temperature profile: 10 min at 95 °C followed by 40 cycles of 15 sec dissociation at 95 °C and one minute annealing and elongation at 60 °C. Melting curve analysis was performed after each run to check for unwanted PCR side products. The primer sequences of the candidate genes are presented in table 4.3 and the primer sequences of the housekeeping genes can be found in table 4.4.

Table 4.3.: Primer sequences of the candidate genes

Gene	Primer forward	Primer reverse
Adpgk	CCAGCCTGACCTGGTGGTCCTT	CCCAAGAGAAGCCACTGCGGG
Akap5	GTGGTTTGGGTCCGTGCTCCT	AGCTGTTCACGGCGTTGTCTC
Cacng3	GGTGGGCACGGACTACTGGC	CTGCGGTGGAACTCGCTGGC
Dusp19	CTCAACGTCGCGTATGGAGT	CCCTGCGTTACAGTGGACAA
Frzb	TGACGTGACCGCCATTGTGGA	AGGAGGGAGTCCGTGGGTGT
Gna11	TGCCCACAACTGGCATCATC	TTCTCGTTGTCCGACTCCAC
Gprc5b	CACGCCATTCCAGAGATCCA	TAGTCCCACCATTGAGCACG
Gucy1a3	TGTCCCTGATGCCTCCCTGCT	ATCTGTCCACACACGGCGACC
Jdp2	TGCAGCCAGATGCCGGAACA	CGATACAGGTGGGCCGGTGG
Map4k5	AGCACAGGCTCCACAGTTAC	GCAGAATGCGGTCAGGAAAC
Olfml1	CCACTACAACATGCCCAGGA	TGCCAGCATCCTTCATCCAA
P2ry12	AGGGCTTTGGCAACGAAACCA	GTTGGCACCAGGCACCTCCA
Pde1a	ACATGTGTGGATTGCTCCCGTG	ACAGCGTGGACAATGCTGCGA
Rasd1	CCCAGCAGAGGGTTAGTTGG	AATGCCCTGTTAGTCCTGGC
Rasgrp3	CCTCGGGAAAGCGGCGACAC	CCCGGAACTCCTCAGTCATGCG
Rasl10a	CGACATCTGCAGCCCGGACA	GATGGGTGCCTCTGGCGCTC
Rnd3	GTGGGCGACAGCCAGTGTGG	TGGCTGCTCCGATCTGCTTCG
Sept4	CAGAGTGCTGGAGGCCCGTG	ATCCAATGGCCGGAGCCCGT
Taok1	CGAGCGCCAGGCCAGAGAAA	TGCCCTGTTCCGTCCGTCCC

Table 4.4.: Primer sequences of the housekeeping genes

Gene	Primer forward	Primer reverse
Actb	GCGTCCACCCGCGAGTACAAC	CGACGACGAGCGCAGCGATA
Rpl6	GAAGAAGCCACTTCGCAAGCCC	TGAGACCGCAGGTAGCCCTGG
Ubb	CCCTGGAGGTGGAGCCCAGT	CACCCCTCAGGCGGAGGACCA

Applied Biosystem's *Sequence Detection System (SDS) Software Version 2.2.2.* was used to analyse the SYBR green fluorescence levels that had been measured during the PCR run. Cycle of threshold (Ct) values were calculated for each well as the theoretical number

of cycles when a defined fluorescence threshold was reached. The triplicate measurements were averaged by arithmetic mean. Reactions were then normalised to the endogenous controls by calculation of ΔCt-values using the following formula:

$$\Delta Ct = Ct_{candidate\ gene} - \sqrt[3]{Ct_{Actb} \cdot Ct_{Rpl6} \cdot Ct_{Ubb}}$$

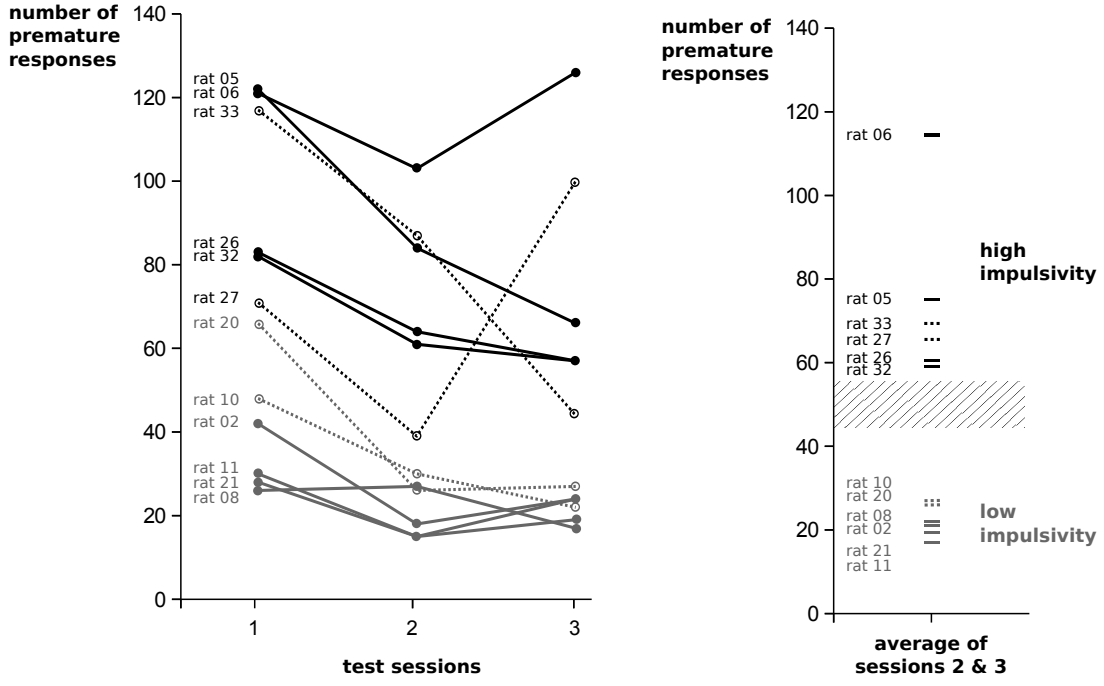
The formula calculated the geometric average of the housekeeping genes following a suggestion from Vandesompele et al. (2002) who had shown that this leads to more consistent average values.

The ΔCt values were then analysed by Student's t-test and fold change in the same way as the microarray data. The analysis was again limited to the data of the four high and four low impulsive animals with the most pronounced phenotypes.

4.3. Results

4.3.1. Results of the 5-choice serial reaction time task (5CSRTT)

In order to study the molecular underpinnings of impulsive behaviour in rat brains, a collaboration had been set up with the research group of Jeff Dalley from the *Cambridge Neuroscience* network (Cambridge, UK). The Cambridge group had set up a bidirectional breeding scheme to generate rats showing extremes of either high or low impulsive behaviour. We received the brains from twelve rats of the sixth generation along with the data of their performance in the 5CSRTT. In order to classify the rats according to their impulsivity phenotype they had undergone three 5CSRTT assessment sessions (figure 4.2a). The number of premature responses in the second and third session were averaged and animals having more than 55 premature responses were classified 'high impulsive' whereas animals scoring below 45 were considered 'low impulsive' (figure 4.2b). All shown animals were used for microarray screening but only animals with unambiguous results in all three 5CSRTT test session (solid lines in figure 4.2a) were used for group comparisons.



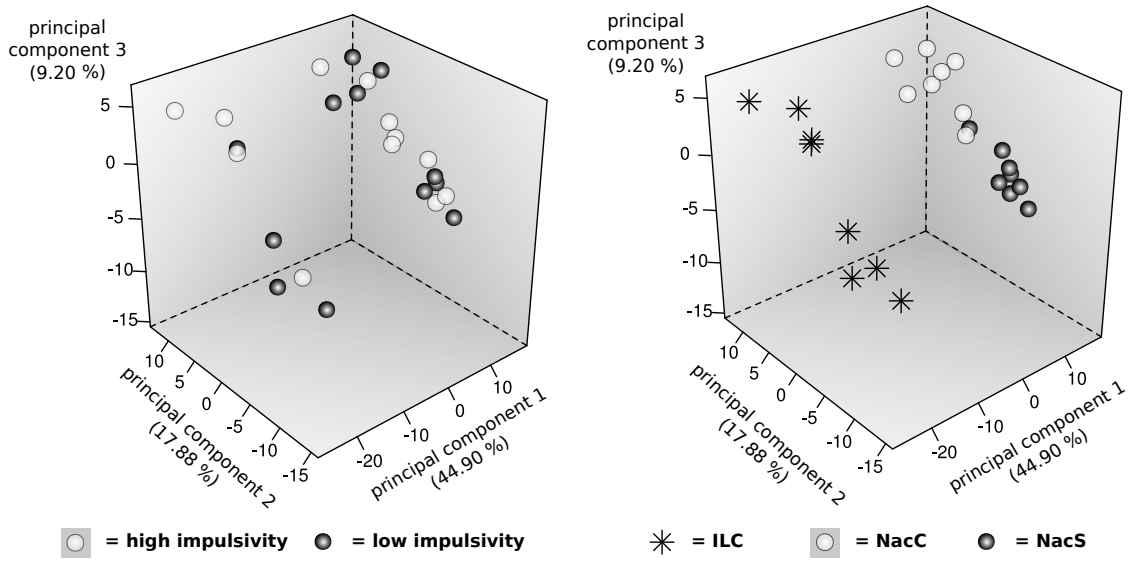
(a) Premature responses per session

(b) Average of session 2 & 3

Figure 4.2.: Number of premature responses in the 5-choice serial reaction time task (5CSRTT). Shown are the data of the rats whose brains were selected for microarray analysis.
Black lines = rats bred for high impulsivity,
grey lines = rats bred for low impulsivity,
dotted lines = rats with ambiguous impulsivity phenotype,
hatched bar in (b) = separation margin (45-55) between impulsivity phenotypes.

4.3.2. Principal component analysis

The normalised gene expression data of all brain regions of the four high and the four low impulsive rats with the most pronounced phenotypes were analysed by *principal component analysis* in order to identify the main sources of variance in the data set. The microarray data sets corresponding each to a brain region of a rat were projected on a three-dimensional Cartesian coordinate system based on the first three principal components. Labelling of the samples according to the behavioural phenotype of the rats led to a random-looking positioning of the high and low impulsivity cases (figure 4.3a). Labelling of the samples according to the brain region led to clearly distinguishable clusters for each brain region (figure 4.3b). The cluster for the infralimbic cortex was the most detached while the nucleus accumbens core and shell lied close to each other.
This showed that brain region but not impulsivity had a profound influence on the overall gene expression pattern in our samples.



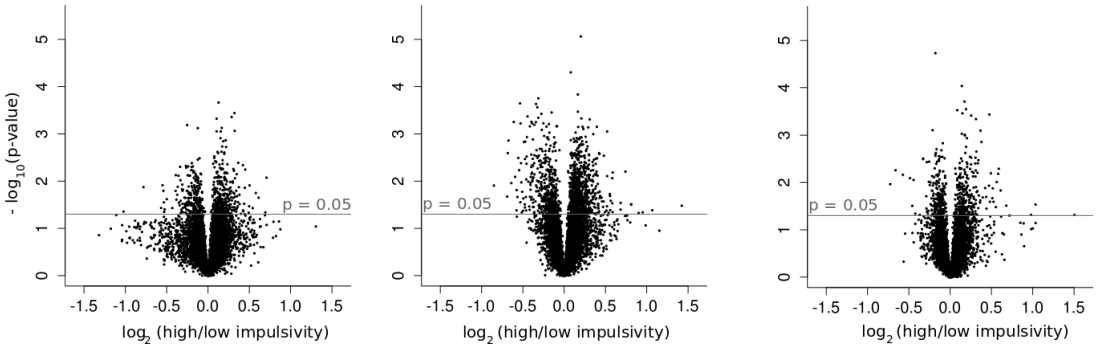
(a) Impulsivity phenotype **(b)** Brain region

Figure 4.3.: Scatter plots of the rats projected on the first three principal components detected by principal component analysis (PCA). (a) Labelling according to the impulsivity phenotype and (b) according to the brain regions. ILC = infralimbic cortex, NacC = nucleus accumbens core, NacS = nucleus accumbens shell.

4.3.3. Volcano plots

In the next step we wanted to see if impulsivity affects the infralimbic cortex and the nucleus accumbens core and shell to the same degree or if one of these brain regions stands out. We therefore displayed the whole expression data for each brain region in Volcano plots, where the x-axis corresponds to the log-transformed ratio of the average expression values for each gene and the y-axis displays the negative decadic logarithm of the p-value of a student's t-test for the gene (figure 4.4).

None of the brain regions exhibited a level of overall differential gene expression noticeable superior to the others but there was a slightly higher differential gene expression in the nucleus accumbens core.

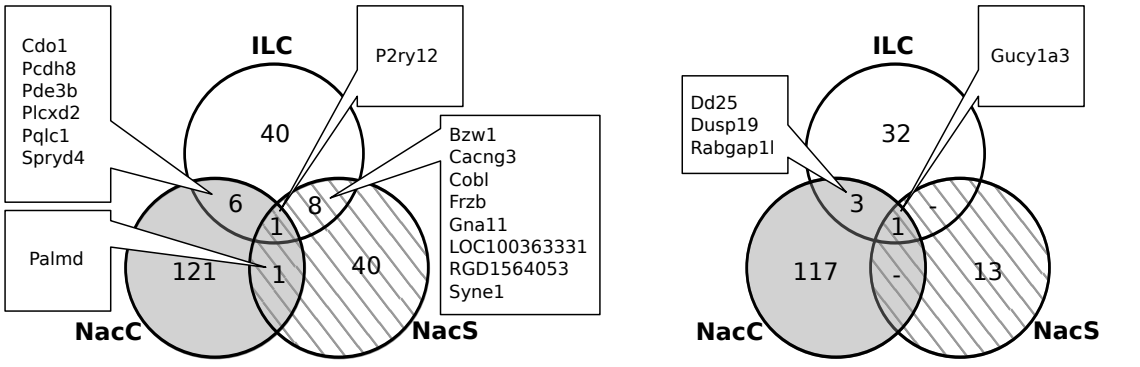


(a) Infralimbic cortex **(b)** Nucleus accumbens core **(c)** Nucleus accumbens shell

Figure 4.4.: Volcano plots showing overall expression differences between high and low impulsive rats. Each dot corresponds to one gene. Dots above the vertical grey line correspond to genes whose differential expression had an uncorrected p-value below 0.05.

4.3.4. Filtering by t-test derived p-values and fold-changes

In order to define differentially expressed genes, the data were filtered according to a p-value cut-off of 0.05 and an absolute value of the fold change of 1.2 (i.e. gene expression ration < 0.833 or > 1.200). As already seen in the volcano plots, the nucleus accumbens core showed the highest number of differentially expressed genes (n = 250 out of 7928 genes in total) followed by the infralimbic cortex (n = 91 out of 7961 genes in total) and the nucleus accumbens shell (n = 64 out of 7926 genes in total). Specific numbers for up- and down-regulation of genes as well as differentially expressed genes that were found in more then one brain region can be found in figure 4.5.



(a) Up-regulated in high impulsivity **(b)** Down-regulated in high impulsivity

Figure 4.5.: Differentially expressed genes in the three investigated brain regions according to t-test (p-value < 0.05) and fold change (absolute value of fold change > 1.2). ILC = infralimbic cortex, NacC = nucleus accumbens core, NacS = nucleus accumbens shell.

A selection of candidate genes from the filtering is presented in figure 4.6: the expression levels of *frizzled-related protein*(*Frzb*) and *G-protein coupled, purinergic receptor P2Y 12* (*P2ry12*) were upregulated in all three brain regions of high impulsive rats although *Frzb* did not reach the significance criterion of a p-value below 0.05 in the nucleus accumbens core. Some genes were only differentially regulated in one brain region as the *G protein-coupled receptor, family C, group 5, member B* (*Gprc5b*) which was down-regulated in the infralimbic cortex of high impulsive rats, the *RAS guanyl releasing protein 3* (*Rasgrf3*) which was up-regulated in the nucleus accumbens core of high impulsive animals and the *ADP-dependent glucokinase* (*Adpgk*) which was down-regulated in the nucleus accumbens shell of high impulsive rats.

4.3.5. KEGG pathways & gene ontology terms

The DAVID Bioinformatics Resources website was used to analyse the t-test and fold change filtered lists of candidate genes for the three investigated brain regions. None of the lists showed significant enrichment of pathways of the *Kyoto Encyclopedia of Genes and Genomes* (KEGG). The screening for enriched *gene ontology* (GO) terms did not reveal any findings which stayed significant after correction for multiple testing with the Bonferroni method. The only GO term which almost retained significance was the *biological process* termed '*intracellular signalling cascade*' in the infralimbic cortex (table 4.5).

Table 4.5.: Enriched gene ontology terms

GO category	gene ontology	number of genes	p-value	fold enrichment	Bonferroni
infralimbic cortex					
biological process	intracellular signalling cascade	14	0.00019	3.3	0.078

4.3.6. Correlation analysis

In a next step, the gene expression measurements were set in relation to the number of premature responses in the 5CSRTT of the corresponding rats. The infralimbic cortex showed a higher number of highly correlated genes (Spearman's rho either > 0.8 or < -0.8) than the nucleus accumbens regions (see table 4.6). A number of genes was found in both the group comparisons by t-test and fold change and the correlation analysis (see table 4.7). Among these genes was the *G-protein coupled, purinergic receptor P2Y 12* (*P2ry12*) which showed an increase in expression levels in relation to the number of premature responses in the the infralimbic cortex and nucleus accumbens core of the rats and was thus positively correlated. Further positively correlated findings in the infralimbic cortex were the *mitogen-activated protein kinase 1* (*Mapk1*) and the *NADP(+) dependent, mitochondrial malic enzyme 3* (*Me3*). In the nucleus accumbens shell, the *voltage-dependent calcium channel gamma subunit 3* (*Cacng3*) was among the up-regulated common findings.

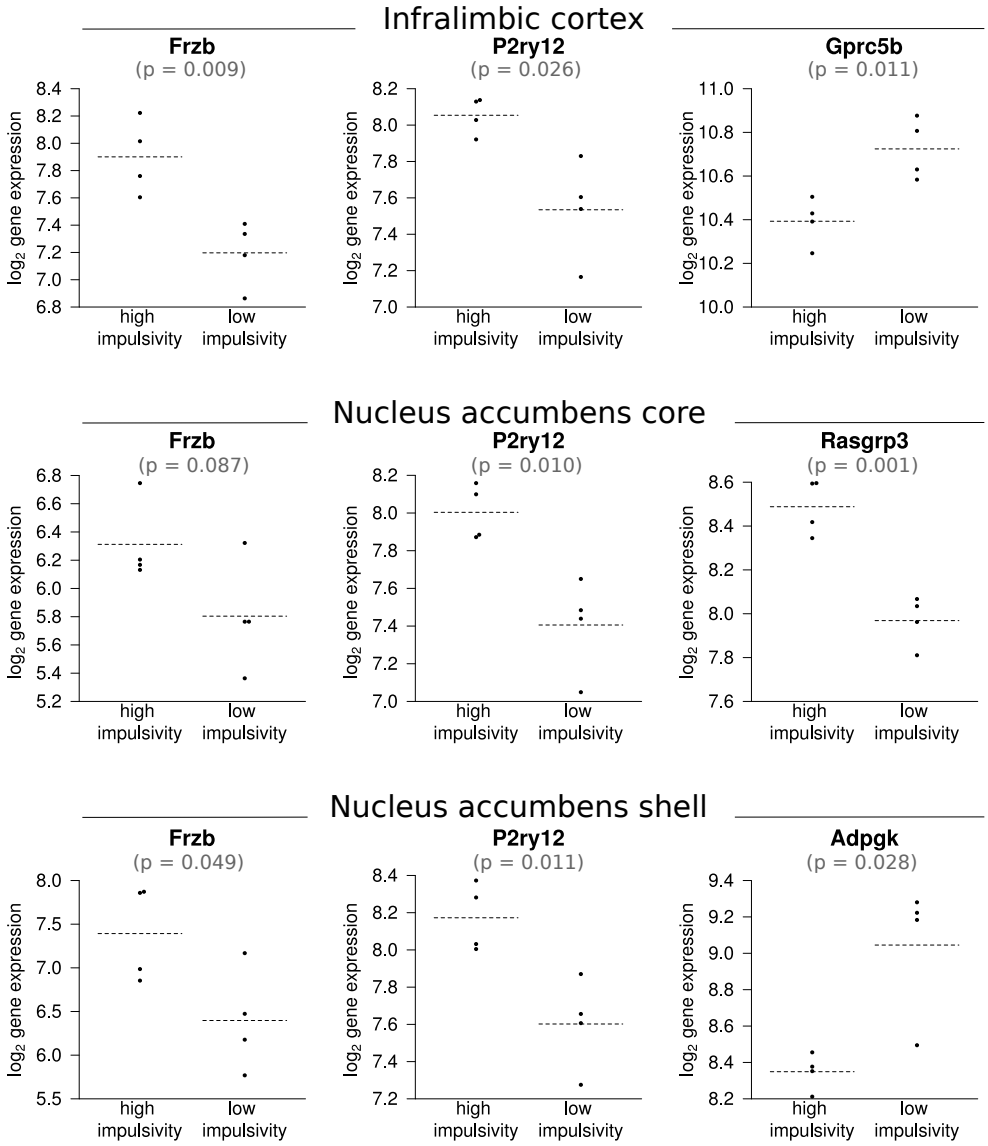


Figure 4.6.: Selection of genes that were differentially regulated between high and low impulsive rats according to the microarray anaylsis. *P2ry12* showed significant expression levels in all three brain regions and *Frzb* in two brain regions. The expression difference of *Gprc5b*, *Rasgrp3*, and *Adpgk* was each only significant in one brain region.

We had used the non-parametric rank correlation test by Spearman for the correlation analysis as the number of premature responses had markedly deviated from a normal distribution. As this test was only based on the ranking of the gene expression values and the number of premature responses, it was likely to yield a great number of false positives. We therefore used this analysis as an additional corroboration and focused on those genes which came also up in the group comparisons by t-test and fold change.

Table 4.6.: Number of genes correlated with the number of premature responses.

Brain region	positive correlation (Spearman's rho > 0.8)	negative correlation (Spearman's rho < -0.8)
Infralimbic cortex	94 (21)	53 (8)
Nucl. accumbens core	25 (6)	13 (2)
Nucl. accumbens shell	24 (4)	22 (2)

In parentheses: number of genes that also appeared in the list of t-test and fold change filtered results.

4.3.7. Relation of gene expression findings to quantiative trait loci

Our collaborators in Cambridge conducted a QTL analysis on the rats. A segment on chromosome 1 spanning from approximately mega base pair position $126.2 \cdot 10^6$ to $178.3 \cdot 10^6$, corresponding approximately to chromosomal locus 1q31 to 1q35, seemed to be in linkage with impulsivity. The normalised microarray data contained 163 genes that lay in this region. Only a fraction of these genes had been considered significantly differentially expressed between high and low impulsive rats according to t-test and fold change filtering (see table 4.8). No such differentially expressed genes were found in the nucleus accumbens shell.

4.3.8. Validation of microarray results by quantitative PCR

We performed quantitative PCR measurements on several of our top findings from the microarray analysis. A group of four genes was chosen which was analysed in all three brain regions. The other candidate genes were specifically selected for one brain region and only tested there.

While most of the selected genes failed to be significantly differentially expressed in the three brain regions, we could confirm the major trends of the microarray experiment as gene ratios almost exclusively went in the right direction. *Frzb* and *P2ry12* were confirmed to be higher expressed in all three analysed brain regions in high impulsive animals by the quantitative PCR although the result of *P2ry12* was not significant in the infralimbic cortex. We could also confirm the regulation pattern of *G protein-coupled receptor, family C, group 5, member B* (*Gprc5b*) and *septin 4* (*Sept4*) in this brain region, while *voltage-dependent calcium channel gamma subunit 3* (*Cacng3*) and *Rho family GTPase 3* (*Rnd3*) showed almost significant differential regulation. In the nucleus accumbens core, we had an almost significant result for the *Ras guanyl releasing protein 3* (*Rasgrp3*) which was up-regulated in high impulsive rats. In the shell region of the nucleus accumbens we could

Table 4.7.: Overlap between the correlation analysis and the group comparison by t-test and fold change.

infralimbic cortex		nucl. accumbens core		nucl. accumbens shell	
up[1]	down[2]	up[1]	down[2]	up[1]	down[2]
Ankrd33b	Cdc42ep1	P2ry12	Ankrd35	Aldh3b1	Fundc1
Bzw1	Cdc42ep2	Pdcd4	Zfp212	Cacng3	Larp6
Fam13c1	Fem1c	Sphkap		Klf13	
Gipc1	Fndc3a	Spryd4		LOC500300	
Gna11	Mpp6	Tfpi			
Gramd1b	Pmp22	Tmem176			
Hsd11b1	Rdx				
Icam5	Taf15				
Kbtbd3					
Kctd13					
Limk2					
Mapk1					
Me3					
P2ry12					
Pcdh8					
Plcxd2					
Prkd2					
Rab3ip					
Rnf217					
Suds3					
Syne1					

[1] up-regulated and [2] down-regulated in high impulsive rats

Table 4.8.: Genes with significantly differential expression that lie in the impulsivity-associated quantitative trait loci region on chromosome 1

Brain region	gene symbol	position in 10^6 base pairs	t-test p-value	fold change high/low impulsivity
infralimbic cortex	Gprc5b	177.2	0.011	0.79
	Me3	146.4	0.027	1.20
	Pde3b	172.6	0.037	1.25
nucleus accumbens core	Chd2	128.7	0.018	0.81
	Olfml1	165.0	0.006	1.21
	Pde3b	172.6	0.004	1.40
	Tm6sf1	138.1	0.049	1.45

confirm a down-regulation of the *ADP-dependent glucokinase* (*Adpgk*) in high impulsive animals.

Nonetheless, the confirmation of some genes failed completely as even the fold change directions were conflicting between the microarray and the quantitative PCR data: The *dual specificity phosphatase 19* (*Dusp19*) and the *guanine nucleotide binding protein alpha 11* (*Gna11*) could not be confirmed in any of the analysed brain regions. The *mitogen-activated protein kinase kinase kinase kinase 5* (*Map4k5*) was not confirmed in the infralimbic cortex and the nucleus accumbens core, the two brain regions where it had been analysed. In addition, the *soluble guanylate cyclase 1 alpha 3* (*Gucy1a3*) and the *TAO kinase 1* (*Taok1*) were not detected as differentially expressed in the striatal core region. A differential expression of *Cacng3* in the striatal shell region could also not be confirmed.

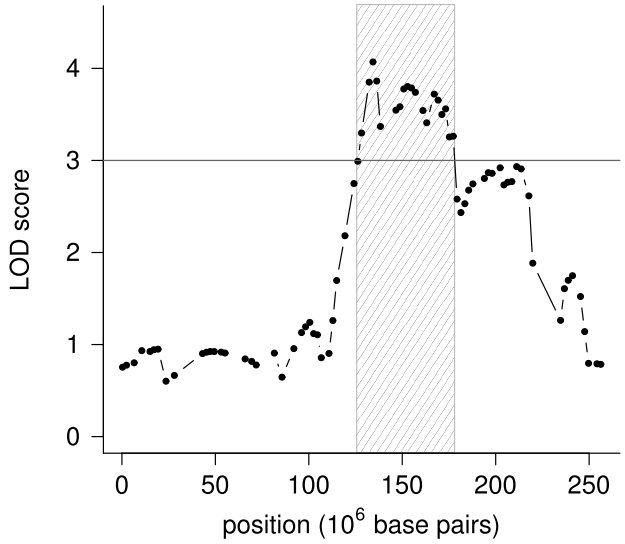


Figure 4.7.: Quantitative trait loci on chromosome 1 which were linked to the number of premature responses in high and low impulsive rats. LOD scores higher than 3, i.e. above the grey line, were considered to be in linkage with the trait and were underlined with a hatched box. Data from Jeff Dalley (University of Cambridge) and Silvia Pitzoi (Imperial College London).

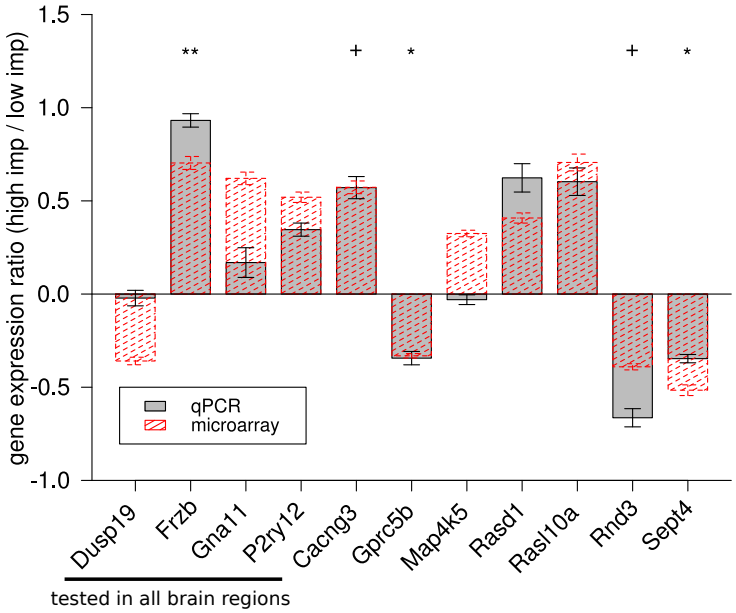
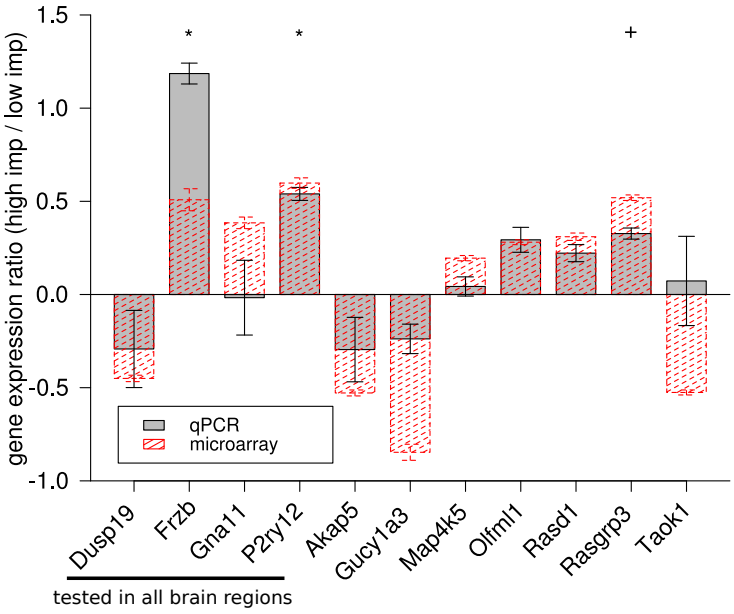
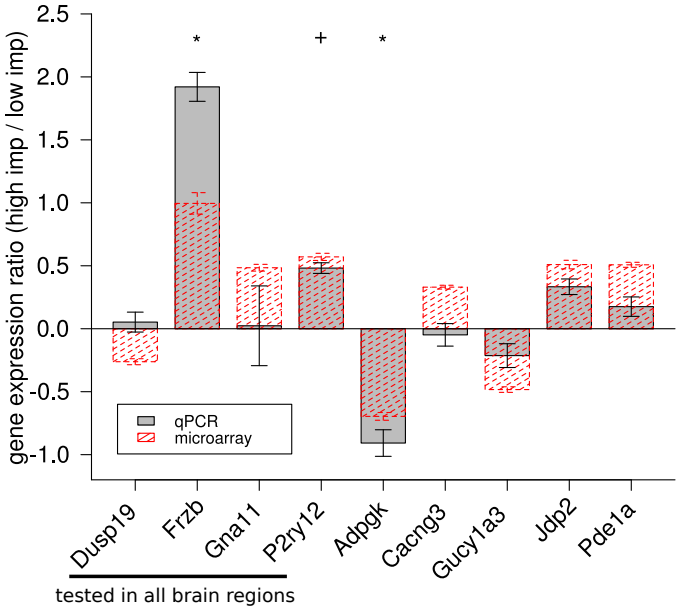


Figure 4.8.: Infralimbic cortex

Figure 4.9.: Results of the quantitative PCR measurements (grey bars) in relation to the microarray data (dashed red bars) in the ***infralimbic cortex***. Indicated are the levels of significance of the qPCR results: $+ < 0.1$, $* < 0.05$, $** < 0.01$.



(a) Nucleus accumbens core



(b) Nucleus accumbens shell

Figure 4.10.: Results of the quantitative PCR measurements (grey bars) in relation to the microarray data (dashed red bars) in the ***nucleus accumbens core*** and ***shell***. Indicated are the levels of significance of the qPCR results: $+ < 0.1$, $* < 0.05$.

4.4. Discussion of the study

In the present study we analysed genome-wide gene expression in selected brain areas of rats bred for extremes in high and low impulsive behaviour. In a first approach, we were locking at the overall amount of gene expression differences between the two groups and not at the level of individual genes.

Principal component analysis

The projection of the rats according to the first three principal components showed a clear separation according to the brain regions but no separation according to the impulsivity phenotype. While brain anatomy was therefore responsible for the largest part of the overall variance in the gene expression data set, only a small portion of genes was involved in the behavioural trait of impulsivity. This was not surprising as the gene expression differences underlying the functional and anatomical differences of the brain regions should be much more pronounced than the subtle changes in neuronal receptor densities and signalling cascades which might influence the degree of impulsive behaviour. The separation of the tissue samples according to the brain regions reflected the architectural differences as the nucleus accumbens core and shell regions are separated but closely linked regions while the infralimbic cortex is situated further apart.

Volcano plots and candidate gene lists

The presentation of the t-test p-values and fold changes of all genes in the form of volcano plots did not show a noteworthy greater influence of any of the brain regions on impulsivity. All analysed brain regions showed a number of differentially regulated genes but their number and location was about the same. Nonetheless, the list of differentially expressed genes contained approximately three times more genes for the core region of the nucleus accumbens than for the shell region or the infralimbic cortex. This suggests that the nucleus accumbens core showed the greatest cellular differences between the phenotypes. This finding was in line with lesion studies performed by Christakou et al. (2004) and by Pothuizen et al. (2005) which had already stressed the importance of the core region in impulsive behaviour.

On the other hand, the correlation of our gene expression data with the number of premature responses of the corresponding animals had shown the greatest number of candidate genes in the infralimbic cortex. This finding received also support from the literature (Dalley et al., 2011). Chudasama et al. (2003) had shown that lesions of the infralimbic cortex led to higher impulsivity. A similar effect can be provoked with the administration of a NMDA receptor antagonists in this brain region (Murphy et al., 2005).

Individual gene findings

The analysis of the data set by t-tests and fold change filtering yielded a list of candidate genes for each analysed brain region. The ***G-protein coupled purinergic P2Y receptor 12*** (***P2ry12***) was significantly up-regulated in all three brain regions in the high impulsive

rats and this result was confirmed by quantitative PCR analysis. In the infralimbic cortex and nucleus accumbens core, *P2ry12* was also significantly positively correlated with the number of premature responses. The gene codes for a G protein-coupled receptor which is activated by adenosine diphosphate (ADP). It has first been described in the context of haemostasis research which showed that extracellular nucleotides such as ADP can act as messenger molecules and activate purinergic receptors on blood cells which will cause platelet aggregation (Nicholas, 2001). It has since been discovered that extracellular nucleotide signalling and *P2ry12* play a role in multiple cellular functions. Haynes et al. (2006) showed in a mouse knock-out model that *P2ry12* is essential for microglial activation as a response to cortical damage and in line with this finding Amadio et al. (2010) reported that loss of this receptor is an aggravating factor in grey matter lesions caused by multiple sclerosis. But no link has been established between the receptor and impulsivity or any psychiatric disorder so far. We could therefore have come across a yet unknown role of this receptor in brain function. Another possibility is that we face an instance of random genetic drift and that the differences in *P2ry12* gene expression levels were enriched by the breeding program although the gene is not linked to impulsivity. Subjecting the knock-out mouse model from Haynes et al. (2006) to the 5-choice serial reaction time task (5CSRTT) would be a good way to see whether this gene has an effect on impulsive behaviour.

Another gene which was significantly up-regulated in all three brain regions was the ***frizzled-related protein*** (***Frzb***). This protein was first described based on its analogy to *Drosophila's frizzled* protein (Hoang et al., 1996) and functionally characterised as an secreted antagonist in the Wnt signalling pathway (Leyns et al., 1997; Lin et al., 1997). Interestingly, *catenin (cadherin associated protein), beta 1* (*Ctnnb1*), a downstream effector in the *Frzb*-mediated part of the Wnt signalling pathway, was also significantly up-regulated in the infralimbic cortex (p-value = 0.049, ratio = 1.13). It was shown that Wnt signalling is involved in axon remodelling and synaptic differentiation (Hall et al., 2000). Ataman et al. (2008) showed that Wnt signalling plays a role in pre- and post-synaptic modifications as a result of synaptic activity. It should also be noted that *Frzb* had already been connected to impulsivity in a study on inbreed mouse strains (Loos et al., 2009). Given these facts, we believe that it is unlikely that *Frzb* was only linked to impulsivity as a result of random drift. The gene would therefore be a core candidate for functional validation. Unlike in the case of *P2ry12*, no established transgenic knock-out model for the frizzled-related protein exists to the best of our knowledge. It would be therefore preferable to alter the *Frzb* activity by virus-mediated RNA interference which allows to silence the gene in adult animals without the need to consider a possible role of the gene during the embryonic development. The impulsivity of the thus-treated animals could be tested with the 5CSRTT. Local injection of the viral vector containing the *Frzb* RNA-complementary sequence would allow to correlate the degree of impulsivity more specifically to the activity of this gene in brain areas such as the infralimbic cortex and the nucleus accumbens.

The list of quantitative PCR validated, differentially regulated genes also contained the ***G protein-coupled receptor, family C, group 5, member B*** (***Gprc5b***) which was down-regulated in the infralimbic cortex of high impulsive rats. This receptor, which is activated by retinoic acid (Bräuner-Osborne and Krogsgaard-Larsen, 2000), shows high

expression levels in the neocortex (Robbins et al., 2002). It has been suggested that the receptor interacts with the frizzled G protein of the Wnt signalling pathway (Harada et al., 2007) and thus acts on the same pathway as the *Frzb* protein described above.

A further finding in the infralimbic cortex was a significant down-regulation of ***septin 4*** (***Sept4***), a member of a group of guanosine triphosphate binding cell structure proteins which has been linked to neuro-degenerative diseases but not to psychiatric disorders or impulsivity so far (Sitz et al., 2008).

Two further genes of the infralimbic cortex candidate list reached almost significance in the quantitative PCR experiment. We found an up-regulation of the ***voltage-dependent calcium channel gamma subunit 3*** (***Cacng3***) and a down-regulation of the ***Rho family GTPase 3*** (***Rnd3***) in high impulsive rats. While both genes are implicated in signalling the have not been linked to impulsivity previously.

A further gene which reached almost significance in the nucleus accumbens core in the quantitative PCR was the ***RAS guanyl releasing protein 3*** (***Rasgrp3***) which was up-regulated in high impulsive rats. Like *Rasgrf2*, which had been linked to alcohol intake in a meta-analysis of genome-wide association studies (Schumann et al., 2011) and whose expression level was analysed in one of our post-mortem tissue studies (see section 5.1.3 and figure 5.2 on page 96), *Rasgrp3* codes for a guanine nucleotide exchange factor and is thus implicated in G protein coupled signalling. Both genes are situated next to each other in the *mitogen activated kinase-like protein* (*MAPK*) signalling pathway of the *Kyoto Encyclopedia of Genes and Genomes* (KEGG). As our laboratory already has a transgenic animal model consisting of *Rasgrf2* $^{-/-}$ mice (Stacey et al., 2012), these animals could be tested in the 5CSRTT to further assess the influence of the *MAPK* pathway on impulsivity.

The quantitative PCR of the shell region also confirmed a significant down-regulation of the ***ADP-dependent glucokinase*** (***Adpgk***) in high impulsive rats. But while this gene had been previously linked to hypoxia, no such connection to psychiatric disorders or impulsivity had been brought forward till today (Ronimus and Morgan, 2004).

KEGG Pathways and enriched gene ontology terms

There had been no enriched biological pathways of the *Kyoto Encyclopedia of Genes and Genomes* in our lists of candidate genes but a *gene ontology* term of the category '*biological process*' had been detected with an almost significant corrected p-value in the infralimbic cortex: '*intracellular signalling cascade*'. This finding suggests that the communication between cells is altered in this brain region according to the level of impulsivity of the rat. This would be in line with our original hypothesis that we would find differences in neurotransmitter signalling systems.

Based on the strong differential regulation of *Frzb*, it is noteworthy that the Wnt signalling pathway was also not discovered as an enriched feature. In addition, the pathway analyses did not reveal a specific enrichment of genes belonging to the dopaminergic and serotonergic signalling. We can therefore not confirm our hypothesis form the beginning that it would be this kind of biological mechanisms which would be implicated in the generation of impulsivity in the infralimbic cortex and the nucleus accumbens.

Genes linked to a quantitative trait loci on chromosome 1

We also screened if some of the candidate genes lay in a section of chromosome 1 (1q31 - 1q35) that had been found to be linked to the trait of impulsivity by our collaborators in Cambridge. This trait has not been analysed by a genome wide association study so far and the only available rat data for comparison originate from attention deficit hyperactivity disorder rat models which analysed different sub-features and brought up different loci (Moisan et al., 2003; Vendruscolo et al., 2006). One of our main findings in the infralimbic cortex had been *Gprc5b* which had also been confirmed in the quantitative PCR experiment and which had been described above. Further candidate genes from this brain region, that were situated in this chromosomal section, included the *NADP-dependent mitochondrial malic enzyme 3* (*Me3*) which had been linked to neonatal lesions in an animal model of schizophrenia but not to impulsivity or ADHD so far (Wong et al., 2005). The *cGMP-inhibited phophodiesterase 3B* (*Pde3b*) which catalyses the hydrolysis of cyclic nucleotide monophosphats and which is situated in this chromosomal region was up-regulated in both infralimbic cortex and nucleus accumbens core. While *Pde3b* was brought in connection with hypothalamic leptin-signalling it has not been linked to impulsivity or any psychiatric disorder before (Sahu, 2011). There were three other genes which came up only in the nucleus accumbens core: The *chromodomain helicase DNA binding protein 2* (*Chd2*), a chromatin remodelling protein which plays a vital role in embryogenesis (Marfella et al., 2006). The *olfactomedin-like 1* (*Olfml1*), whose zebrafish homologue was reported to be involved in retinal axon elongation via the Wnt signalling pathway (Nakaya et al., 2008). *Olfml1* was among the genes re-analysed by quantitative PCR. While the gene's expression levels showed a similar ratio as in the microarrays the statistical difference of expression was not confirmed. And finally the *transmembrane 6 superfamily member 1* (*Tm6sf1*), a gene discovered by a sequencing project with no known protein function until now (Carim-Todd et al., 2000). There had been not a single gene lying in the specific chromosomal region which was significantly differentially regulated in the nucleus accumbens shell data.

Taken together, the relation of our gene expression data to the quantiative trait loci on chromosome 1 yielded only few genes and most of these genes seemed to have no apparent relation to impulsivity or psychiatric disorders in general. A possible explanation would be that the associated SNPs are related to trans-acting transcription regulators which exert their influence somewhere else in the genome. Candidate genes like the *Tm6sf1* could therefore just be false-positives contained in the list of gene expression findings. In addition, it has to be noted, that the selected quantitative trait loci had moderate LOD scores in the range of 3 to 4. While such scores are generally considered to indicate an association between the chromosomal region and the analysed quantitative trait, the association has nonetheless to be considered rather weak.

Limitations of the study

The present study had several limitations. While we had originally planned to compare six high to six low impulsive animals, we limited the group size to four for the t-test and fold change calculations due to ambiguous behavioural results in the other rats. Such a

low number of replicates per group limited the ability of the statistical test to detect subtle but otherwise relevant gene expression differences.

Our study was also not replicated in another cohort of bidirectionally bred impulsive rats. Such a repetition would have allowed to differentiate chance findings from genes that are truly involved in the phenotype.

And finally, the rats of the high impulsivity group did not differ very strongly in the number of premature responses in the 5-choice serial reaction time task from their low impulsive counterparts. The behavioural phenotype was therefore only weakly expressed and results of the groups even overlapped between the test sessions in some cases. The behavioural differentiation appears especially weak when compared to rat lines bred for high and low levels of voluntary alcohol consumption. In these rat lines, the alcohol-preferring animals consume up to ten times more alcohol than their non-preferring counterparts (Rodd et al., 2004; Björk et al., 2010; Crabbe et al., 2010).

Follow-up experiments

As already mentioned during the discussion of the candidate genes, it is not possible to determine from the microarray data if a gene finding is really implicated in the analysed phenomena or if it is just a false positive. This determination can only be achieved by functional validation. That means either silencing or artificially enhancing the expression of the candidate gene and observing the effect that it has on the queried phenotype, which was the level of impulsivity in this study. Based on our results and the available literature, *P2ry12*, *Frzb* and *Gprc5b* present the most promising candidates for such an approach.
Another option would be to repeat parts of this experiment with more homogeneous brain tissue. A technique such as laser capture microdissection could be used to extract only one type of neurons such as the medium spiny neurons of the nucleus accumbens. The low amount of such tissue would probably forbid the analyses by microarrays but the analysis of a low number of genes by quantitative PCR would be possible. This would allow to correlate our findings more specifically to unique neuronal cell types.

Conclusion

In the present study, we used microarray analysis to profile gene expression in the infralimbic cortex and the nucleus accumbens core and shell of high and low impulsive rats selected from the sixth generation of an impulsivity breeding program. Our data did not show a clear dominance of one of these brain regions in the association of their gene expression to the impulsivity but suggested that their influence diminished from infralimbic cortex via the core to the shell region of the nucleus accumbens. Several candidate genes were discovered in these regions such as *P2ry12*, *Frzb* and *Gprc5b* with the latter gene being situated in a chromosomal region associated with impulsive behaviour. Those candidate genes led to the hypothesis that modification of the Wnt signalling pathway might play a role in the modulation of impulsivity levels.
We thus showed that a purely behavioural, non-pathological phenotype such as impulsivity can be associated with characteristic gene expression pattern in the brain. This implies

that this personality trait reverberates on the cellular level. Part of the discovered candidate genes might therefore hint at new pharmacotherapeutic targets for the treatment of ADHD, drug addiction and other psychiatric disorders in which impulsivity plays a pivotal role.

5. Candidate gene expression studies in human post-mortem brain tissue

5.1. Introduction to candidate gene studies

5.1.1. Measuring gene expression in human post-mortem brain tissue

The key to a better understanding of addictive disorders lies in the human brain. Biomedical research is therefore in need of brain tissue to learn more about the connection between the behavioural phenotype of addiction and underlying alterations on the cellular level. Taking brain tissue samples from living persons is not feasible as this would require brain surgery which is always a delicate and risky medical intervention. The risk is not justified as addictions don't constitute an acutely live-threatening danger as for example a brain tumour. In addition, the brain areas involved in addiction show cellular alterations but they are far from being defunct and their integrity is needed for the well-being of the patient.

The gene expression studies presented in the previous chapters circumvented this problem by analysing brain tissue from animals or peripheral tissue from humans in the hope that the results can be extrapolated to the human brain. Another option is to take human brain tissue samples after the death of a person. This approach has already been applied by Paul Broca (1824 - 1880) in the 19th century (Broca, 1861). While Broca based his analysis on visually distinguishable anatomical differences in brain areas of patients the present study focused on subtle molecular modifications which should be detectable by measuring messenger RNA concentrations.

It is important to keep in mind that such analyses are subjected to a number of limitations which lie in the nature of the post-mortem tissue. While the primary goal is to link different expression levels of a candidate gene to the disease status or genotype of the brain donor, the difference in mRNA concentration can also be caused by a number of other factors (Stan et al., 2006):

- **Post-mortem interval**: As a person dies the body's tissue is cut-off from oxygen and nutritional supply which is vital for proper cell function and the maintenance of intact cell bodies. As a consequence, the cells can no longer protect their RNAs from the abundantly present RNases which leads to a rapid degeneration of the ribonucleic acids. The interval between time of death and the autopsy has therefore a great influence on RNA concentration. This effect is also dependent on the ambient temperature as tissue decomposition and RNA decay proceed more quickly at higher temperatures.

- **Death struggle**: The way of dying has an important impact on gene expression

levels. A rapid death, as in the case of a sudden cardiac death, is less likely to cause widespread gene expression changes than a slow and agonising death struggle.

- **Comorbidities and medical treatments:** RNA levels can further be influenced by the history of medical treatment and the presence of prescription as well as illegal drugs in the body at the time of death. Additional diseases can also influence the RNA composition. Especially in the group of control subjects, which are often selected to have a similar age of death as the diseased cases, death was often caused by a non-psychiatric medical condition such as cardiac failure.

- **Heterogeneity of the tissue**: While it is possible to locate and extract tissue from specific brain areas, the punched-out samples will still be made up by a mixture of neurons and glia cells and possibly even some other cell types originating from tiny blood vessels or belonging to the immune system. This makes it difficult to draw conclusions about gene expression levels in specific cell types.

There are several ways to address these limiting factors. Increasing the sample size is likely to reduce the impact of confounding factors on the overall result under condition that these factors affect disease and control cases randomly. Statistical analysis of the mRNA concentrations in the samples with a regression analysis can take these factors into account as covariates. Another option is to select a core set of samples excluding cases whose post-mortem interval exceeded a set limit, who were linked to confounding disease or whose toxicological examination revealed the presence of prescription or illegal drugs. The heterogeneity of the brain tissues can be addressed by using laser capture microdissection or similar methods for the isolation of specific cell types. But this is only possible if the cell types can be distinguished from each other and if the dissection yields enough high quality mRNA for subsequent quantification.

Nonetheless, post-mortem tissue samples can be used to validate research findings from animal studies and as a follow up of genetic epidemiology studies. With the advent of *genome-wide association studies* (GWAS), large numbers of associations between specific variations of *single-nucleotide polymorphisms* (SNPs) and disease have been discovered. However, most associated SNP variants do not result in a change of the amino acid chain of a protein but lie outside of exon regions. It is therefore likely that the disease-related SNP variants modify the interaction between the DNA and transcription factors thus influencing the transcription rates of surrounding genes (Manolio, 2010). Post-mortem brain samples permit to investigate the expression levels of such linked genes in brain areas relevant to psychiatric disorders.

5.1.2. Investigated brain regions

The present study was based on the analysis of tissue from four different brain regions: The *anterior cingulate cortex*, *Brodmann's area 9*, the *caudate nucleus* and the *ventral striatum*. These structures shall be briefly introduced here.

Anterior cingulate cortex

The corpus callosum, which connects the two brain hemispheres, is coated from above by each cerebral hemisphere with a cortical structure called the *cingulate cortex* (from the Latin word *cingulum* = belt as it girds the corpus callosum like a belt). This structure belongs to the phylogenetically oldest part of the cerebral cortex, the *archicortex*. It forms, together with the hippocampus, the amygdala, the fornix fibres and parts of the thalamus, the limbic system which is the centre of emotion processing.

The cingulate cortex can be cytoarchitectonically and functionally divided into three parts: the *anterior cingulate cortex* (figure 5.1a) with its characteristic spindle neurons (Allman et al., 2001), the *midcingulate cortex* and the *posterior cingulate cortex* (Vogt et al., 2004; Vogt and Paxinos, 2012). Both the anterior and the posterior cingulate cortex receive their main input from the thalamus and have output projections to prefrontal areas. The ventral part of the anterior cingulate cortex also sends projections to the amygdala (Vogt et al., 1987; Vogt and Pandya, 1987).

While the more posterior structures were linked to episodic memory retrieval and the perception of physical pain (Nielsen et al., 2005), the anterior cingulate cortex seems to be crucial for the regulation of emotion and cognitive processing (Bush et al., 2000). It comes as no surprise that cytoarchitetonical alterations in the anterior cingulate cortex have been linked to psychiatric disorders such as schizophrenia (Yan et al., 2012). The anterior area has also been linked to drug addiction and more specifically alcohol abuse (Goldstein and Volkow, 2002). It has been proposed that cellular modifications in the anterior cingulate cortex are crucial for the maintenance of addiction in long-term alcoholics (Kalivas and Volkow, 2005).

Brodmann's area 9

The second region investigated in our studies is a cytoarchitecturally homogeneous area of the frontal lobe which was first characterised by Korbinian Brodmann (1868 - 1918) as *area frontalis granularis* (Brodmann, 1909). This area, which is commonly referred to as *Brodmann's area 9* (figure 5.1b), shows the classical six layered structure of the cerebral cortex with the exception that layer V can be visually separated into an outer, neuron rich and an inner sparsely populated layer (Brodmann, 1909). The pyramidal cells from this layer are the main source of efferents to the striatum (Patestas and Gartner, 2006).

Brodmann's area 9 has been reported to be in connection with a number of psychiatric disorders, including major depression, bipolar disorder and schizophrenia (Fitzgerald et al., 2006; Kim et al., 2010; Uranova et al., 2004). The region is also of interest in alcoholism research. Dao-Castellana et al. (1998) showed that chronic alcohol-drinking patients had specific metabolic abnormalities in the dorsolateral prefrontal cortex, which roughly corresponds to Brodmann's area 9, and linked these alterations to neuropsychological and behavioural impairments often found in alcoholics. George et al. (2001) reported that this brain region showed increased activity in alcohol-dependent subjects while viewing alcohol-related cues. A molecular analysis of this brain region using mass spectrometry revealed concentration differences of sets of proteins in post-mortem tissue of alcohol-dependent sub-

jects (Alexander-Kaufman et al., 2006, 2007). These cellular alterations in Brodmann's area 9 might lead to an impairment of impulse control which could explain their connection to addictive behaviour (Koob and Volkow, 2010).

Caudate nucleus

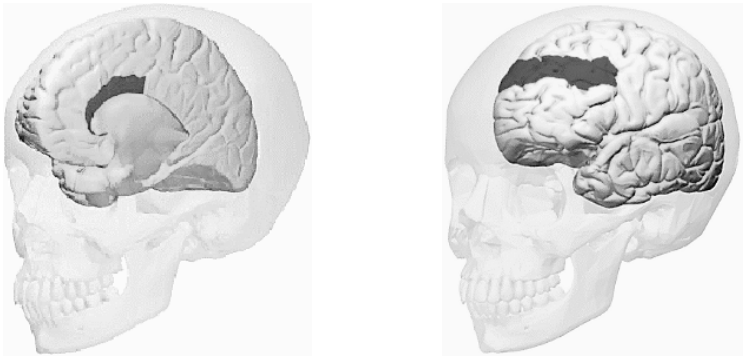
The *caudate nucleus* (figure 5.1c) is a cluster of neurons which stretches in a semicircle from the putamen to the amydala within each cerebral hemisphere. It is part of a functionally tightly connected network of nuclei, called the *basal ganglia*, which is responsible for voluntary motor control. The caudate nucleus receives its primary input from dopaminergic neurons originating in the midbrain structures of the ventral tegmental area and the substantia nigra (Smith and Parent, 1986; Packard and Knowlton, 2002). A large portion of its efferents go to the primary motor cortex but it also contains a significant number of neurons which send their signal back to the midbrain (Royce and Laine, 2004).

The caudate nucleus has been associated with learning and memory formation as well as the control of movements and cognition (Packard and Knowlton, 2002; Graybiel, 2005). Anatomic and metabolic alterations in this area have been linked to psychiatric conditions such as obsessive compulsive disorder (Hansen et al., 2002; Radua and Mataix-Cols, 2009). The caudate nucleus is also connected to reward processing (Nakamura and Hikosaka, 2006). Not surprisingly, alterations in this region have been associated with alcohol addiction (Weiner et al., 1980; Acheson et al., 2009). Gilman et al. (2012) showed in a study based on a behavioural test combined with a functional magnetic resonance scan that alcohol increased risk-taking behaviour in healthy social drinkers while it decreased the activity level of the caudate nucleus at the same time.

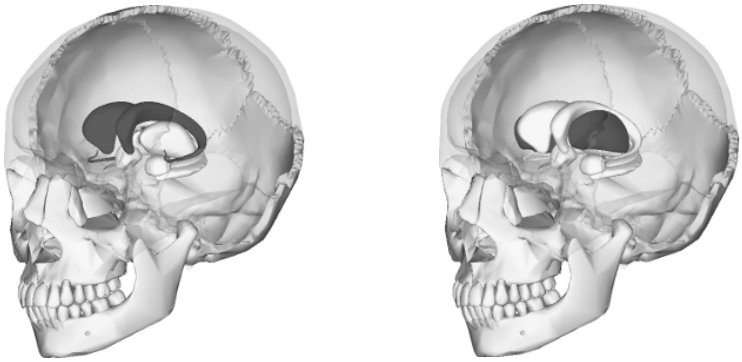
Ventral striatum

Finally, we also analysed post-mortem tissue from the *ventral striatum*. The *striatum* is a core structure of the basal ganglia system which consists of the above-mentioned caudate nucleus and the *putamen* (figure 5.1d). In the most ventrorostral part of the striatum, where the caudate nucleus and the putamen merge, lies a small region which is called the *nucleus accumbens* (Trepel, 2008). This nucleus had already been the region of interest in the study on the influence of alcohol exposure and circadian rhythmicity on gene expression in the rat brain and the study which profiled gene expression in high and low impulsive rats. As it is difficult to identify and isolate exclusively this small nucleus in the human brain, the pathologists provided us with tissue from a region which they labelled *ventral striatum*. This term encompasses primarily the nucleus accumbens but the tissue could also contain the adjacent olfactory tubercle as well as further ventrorostral tissue of the putamen and the caudate nucleus (Heimer and Wilson, 1975; Ubeda-Bañon et al., 2007).

More then 90 % of the neurons of the ventral striatum are medium spiny neurons whose neurotransmitter is γ-aminobutyric acid (GABA) (Lape and Dani, 2004; Sazdanović et al., 2011). The neurons are responsible for the main output of the ventral striatum. Their axons project to the *globus pallidus* and the *substantia nigra pars reticulata* from where the signal is further transferred to the thalamus and then to the motor cortex. Dopaminergic



(a) Anterior cingulate cortex **(b)** Brodmann's area 9



(c) Caudate nucleus **(d)** Putamen*)

Figure 5.1.: Analysed brain regions. The region of interest is coloured in black in each figure. *) For purpose of presentation, the putamen is shown instead of the ventral striatum which was analysed in this study. The later structure lies at the ventrorostral end of the putamen (see text for details). [1]

neurons projecting from the ventral tegmental area and the substantia nigra pars compacta, both situated in the midbrain, form the main input to the ventral striatum (Lobo and Nestler, 2011).

The ventral striatum plays a central role in the sensation of reward and pleasure. It has been shown that those sensations correlate with an increase in dopamine signaling from the ventral tegmental area to the ventral striatum (Arias-Carrión et al., 2010). Given the rewarding effect of drugs of abuse, the ventral striatum plays a key role in the establishment and maintenance of drug addiction (Everitt et al., 1999), including the addiction to alcohol (Koob, 2013).

[1] All figures are made from content published under the *Creative Commons Attribution 2.1 Japan license* on the BodyParts3D/Anatomography website (`http://lifesciencedb.jp/bp3d/?lng=en`) by the Database Center for Life Science (Tokyo, Japan). Figures 5.1a and 5.1b were modified from versions originally published on wikipedia (`en.wikipedia.org/wiki/Anterior_cingulate_cortex`, `en.wikipedia.org/wiki/Brodmann_area_9`) and figures 5.1c and 5.1d were self-made on BodyParts3D/Anatomography.

5.1.3. Candidate genes

The present study analysed four candidate genes which shall be briefly introduced here.

AUTS2

The first gene of interest was the *autism susceptibility gene 2* (*AUTS2*). Sultana et al. (2002) were the first to report on this gene which, as the authors suggest, might be disrupted in rare cases of autism. It is situated on chromosome 7q in a region which has previously been linked to autism (International Molecular Genetic Study of Autism Consortium (IMGSAC), 2001). Recently, this gene has gained attention from addiction research, as a SNP in the fourth intron of *AUTS2* was associated with the amount of daily alcohol consumption in a meta-analysis of genome-wide association studies: rs6943555 showed a significant linkage (p-value = $4.2 \cdot 10^{-8}$) with its minor A allele associated with a 5.5 % lower alcohol consumption (Schumann et al., 2011).

In spite of these reports that link *AUTS2* to psychiatric disorders the function of the gene remains hitherto unknown. Neither the fact that *AUTS2* has homologs in various vertebrate species nor its DNA sequence have yielded any information on this matter.

The present study did not address this question but aimed to investigate if the allelic variation at SNP rs6943555 leads to a difference in *AUTS2* gene expression. To analyse this research question, *AUTS2* mRNA concentration was measured in post-mortem tissue of Brodmann's area 9 by quantitative PCR and related to the allelic variation in SNP rs6943555.

RASGRF2

The same genome-wide association study which brought up *AUTS2* in connection with alcohol dependence suggested a connection of another gene to this disorder: the *Ras protein-specific guanine nucleotide-releasing factor 2* (*RASGRF2*). This protein transforms an inactive small GTPase from the RAS family to its active form by exchanging its bound guanosine diphosphate (GDP) with a guanosine triphosphate (GTP) (figure 5.2). *RASGRF2* acts thus as an upstream modulator of the MAPK/ERK pathway. This effect has been shown to take place in the medium spiny neurons of the striatum and was linked to long-term behavioural responses to cocaine (Fasano et al., 2009; Fasano and Brambilla, 2011).

Schumann et al. (2011) reported a possible connection between SNP26907 lying in the *RASGRF2* gene and alcohol consumption: The minor T allele is associated with a 2.6 % decrease in alcohol consumption with a nominal p-value of $2.2 \cdot 10^{-4}$. The SNP26907 lies in an intronic region between exon three and four of the full-length transcript. Another guanine nucleotide exchange factor, *RASGRP3*, which is closely related to *RASGRF2*, had been differentially regulated in our study on impulsive behaviour in rats (see figure 4.6 on page 79 and figure 4.10a on page 84). As outlined in this previous study, impulsivity is a risk factor for alcohol addiction.

This study therefore investigated whether the presence of the minor T allele of SNP26907 influences alcohol consumption by modifying the expression level of *RASGRF2*. To analyse

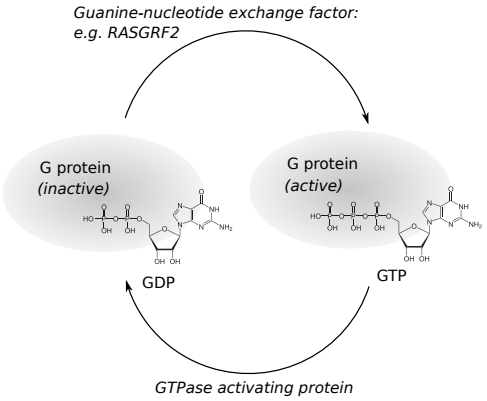


Figure 5.2.: Activation cycle of G proteins. *RASGRF2* acts as an guanine nucleotide exchange factor activating a small GTPase of the RAS family.

this research question, *RASGRF2* mRNA concentration was measured in post-mortem tissue of Brodmann's area 9 by quantiative PCR and related to the allelic variation in SNP26907.

TACR1

In 1931, Ulf von Euler and John H. Gaddum reported the discovery of a substance in rabbit intestine and brain tissue which had the ability to cause slow contractions of the longitudinal muscle of the rabbit's intestines but which was not inhibited by atropine (von Euler and Gaddum, 1931). The causative agent was subsequently named *substance P* with the "P" refering to the fact that it was first isolated in the form of a powder. The substance is a unadecapeptide made up of the following amino acid chain: *Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-*NH_2. The *Phe-X-Gly-Leu-Met-*NH_2 sequence, with *X* standing for an either aliphatic or aromatic amino acid, at the C-terminal region is the characteristic identifying sequence of a whole family of neuropeptides called *tachykinins*, to which substance P belongs.

Substance P is the endogenous ligand for the tachykinin receptor 1 (*TACR1*), which is also named neurokinin 1 receptor (*NK1R*). It's a G protein coupled receptor (Takeda et al., 1991) which acts at least via the following three second-messenger systems: (1) stimulation of phosphatidyl inositol via phospholipase C, (2) mobilisation of arachidonic acid via phospholipase A2 and (3) increase of cAMP levels via stimulation of adenylate cyclase (Quartara and Maggi, 1997). Besides *TACR1*, substance P also binds with a weaker affinity to the two other tachykinin receptors, *TACR2* and *TACR3*.

There is vast evidence linking substance P to the processing and perception of pain information (e.g. Carr and Lipkowski 1990; Otsuka and Yanagisawa 1990) but from the perspective of addiction research it is most notable that substance P seems to enhance glutamate-mediated excitatory neurotransmission via an upregulation of glutamatergic NMDA receptor function (Randić et al., 1990). It was suggested that substance P plays a noticable role in the body's reaction to psychological stress and that pharmacological inhibition of the tachykinin receptor 1 can alleviate anxiety- and depression-related responses

in rodents (Holmes et al., 2003) and in humans (Furmark et al., 2005).

George et al. (2008) provided evidence that pharmacological inhibition of the tachykinin 1 receptor might be a therapy for the treatment of alcohol dependent patients as this inhibition decreased the behavioural sensitivity to stress. In addition, our collaborators from the *National Institute on Alcohol Abuse and Alcoholism* (NIAAA) found a possible link between SNP rs3771807 situated in the intronic region between exon 2 and 3 of the *TACR1* and brain activation in response to alcohol cues (unpublished data).

We therefore hypothesised that, given the implication of tachykinin 1 receptor in behavioural sensitivity to stress and indirectly alcohol addiction, *TACR1* is differentially expressed in the brain of alcohol dependent patients when compared to control subjects and we carefully speculate that the expression levels of TACR1 is also dependent on the genotype of SNP rs3771807 in the TACR1 gene.

To analyse our research question, we performed *TACR1* mRNA concentration measurements by quantitative PCR in the anterior cingulate cortex, Brodmann's area 9, caudate nucleus and ventral striatum of deceased alcoholics and control subjects and related the findings to the disease status. Additional quantitative PCR measurements of *TACR1* as well as genotyping of SNP rs3771807 was carried out in Brodmann area 9 of a larger, non-alcohol related sample set and the association between gene expression and genotype was subsequently analysed.

GRIN3A

Alcohol interacts with a large variety of receptors in the human body. One of the strongest interactions is an inhibition of the N-methyl-D-aspartate (NMDA)-responsive ionotropic glutamate receptors (Spanagel, 2009). This group of glutamate-activated receptors, which is defined by the fact that its members are also activated by the artificial agonist NMDA, are heterotetramers made up of a combination of the subunits presented in table 5.1 (Pachernegg et al., 2012; Andersson et al., 2001).

Table 5.1.: Subunits of the NMDA glutamate receptors

Gene name	Subunit variant	Description
GRIN1	NR1	glutamate receptor, ionotropic NMDA1
GRIN2A	NR2	glutamate receptor, ionotropic NMDA2A
GRIN2B	NR2	glutamate receptor, ionotropic NMDA2B
GRIN2C	NR2	glutamate receptor, ionotropic NMDA2C
GRIN2D	NR2	glutamate receptor, ionotropic NMDA2D
GRIN3A	NR3	glutamate receptor, ionotropic NMDA3A
GRIN3B	NR3	glutamate receptor, ionotropic NMDA3B

The *GRIN2* subunits are responsible for binding the neurotransmitter glutamate while the *GRIN1* subunit binds the co-agonist glycine.

A consequence of alcohol's inhibition of the NMDA receptor mediated signalling is an increased cell-surface density of those receptors in the brain after chronic ethanol exposure (Freund and Anderson, 1996; Qiang et al., 2007). NMDA antagonists have therefore

been proposed as a post-withdrawal treatment for alcohol dependent patients. But clinical trials of neramexane, a noncompetitive NMDA receptor antagonist, did not show any effect (Spanagel and Kiefer, 2008). Tests in rat lines bred for high alcohol consumption showed that neramexane suppresses alcohol consumption in *ALKO Alcohol Accepting* (AA) rats but not in *Marchigian Sardinian alcohol-preferring* (msP) rats (Spanagel, 2009). Microarray-based gene expression profiling in cortical tissue of both rat lines revealed a significant upregulation of *Grin3a* in msP rats compared to AA rats (Björk et al., 2010).

We therefore hypothesised that *GRIN3A* is significantly differentially expressed in the brain of alcohol dependent individuals. *GRIN3A* mRNA concentration measurements were carried out in the anterior cingulate cortex, Brodmann's area 9, caudate nucleus and ventral striatum of deceased alcoholics and control subjects by quantitative PCR and the findings were related to the disease status of the subjects.

5.2. Materials and Methods

The human candidate gene studies were based on tissue samples from two different brain banks. Table 5.2 shows the available brain regions from these two tissue resources.

Table 5.2.: Available post-mortem tissue

Brain region	Human Brain Tissue Bank (HBTB)	New South Wales Tissue Ressource Centre (NSW TRC)
Comparison	suicide *vs.* control	alcoholic *vs.* control
Anterior cingulate cortex	–	✔
Brodmann's area 9	✔	✔
Caudate nucleus	–	✔
Ventral striatum	–	✔

5.2.1. Post-mortem samples from the Human Brain Tissue Bank

Tissue samples were obtained from the *Human Brain Tissue Bank* (HBTB) from the Department of Anatomy, Semmelweis University, Budapest, Hungary (humanbraintissuebank-budapest.sote.hu). The brain bank offers tissue from suicide victims and control subjects who died of various other causes. In addition, the following data are given for each subject: sex, age at time of death, time-span between estimated time of death and the autopsy (post-mortem interval), exact cause of death according to post-mortem examination.

At the beginning of our studies, we possessed 104 samples from Brodmann area 9. Seventy-five samples (45 male/30 female) were from control subjects and 29 (19 male/10 female) from suicides (see table A.1 on page 119 in the annex).

5.2.2. Post-mortem samples from the New South Wales Tissue Resource

Tissue samples were obtained from the New South Wales Tissue Resource Centre (NSW TRC) at the University of Sydney, Australia (sydney.edu.au/medicine/pathology/trc/index.php). The brain bank provides tissue from deceased patients with a history of alcohol dependence and from age-matched control subjects (Sheedy et al., 2008). Alcohol dependent subjects were diagnosed retrospectively using the *Diagnostic Instrument for Brain Studies - Revised (DIBS-R)* which is a post-mortem analysis based on medical records and interviews with persons of reference. The classification criteria are in line with the Diagnostic and Statistical Manual for Mental Disorders, fourth edition (DSM-IV) (Dedova et al., 2009). Similar to the Hungarian Brain Bank, the New South Wales Tissue Resource Centre also provides information on age of death and sex but also on ethnic origin, brain pH, smoking habits, time-span between estimated time of death and autopsy as well as a report on the cause of death and the results of the toxicological analysis.

For our studies, we used tissue from 20 male subjects of European descent consisting of 10 alcohol dependent and 10 control cases. All alcohol dependent subjects had consumed more

than 80 g of alcohol per day while the control cases had all a daily consumption below 20 g. Except from one suicide in the control group, all subjects died of non-psychiatric medical conditions. The post-mortem interval of all selected subjects was below 36 hours and no blood alcohol or significant amounts (concentration > 1.0 mg/l) of psychiatric medication had been reported (see table A.2 on page 123 in the annex).

5.2.3. DNA isolation and purification

Brain samples from the Human Brain Tissue Bank (HBTB) were used to relate the genotype of SNPs lying in the vicinity of *AUTS2*, *RASGRF2* and *TACR1* to the gene expression of these genes.

For DNA extraction, small pieces of the brain samples, corresponding roughtly to 25 mg of tissue, were cut off with a scalpel at an ambient temperature of $-20\,^{\circ}$C in a Leica CM 3000 Cryostat (Leica, Wetzlar, Germany). DNA was then extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

5.2.4. Single nucleotide-polymorphism genotyping

For the *TACR1* study, the genotype of SNP rs3771807 was detected by quantitative PCR using the TaqMan® Universal PCR Master Mix and the TaqMan® SNP Genotyping Assay with the product number '*C___2974562_10*' (both: Life Technologies, Darmstadt, Germany) consisting of a pair of primers and two differentially labelled fluorescent probes which bind to either of the two variants of the investigated SNP. Master mix, genotyping assay and the samples were mixed in a total volume of 25 µl according to the manufacturer's protocol. The genotyping PCR was performed on an Applied Biosystems 7900 HT Real-time PCR system (Life Technologies) using the following temperature profile: 10 min at $95\,^{\circ}$C followed by 40 cycles of 15 sec dissociation at $92\,^{\circ}$C and one minute annealing and elongation at $60\,^{\circ}$C. Genotypes were called by *Applied Biosystems' Sequence Detection System (SDS) Software Version 2.2.2.*

The genotyping of SNP rs6934555 in the vicinity of *AUTS2* and rs26907 in the vicinity of *RASGRF2* was done by our collaborators at the Institute of Psychiatry, King's College London (London, UK).

5.2.5. RNA isolation and purification

Small pieces of the brain samples, corresponding to roughly 50 mg of tissue, were cut off with a scalpel at an ambient temperature of $-20\,^{\circ}$C in a Leica CM 3000 Cryostat (Leica, Wetzlar, Germany). Samples were kept at $-80\,^{\circ}$C until further use when the tissue samples were homogenised by repeated trituration in 1 ml of TRIzol® Reagent (Life Technologies, Darmstadt, Germany) using first a 20 gauge and then a 22 gauge needle. Samples were subsequently filled up with 200 µl of chloroform, mixed and centrifuged to separate the RNA containing aqueous upper phase from the organic lower phase. The upper phases were carefully collected and purified with an RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany).

The concentration of the RNAs was determined with a Nanodrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany). RNA quality was assessed on the basis of the 260 nm to 280 nm absorption ratio and further analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

For the Human Brain Tissue Bank collection, only RNA samples with a RNA integrity number (RIN) above 6.5 were taken into account and the RIN values were included as a co-factor in the statistical analysis. In the case of the New South Wales Tissue Resource Centre collection, all RNA samples had RIN values above six and were therefore included in the quantitative PCR analysis.

5.2.6. Quantitative PCRs

The messenger RNA concentration of all four candidate genes (*AUTS2*, *RASGRF2*, *TACR1* and *GRIN3A*) was assessed by quantitative PCR.

Experimental procedures

For the HBTB samples, a DNase treatment was performed before the reverse transcription to reduce the contamination of the RNA samples with DNA. 1.5 µg of each RNA sample was treated with RQ1 RNase-free DNase (Promega, Mannheim, Germany) according to the manufacturer's recommendations. 1.0 µl of the 10 µl reaction volume were subsequently used for reverse transcription which was performed using the High Capacity RNA-to-cDNA Kit (Life Technologies, Darmstadt, Germany) following the manufacturer's protocol. For the NSW-TRC samples it was not necessary to perform a DNase digestion step as the primers had been specifically designed to fit only to messenger RNA molecules after the splicing-out of the introns. Therefore, 100 ng of each NSW-TRC RNA sample were directly transcribed using the High Capacity RNA-to-cDNA Kit. All cDNA samples from both tissue collections were diluted 1:5.

The expression levels of the candidate genes were measured on an Applied Biosystems 7900 HT Real-time PCR system (Life Technologies) using the Power SYBR® Green PCR Master Mix (Life Technologies). Reactions were performed in a total volume of 20 µl with 5 µl diluted cDNA and a final concentration of 400 nmol/l of both the forward and the reverse primer. *GAPDH* was used as a housekeeping gene and run in duplicates while all candidate genes were tested in triplicates. The PCRs of the *AUTS2* and *RASGRF2* assays were performed with the following temperature profile: 15 min at 95 °C followed by 40 cycles of 30 sec at 95 °C and 30 sec annealing and elongation at 60 °C. The PCRs of the *GRIN3A* and the *TACR1* assays were performed with the following temperature profile: 10 min at 95 °C followed by 40 cycles of 15 sec dissociation at 95 °C and one minute annealing and elongation at 60 °C. Melting curve analysis was performed after each run to check for unwanted PCR side products. Data were normalised to the endogenous control by subtraction. Primer sequences of the candidate genes and GAPDH are presented in table 5.3.

Table 5.3.: Primer sequences of the genes of interest

Gene	Primer forward	Primer reverse
AUTS2	CGAGAAAATGACCGCAATCT	GCTGTTCTGTCCTGGCTTGA
GAPDH*	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT
GRIN3A	TCATGTGGCCACTCCACTGGACA	TCGCCCTTGGGAGTCAAACCAA
RASGRF2	GCTTGTATACCTCTCTCTCCTGGT	ACGCCCATTTTCCAGGTA
TACR1	TCAATGACAGGTTCCGTCTGGGCTT	ATAGTCGCCGGCGCTGATGA

*) Reference (= housekeeping) gene

Normalisation

Applied Biosystems' Sequence Detection System (SDS) Software Version 2.2.2. was used to analyse the SYBR green fluorescence levels that had been registered during the PCR run. Cycle of threshold (Ct) values were calculated for each well as the theoretical number of cycles when a defined fluorescence threshold was reached. Duplicates (housekeeping gene) or triplicates (candidate genes) were averaged by arithmetic mean. Reactions were normalised to the housekeeping gene by calculation of the ΔCt value which was defined as:

$$\Delta Ct = Ct_{candidate\ gene} - Ct_{housekeeping\ gene}$$

5.2.7. Statistical analyses

All data were analysed with R statistical programming language version 2.14.2.

Analysis of Human Brain Tissue Bank samples

In the case of the quantitative PCR data acquired from the Human Brain Tissue Bank samples, the influence of potentially confounding co-factors was preliminarily assessed by Student's t-tests for categorical variables and Pearson correlation tests for continuous variables and subsets of the original data sets were taken where necessary.

As the residuals of the ΔCt values showed a substantial deviation from a normal distribution, we quantile transformed the data to a normally distributed set of random data with $\mu = 0$ and $\sigma = 1$ (Bolstad et al., 2003; Peng et al., 2007). The transformed data were then analysed with a general linear model with the quantile normalised ΔCt values as the dependent variable and the gene dose of the investigated SNP as the predictor variable. Depending on the preliminary t-tests and the sample subset further potentially confounding predictor variables were included in the models (see result section for details).

The statistical analysis of the *AUTS2* and *RASGRF2* data was originally done by our collaborator Anbarasu Lourdusamy (Institute of Psychiatry, King's College London, London, UK) and his version was used for the publication of the results in Schumann et al. (2011) and Stacey et al. (2012). I reproduced these analyses for this thesis obtaining the same outcome which is described in the results section.

Analysis of New South Wales Tissue Resource Centre samples

In contrast to the Human Brain Tissue Bank which had provided us with tissue of one brain region from 104 subjects, the New South Wales Tissue Resource Centre had given tissue of four brain regions but only from 20 subjects (10 diseased/10 control) which had been selected with the intent to minimise the number of confounding factors. Taken this different prerequisites into account, we analysed the New South Wales Tissue Resource Centre samples with Student's t-tests.

5.3. Results

In the context of our post-mortem gene expression study we measured *AUTS2*, *RASGRF2*, *TACR1* and *GRIN3A* mRNA in our brain banks (see table 5.4).

Table 5.4.: Overview of qPCR experiments in human samples

Candidate gene	Human Brain Tissue Bank (HBTB)	New South Wales Tissue Ressource Centre (NSW TRC)
Brain region	BA9	ACC, BA9, CAU, VST
AUTS2	✔	–
RASGRF2	✔	–
TACR1	✔	✔
GRIN3A	–	✔

ACC = anterior cingulate cortex, BA9 = Brodmann's area 9,
CAU = caudate nucleus, VST = ventral striatum

5.3.1. AUTS2

AUTS2 mRNA was quantified by quantitative PCR in tissue from Brodmann's area 9. Table 5.5 shows the average of the Ct values for *AUTS2* and the reference gene *GAPDH* as well as for the normalised ΔCt values (=*AUTS2–GAPDH*) for each allelic combination at rs6934555.

Table 5.5.: *AUTS2* gene expression:
mean Ct value ± standard deviation for the three genotypes of rs6934555

Genotype	Count	*AUTS2*	*GAPDH*	*AUTS2–GAPDH*
AA	57	25.78 ± 0.91	19.03 ± 0.98	6.75 ± 0.82
AT	33	25.79 ± 1.04	19.21 ± 1.44	6.59 ± 0.93
TT	6	25.50 ± 1.24	19.30 ± 1.96	6.20 ± 1.00

The data were then quantile transformed to allow for the statistical analysis of the influence of the genotype on *AUTS2* gene expression with a general linear model. The following variables were included in the model: rs6934555 genotype, sex, age, post-mortem interval and RNA integrity number. Genetic ancestry (p-value = 0.819) and cause of death (suicide versus control; p-value = 0.773) had no influence on *AUTS2* gene expression and were not included in the regression analysis. The statistical analysis showed that the minor A allele of SNP rs6943555 led to a significant increase in *AUTS2* mRNA concentration (p-value = 0.023). RNA integrity also affected the *AUTS2* mRNA levels significantly. This is expected as the amount of detectable mRNA decreases with increased sample degradation. See table 5.6 and figure 5.3 for details.

Table 5.6.: Influence of predictor variables on *AUTS2* gene expression

Variable	Estimate	Std. Error	p-Value
Gene dose	-0.326	0.140	0.023 *
Sex(male)	0.112	0.185	0.548
Age	-0.006	0.005	0.294
PMI[1)]	-0.058	0.035	0.103
RIN[2)]	0.369	0.127	0.005 **

* < 0.05, ** < 0.01

[1)] PMI = post-mortem interval

[2)] RIN = RNA integrity number

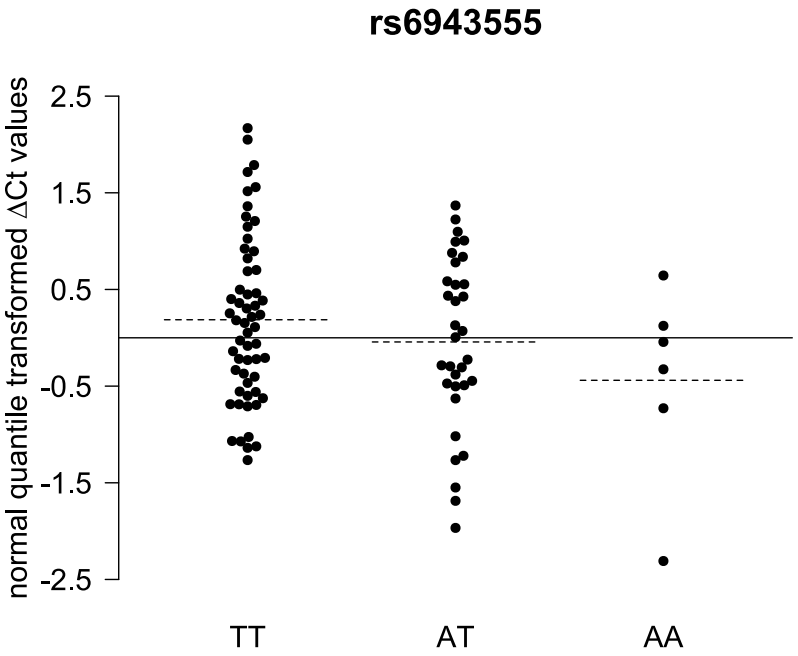


Figure 5.3.: Genotype-specific *AUTS2* messenger RNA levels in post-mortem human prefrontal cortex tissue (Brodmann Area 9). Lower normalised ΔCt values indicate higher expression levels. Dotted horizontal lines indicate group averages.

5.3.2. RASGRF2

RASGRF2 mRNA was quantified by quantitative PCR in tissue from Brodmann's area 9. Table 5.7 shows the average of the Ct values for *RASGRF2* and the reference gene *GAPDH* as well as for the normalised ΔCt values (=*RASGRF2–GAPDH*) for each allelic combination at rs26907.

Table 5.7.: *RASGRF2* gene expression:
mean Ct value ± standard deviation for the three genotypes of rs26907

Genotype	Count	*RASGRF2*	*GAPDH*	*RASGRF2–GAPDH*
AA	3	29.27 ± 1.33	20.72 ± 2.56	8.55 ± 1.43
AG	24	26.94 ± 1.05	19.08 ± 1.06	7.86 ± 0.57
GG	69	26.97 ± 1.18	19.05 ± 1.17	7.93 ± 0.79

Like in the case of *AUTS2*, data were then quantile transformed to allow for the statistical analysis of the influence of the genotype on gene expression with a general linear model. The analysis showed an almost significant influence of cause of death (suicide versus control; p-value = 0.083) on *RASGRF2* gene expression. As we detected an additional significant influence of sex (p-value = 0.019), we restricted the regression analysis to the male control samples. A linear model of *RASGRF2* gene expression based on rs26907 genotype, age, post-mortem interval and RNA integrity number showed no influence of the genotype on gene expression (p-value = 0.859). Genetic ancestry had no influence on *RASGRF2* gene expression (p-value = 0.660) and was not included in the analysis. See table 5.8 for details.

Table 5.8.: Influence of predictor variables on *RASGRF2* gene expression

Variable	Estimate	Std. Error	p-Value
Gene dose	-0.048	0.267	0.859
Age	-0.007	0.012	0.564
PMI[1)]	-0.031	0.083	0.706
RIN[2)]	0.075	0.230	0.745

[1)] PMI = post-mortem interval
[2)] RIN = RNA integrity number

5.3.3. TACR1

TACR1 mRNA was quantified in post-mortem tissue samples of alcohol addicted patients and control subjects. We analysed the expression of this gene in the anterior cingulate cortex, Brodmann's area 9, the caudate nucleus and the ventral striatum. Table 5.9 shows the Ct values for *TACR1*, the reference gene *GAPDH* and the normalised ΔCt values (=*TACR1–GAPDH*) of each brain region for the ten control and the ten alcohol addicted subjects.

Only the anterior cingulate cortex had a significantly differential expression of *TACR1*: gene expression was up-regulated in this brain region in the alcohol-dependent patients

Table 5.9.: *TACR1* gene expression:
mean Ct value ± standard deviation for alcoholics and control subjects

Brain region	Disease status	*TACR1*	*GAPDH*	*TACR1–GAPDH*	p-Value
ACC	control	29.76 ± 1.72	19.13 ± 1.51	10.63 ± 1.14	0.017 *
	alcohol	29.09 ± 1.09	20.75 ± 2.24	8.34 ± 2.51	
Ba9	control	28.26 ± 0.38	19.02 ± 0.19	9.24 ± 0.27	0.348
	alcohol	28.81 ± 0.86	19.43 ± 0.84	9.38 ± 0.41	
CAU	control	26.80 ± 0.55	19.45 ± 0.33	7.35 ± 0.48	0.589
	alcohol	27.14 ± 0.99	19.68 ± 0.88	7.47 ± 0.49	
VSt	control	26.62 ± 0.81	19.11 ± 0.58	7.51 ± 0.54	0.543
	alcohol	27.27 ± 1.07	19.56 ± 0.92	7.71 ± 0.83	

ACC = anterior cingulate cortex, Ba9 = Brodmann's area9,
CAU = caudate nucleus, VSt = ventral striatum, * < 0.05

(Student's t-test: p-value = 0.017). The expression values are depicted in figure 5.4.

TACR1 mRNA was also quantified in the collection of Brodmann's area 9 post-mortem samples from the Human Brain Tissue Bank in Budapest. A preliminary assessment with Student's t-tests showed an almost significant influence of cause of death (suicide versus control; p-value = 0.062) on *TACR1* gene expression. We therefore restricted the regression analysis to the control samples. Table 5.10 shows the average of the Ct values for *TACR1* and the reference gene *GAPDH* as well as for the normalised ΔCt values (=*TACR1–GAPDH*) in the control samples for each allele combination at rs3771807.

Table 5.10.: *TACR1* gene expression in the control samples:
mean Ct value ± standard deviation for the three genotypes of rs3771807

Genotype	Count	*TACR1*	*GAPDH*	*TACR1–GAPDH*
CC	47	31.50 ± 1.11	20.91 ± 1.23	10.59 ± 0.60
CG	21	31.31 ± 1.02	20.93 ± 1.21	10.38 ± 0.68
GG	0	–	–	–

Like in the case of *AUTS2* and *RASGRF2*, data were then quantile transformed to allow for the statistical analysis of the influence of the genotype on gene expression with a general linear model. The model relating *TACR1* gene expression to the rs3771807 genotype, age, post-mortem interval, RNA integrity number and ancestry showed no influence of genotype on gene expression (p-value = 0.681). Ancestry had been included in the model as a stand-alone t-test had shown an almost significant influence (p-value = 0.081). This effect was no longer present in the model (p-value = 0.219). Sex had no influence and was not included in the analysis. See table 5.11 for details.

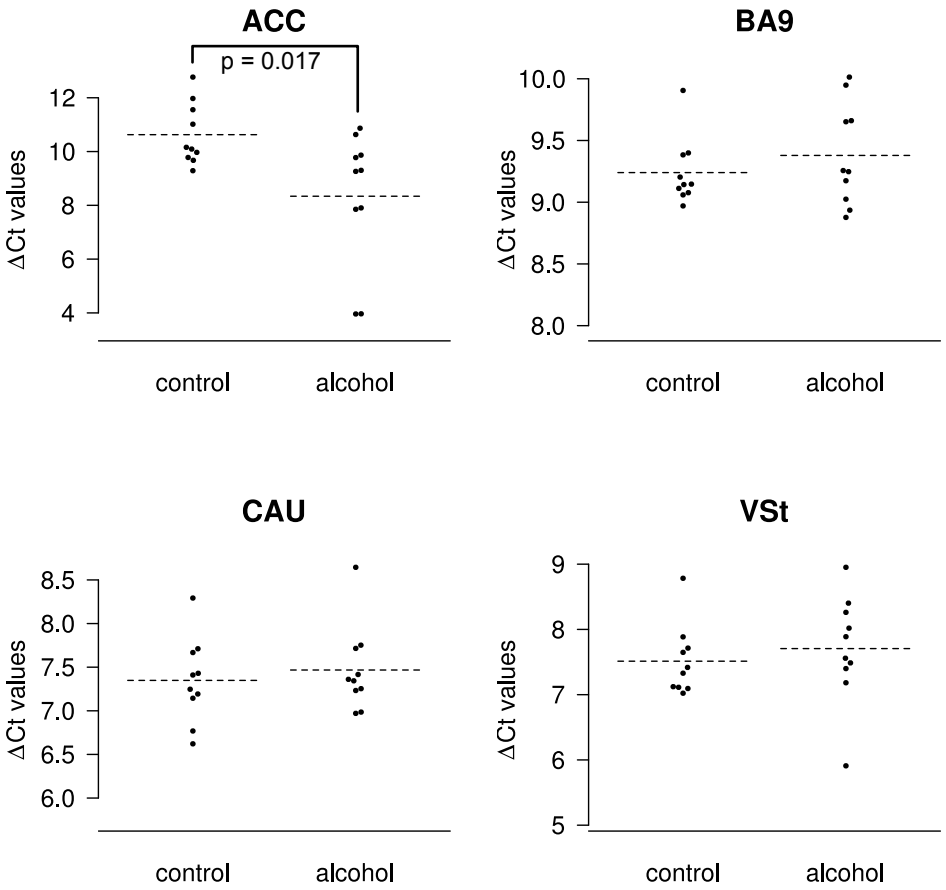


Figure 5.4.: *TACR1* gene expression in post-mortem tissue samples of alcohol dependent patients and control subjects. ACC = anterior cingulate cortex, BA9 = Brodmann's area 9, CAU = caudate putamen, VSt = ventral striatum. Lower normalised ΔCt values indicate higher expression levels. Dotted horizontal lines indicate group averages.

Table 5.11.: Influence of predictor variables on *TACR1* gene expression

Variable	Estimate	Std. Error	p-Value
Gene dose	0.105	0.254	0.681
Age	-0.013	0.008	0.114
PMI[1)]	0.081	0.063	0.203
RIN[2)]	0.012	0.185	0.949
ANCESTRY	-0.690	0.554	0.219

[1)] PMI = post-mortem interval

[2)] RIN = RNA integrity number

5.3.4. GRIN3A

We also assessed *GRIN3A* gene expression in the post-mortem tissue samples of alcohol addicted patients and control subjects. Table 5.12 shows the Ct values for *GRIN3A*, the reference gene *GAPDH* and the normalised ΔCt values (=*GRIN3A–GAPDH*) of each brain region for the ten control and the ten alcohol addicted subjects.

Table 5.12.: *GRIN3A* gene expression:
mean Ct value ± standard deviation for alcoholics and control subjects

Brain region	Disease status	*GRIN3A*	*GAPDH*	*GRIN3A–GAPDH*	p-Value
ACC	control	30.35 ± 3.76	19.13 ± 1.51	11.22 ± 2.44	0.052 $^{+}$
	alcohol	29.49 ± 2.42	20.75 ± 2.24	8.74 ± 2.86	
Ba9	control	26.95 ± 0.47	19.02 ± 0.19	7.92 ± 0.42	0.024 *
	alcohol	28.04 ± 1.46	19.43 ± 0.84	8.62 ± 0.78	
CAU	control	31.68 ± 0.57	19.45 ± 0.33	12.22 ± 0.61	0.770
	alcohol	31.81 ± 1.51	19.68 ± 0.88	12.13 ± 0.78	
VSt	control	30.52 ± 1.72	19.11 ± 0.58	11.41 ± 1.81	0.772
	alcohol	30.72 ± 2.58	19.56 ± 0.92	11.16 ± 2.10	

ACC = anterior cingulate cortex, Ba9 = Brodmann's area9,
CAU = caudate nucleus, VSt = ventral striatum, $^{+} < 0.10$, * < 0.05

The analysis revealed a significant down-regulation of *GRIN3A* in Brodmann's area 9 of alcohol addicted patients and an almost significant up-regulation in the anterior cingulate cortex of those patients. See also figure 5.5.

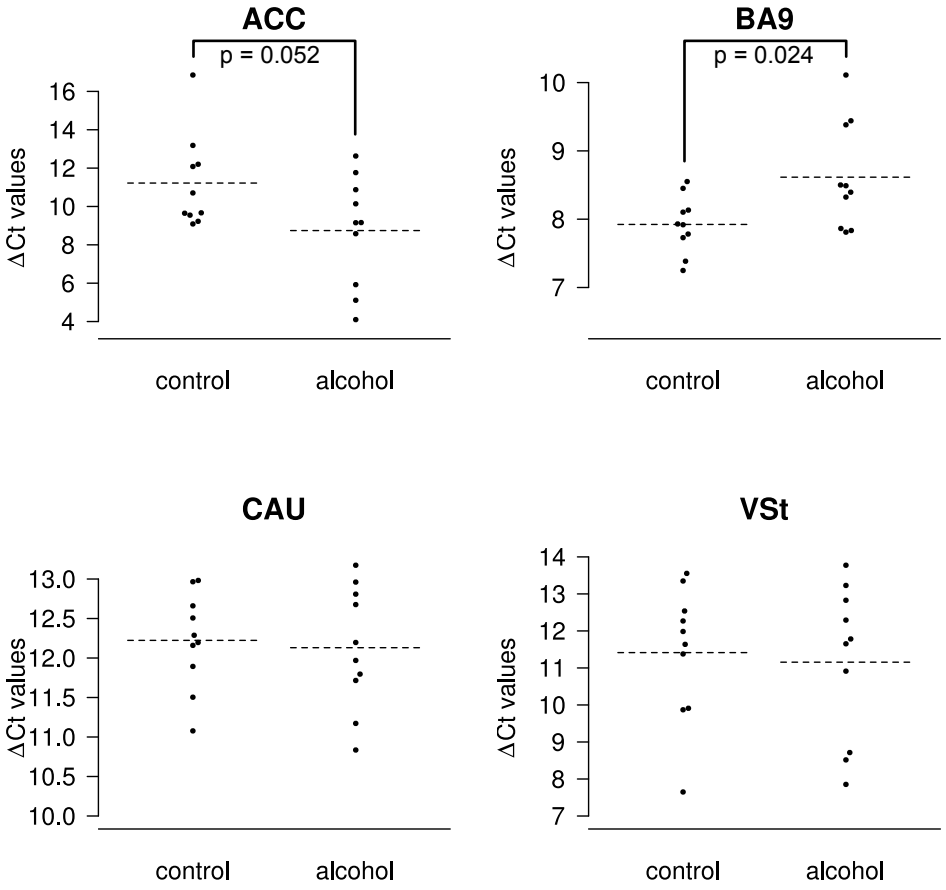


Figure 5.5.: *GRIN3A* gene expression in post-mortem tissue samples of alcohol dependent patients and control subjects. ACC = anterior cingulate cortex, BA9 = Brodmann's area 9, CAU = caudate putamen, VSt = ventral striatum. Lower normalised ΔCt values indicate higher expression levels. Dotted horizontal lines indicate group averages.

5.4. Discussion of candidate gene studies

5.4.1. AUTS2

Our quantitative PCR analysis showed that *AUTS2* gene expression in the dorsal prefrontal cortex is influenced by the genotype of SNP rs6943555, which lies in the fourth intron of the AUTS2 gene. The minor A allele correlated with a decreased *AUTS2* gene expression. Schumann et al. (2011) showed in a genome wide association study meta-analysis that the minor A allele is associated with lower alcohol consumption. We thus speculate, that this allele lies in a sequence which acts as a cis-regulatory element for *AUTS2* gene expression and that the lower concentration of *AUTS2* modifies human behaviour in a way that less alcohol is consumed.

This hypothesis is corroborated by a meta-analysis of gene expression profiles in mouse models with well-established differences in voluntary alcohol consumption rats. *AUTS2* showed significant gene expression differences in whole brain extracts from the different mouse models. Another study showed that downregulation of the *AUTS2* homologous gene *tay* in *Drosophila melanogaster* led to reduced alcohol sensitivity (both studies: Schumann et al., 2011).

Taken together, these results suggest that *AUTS2* is implicated in alcohol use and abuse. The next step in the analysis of the relationship of *AUTS2* to alcohol dependence is a characterisation of the gene function of *AUTS2*. Bedogni et al. (2010) used web-based tools to characterise the protein: *AUTS2* is probably not secreted nor integrated in the cell membrane as no signal peptide was detected. But its N-terminus contains several nuclear localisation sequences (NLS) suggesting that *AUTS2* is a nuclear protein. The authors managed to confirm this localisation by antibody staining.

A possible next step in the characterisation of the *AUTS2* function would be x-ray cristallography to determine the exact form of the mature protein. This might allow to deduce the molecular function from its functional groups. Another option would be the application of two-hybrid assays to determine the molecular partners of this protein.

5.4.2. RASGRF2

The statistical analysis of our quantitative PCR experiment did not reveal a rs29607 genotype-specific regulation of *RASGRF2* in the dorsal prefrontal cortex. We had hypothesised that the known effect of rs26907 on alcohol consumption (Schumann et al., 2011) is mediated by a cis regulatory mechanism targeting the near-by RASGRF2 promoter. Our results do not corroborate this hypothesis.

In contrast to our findings in human brain tissue, *RASGRF2* mRNA levels in total brain extracts from high alcohol-preferring (HAP1) lines were shown to be higher than those of low alcohol-preferring (LAP1) mouse lines (Mulligan et al., 2006; Stacey et al., 2012). Given the technical constraints of human post-mortem tissue, which were discussed in the introduction, we cannot rule out that we missed an existing difference in *RASGRF2* expression due to experimental limitations. It is also possible that a differential expression exists in another not-investigated brain region. Consultation of our own microarray

databases, which are based on rodent models of alcohol consumption, did not reveal a differential regulation of *RASGRF2* upon alcohol exposure, thus supporting our finding in humans.

Stacey et al. (2012) also reported a reduced excitability of dopaminergic neurons in the ventral tegmental area in *RASGRF2*$^{-/-}$ mice. This effect correlated with a pronounced reduction in the alcohol consumption of the animals. The authors speculated that this mechanism is dependent on a disregulation of the ERK pathway as pharmacological inhibition of this pathways mimicked the alterations in dopaminergic neuron excitability which had been observed in the knock-out animals. In addition, the authors showed that a haplotype block surrounding rs26907 was significantly associated with an elevated BOLD signal in the ventral striatum during reward anticipation in adolescents. The fact that the same haplotype was associated with increased alcohol consumption in teenagers confirmed our hypothesis that the genomic area around rs26907 is associated with alcohol consumption.

Our research thus suggests that while there is ample evidence for an implication of SNP rs29607 and the surrounding gene *RASGRF2* in addiction, the mechanism of action is not dependent on altered gene expression levels of this protein in Brodmann's area 9.

As this is partly in disagreement with findings from animal studies it would be interesting to repeat our measurements in another human brain database to obtain more certainty. The already existing *RASGRF2*$^{-/-}$ mouse model could be used to measure phosphorylated versus non-phosphorylated ERK proteins to investigate non-transcription related signalling events in the presence and absence of *RASGRF2*.

5.4.3. TACR1

We could partly confirm our hypothesis that alcohol addiction modifies substance P signalling via a differential expression of *TACR1* but we did not find any correlation between *TACR1* gene expression in Brodmann area 9 and the SNP rs3771807. While Brodmann's area 9, caudate nucleus and ventral striatum showed no signs of differential regulation of *TACR1* we found a significant up-regulation of this gene in the anterior cingulate cortex of alcohol dependent patients. This brain region is crucial for such tasks as emotional self-control and motivation (Allman et al., 2001) which could suffer from an increased substance P driven stress level which is indicated by the elevated *TACR1* receptor expression. The lack of differential expression in the ventral striatum suggests that alcohol-induced modification of substance P signalling and alcohol-related alterations in this area do not interact with each other.

Our findings thus corroborate the results of previous studies. Seneviratne et al. (2009) reported that binding of an antagonist to the *TACR1* receptor reduced alcohol self-administration in mice and craving for alcohol in humans. And Baek et al. (2010) showed that miRNA-mediated down-regulation of the *TACR1* receptor reduced alcohol consumption in mice. The fact that we did not find a genotype-specific *TACR1* expression does not corroborate the hypothesis of our collaborators at the *National Institute on Alcohol Abuse and Alcoholism* (NIAAA) that the SNP rs3771807 might be linked to brain activation in response to alcohol cues via a modulation of *TACR1* gene expression.

Our results thus refine previous findings by indicating that only the anterior cingulate cortex but not striatal or frontal cortex regions are affected by an interaction between alcohol and the *TACR1* receptor.

These results suggest that pharmacological inhibition of the tachykinin receptor might be a target for the treatment of alcohol dependent patients. It would be therefore of interest to analyse the effect of *TACR1* inhibitors on drinking levels in rodent models of alcohol consumption.

5.4.4. GRIN3A

We could show that *GRIN3A* is significantly down-regulated in the dorsal prefrontal cortex and almost significantly up-regulated in the anterior cingulate of alcohol dependent patients. Expression levels in the caudate nucleus and the ventral striatum did not show signs of alteration. So we can partially confirm our hypothesis that a chronic alcohol exposure modifies *GRIN3A* expression.

The up-regulation of *GRIN3A* in the anterior cingulate cortex upon chronic alcohol consumption can be interpreted as a compensation: The cells of this region compensate the dampening effect of alcohol on the NMDA receptors by over-production of this receptor subunit (Qiang et al., 2007). The fact that such an effect cannot be seen in the ventrial striatum could be explained by the relative low level of glutamate signalling in this brain structure (Lape and Dani, 2004). In line with this hypothesis, we also found comparably higher *GRIN3A* Ct values, signifying a lower *GRIN3A* mRNA concentration, in this structure in comparison to the other brain regions. The significant down-regulation of *GRIN3A* in the dorsal prefrontal cortex cannot be explained by the compensation for reduced NMDA receptor signalling efficacy and we can only speculate if this is due to an altered NMDA receptor subunit composition in this brain area. The only comparable human analysis is from Mueller and Meador-Woodruff (2004) who showed that *GRIN3A* is strongly up-regulated in the dorsal prefrontal cortex of psychiatric patients suffering from schizophrenia. It is thus interesting to compare our results to studies from rodent models of alcohol consumption which were treated with the NMDA receptor antagonist *neramexane*: lower expression levels of *GRIN3A* were observed in cortical tissue of rats from the *ALKO Alcohol Accepting* (AA) strain which is susceptible to *neramexane* treatment compared to the non-responsive *Marchigian Sardinian alcohol-preferring* (msP) rat strain (Spanagel, 2009; Björk et al., 2010). This might suggest that low cortical *GRIN3A* levels are favourable for a *neramexane* based therapy of alcohol abuse.

Our study therefore delivered supporting evidence for a modulation of *GRIN3A* gene expression by alcohol addiction and led to a more precise characterisation of these alterations.

But the down-regulation of *GRIN3A* in the dorsal prefrontal cortex of alcohol dependent patients demands further investigation. It would be therefore of great interest to measure the other NMDA receptor subunits in post-mortem tissue of alcohol addicted and control subject.

6. Overall conclusion

This thesis consisted of three microarray studies and four candidate gene expression measurements in the context of alcohol addiction and its risk factors.

The first study analysed how alcohol consumption changed the daily course of gene expression in the nucleus accumbens of short-term and long-term drinking rats. The study was based on overall gene expression measurements by microarray technology and its main conclusion was that the data did not support a specific disruption of circadian gene expression by alcohol.

The second study, also based on a microarray experiment, established diurnal gene expression curves in the blood of healthy human volunteers. As in the first study, the focus was on genes with circadian oscillating expression levels. The obtained expression profiles can now serve as a baseline for future studies assessing the impact of chronic alcohol abuse on circadian gene expression in human blood.

The third study, again based on a microarray experiment, had focused on gene expression differences in the prefrontal cortex and nucleus accumbens of rats selectively breed for high and low levels of impulsivity. We discovered several candidate genes, notably *P2ry12*, *Frzb* and *Gprc5b*, which were significantly associated with the degree of impulsivity in the rats. These candidate genes can now be further validated by screening the degree of impulsivity in rodents where the genes have been silenced.

In the forth study, we analysed the expression levels of *AUTS2*, *GRIN3A*, *RASGRF2* and *TACR1* in human post-mortem brain tissue by quantitative PCR. We found significant expression differences in *AUTS2* related to the single nucleotide polymorphism (SNP) rs6943555 which had been associated with alcoholism in a genome-wide association study. No such differences were found for *RASGRF2* related to SNP rs26907 which had also come up in the same genome-wide association study. *TACR1* was significantly up-regulated in the anterior cingulate cortex of alcohol-dependent patients but its expression was not linked to SNP rs3771807 which had also been associated with alcoholism. *GRIN3A* was significantly down-regulated in the dorsal prefrontal cortex of alcohol-dependent patients.

6.1. Study limitations

All subprojects of this thesis were based on the measurement of messenger RNA concentrations. The focus was either on preselected candidate genes or on a hypothesis-free screening of genome-wide gene expression. During this studies, we faced a number of limiting factors which are typical for gene expression studies in psychiatric research.

In general, we found only small to moderate changes in gene expression resulting in weak p-values and small fold changes between the different conditions. This is not surprising as

psychiatric disorders are associated with subtle changes in neuronal function which become evident in slightly altered expression of genes involved in neurotransmitter production, secretion and reception, synaptic function and cellular signal processing.

6.1.1. Microarray studies

Our microarray findings can therefore not be expected to show the same level of differential expression as it is the case in studies on cancer (Zhang et al., 2011) or neurological disorders such as Parkinson's disease (Taccioli et al., 2011). The meaningfulness of our results was also curtailed by the fact that the messenger RNAs were isolated from brain tissue samples consisting of multiple cell-types. If voluntary alcohol consumption increases for example the expression of a gene of interest in medium-spiny neurons of the nucleus accumbens, we might have been unable to detect this effect as the signal could have been blurred by the gene's messenger RNA from other neurons and glia cells.

The weak p-values and limited fold change ratios in psychiatric gene expression studies also aggravate the multiple testing problem. As the expression levels of thousands of genes are compared between two or more treatment conditions, it becomes difficult to distinguish true biological differences from findings due to random chance. A number of methods have been proposed to reduce the number of false positives such as the *Bonferroni correction* (Abdi, 2007) or the *false discovery rate* (FDR) (Benjamini and Hochberg, 1995) procedure but these methods would have eliminated all findings in our microarray data sets. We therefore used an uncorrected threshold of statistical significance of 0.05 combined with an cut-off value for the absolute fold change of 1.2 as an exploratory approach. These values were weaker than the recommended standards of the DAVID bioinformatics project (Huang da et al., 2009a).

Remarkably, the microarray study on impulsive rats led to more pronounced expression differences than the study on alcohol drinking rats (compare figure 4.4 on page 77 with figure 2.12 on page 34). This is surprising as the animals in the impulsivity study did not undergo a pharmacological challenge. A possible explanation is that the selective breeding of the high and low impulsivity rats had a much higher impact on gene expression than the voluntary consumption of alcohol in an inbred rat strain. We can also not rule out that the *Affymetrix* chips used for the impulsivity study had a higher detection efficacy than the *illumina* chips used for the alcohol study.

6.1.2. Candidate gene studies in post-mortem tissue

The candidate gene studies, on the other hand, were not so dramatically affected by the multiple testing problem. Nonetheless, the validity of our gene expression measurements in the post-mortem tissues was also subject to several limitations. As in the microarray studies we had used brain region-specific but not cell type-specific tissue samples. Gene expression can also be heavily influenced by the way of death which means that post-mortem samples could reveal as much about the way of dying of an individual as about his way of living (Tomita et al., 2004). Unlike in studies on animal models, there is no experimental control of confounding factors such as age, sex, comorbidities, diet, life

style and post-mortem interval. While statistical models can help to reduce this problem, they fail to include factors for which data are not available. And last but not least, the neurological foundation of alcohol addiction are likely to vary from one patient to another given the multifarious progress forms of this psychiatric disorder.

6.2. Conclusions for further studies

Despite of these limitations, we had been able to identify new candidate genes and detect significant expression differences in human post-mortem tissues in the subprojects. In addition, several recommendations for future gene expression studies in psychiatric research can be made based on this thesis. As discussed above, the currently available microarray technology is plagued by a variety of issues including the multiple testing problem, the vast amount of probes lacking biological significant annotation and a probe design which reflects an outdated state of knowledge concerning the rodent genome. It is therefore worth considering to abandon the whole genome gene expression measurement approach in favour of testing smaller, previously selected sets of genes. This could be done using techniques such as SYBR Green or TaqMan PCR arrays or RNA *in situ* hybridisation assays which all allow for the parallel testing of up to several hundred genes (Spurgeon et al., 2008; Arikawa et al., 2008; Itzkovitz and van Oudenaarden, 2011).

Using cell type-specific RNA samples for gene expression measurements would significantly increase the biological conclusiveness of the studies. Laser capture microdissection might be an interesting method to achieve this goal (McCullumsmith and Meador-Woodruff, 2011; Pietersen et al., 2011).

6.3. Outlook

While this work dealt with the neurobiological aspects of alcohol addiction, it is important to stay aware of the multifariousness of this disorder. It is an intricate mesh of childhood difficulties, social surroundings, economic background and psychological factors which together with a genetic susceptibility drive individuals into addiction.

Successful treatment of alcoholism will need to take all of these factors into account. Obviously, pharmaceutical intervention with anti-craving drugs has the potential to be a critical component of such a therapy regime and it is the task of psychopharmacological research to pave the way for the development of the appropriate drugs.

In this context, the present work added to the general understanding of the molecular underpinnings of alcohol addiction and showed that neuronal gene expression remains an important field of discovery in psychiatric research. Its scientific outcome as well as the proposed follow-up studies will hopefully contribute its share to the development of more efficient treatment options for alcohol addicts.

A. Appendix - Human brain tissue banks

Table A.1.: Annotation of brains from the **Human Brain Tissue Bank, Budapest**

Brain ID	Classification	Sex	Age (years)	PMI[1] (hours)	Cause of death and other noteworthy medical conditions
1	control	male	63	2	cardiac insufficiency, coronary sclerosis, earlier myocardial infarction
10	control	female	42	2	cardiac insufficiency, coronary sclerosis, chronic myocardial infarction
11	control	male	47	2	acute cardiac insufficiency, chronic myocardial infarction
12	control	male	80	2	acute cardiac insufficiency, senile, hypertensive arteriosclerosis
19	control	male	56	2	acute myocardial infarction
44	control	female	74	6	cardiorespiratory insufficiency
47	control	female	64	2	heart failure
48	suicide	female	72	4	drug overdose
49	control	male	52	4	acute myocardial infarction
50	control	female	33	1	acute myocardial infarction
51	control	female	60	3	acute cardiac insufficiency
52	suicide	male	45	4	drug overdose
53	suicide	male	48	3	drug overdose, alcohol
54	suicide	male	47	6	hanging - asphyxia, acute paralytic stroke
55	suicide	male	66	6	hanging - asphyxia
56	suicide	male	42	3	hanging - asphyxia, acute paralytic stroke
57	suicide	female	28	6	drug overdose, alcohol, acute heart failure
58	control	female	76	3	traffic accident
59	control	male	50	4	acute heart failure, senile, hypertensive arteriosclerosis
60	control	male	46	4	senile, hypertensive arteriosclerosis; acute myocardial infarction
61	control	male	65	1	pulmonary embolism, arteriosclerosis, heart failure

Continued on next page

Table A.1 – continued from previous page

Brain ID	Classification	Sex	Age (years)	PMI[1] (hours)	Cause of death and other noteworthy medical conditions
63	control	male	58	1	acute cardiac insufficiency, chronic myocardial infarction
69	control	male	55	1	acute myocardial infarction, earlier myocardial infarction
71	control	male	59	1	coronary stenosis, acute myocardial infarction
72	control	male	83	1	acute cardiac and acute respiratory insufficiency, chronic heart failure
73	control	male	43	1	traffic accident
74	control	male	68	2	chronic heart failure, coronary sclerosis
75	control	female	38	2	acute cardiac insufficiency, chronic myocardial infarction
76	control	female	89	2	cardiac insufficiency, coronary arteriosclerosis
77	control	male	57	1	acute cardiac insufficiency, cirrhosis
78	control	male	50	1	peritonitis, cardiac and respiratory insufficiency, atrophic cirrhosis
81	control	male	40	1	acute myocardial infarction, hypertensive arteriosclerosis
85	control	male	68	3	acute myocardial infarction, senile, hypertensive arteriosclerosis
93	suicide	female	43	5	hanging - asphyxia
94	control	male	65	1	heart failure, cardiac and respiratory insufficiency
95	control	male	40	4	smoke intoxication
96	control	female	58	1	acute myocardial infarction, arteriosclerosis
98	control	male	74	3	acute myocardial infarction
99	control	male	71	2	acute heart failure, arteriosclerosis
105	suicide	female	42	3	hanging - asphyxia
106	control	male	64	1	acute heart failure, acute cardiac insufficiency, coronary stenosis
108	control	female	63	1	heart failure, chronic myocardial infarction
109	control	male	67	1	pneumonia, senile, hypertensive arteriosclerosis, cirrhosis
111	control	male	55	3	cardiac insufficiency

Continued on next page

Table A.1 – continued from previous page

Brain ID	Classification	Sex	Age (years)	PMI[1] (hours)	Cause of death and other noteworthy medical conditions
112	control	male	45	3	respiratory insufficiency, coronary stenosis, acute myocardial infarction
113	control	male	81	5	acute cardiac insufficiency, previous myocardial infarction
115	control	female	62	6	schizophrenia, Parkinson's disease, pneumonia
118	suicide	male	35	6	hanging - acute respiratory insufficiency
123	control	male	58	2	myocardial infarction, earlier chronic myocardial infarction
136	suicide	male	36	6	hanging - asphyxia
138	suicide	male	52	3	hanging - asphyxia, acute paralytic stroke
139	suicide	male	79	4	hanging - asphyxia
140	suicide	male	49	6	hanging - asphyxia, acute paralytic stroke
143	suicide	male	43	3	hanging - asphyxia
144	suicide	male	42	4	hanging - acute paralytic stroke, acute respiratory insufficiency
145	suicide	female	36	2	drug overdose - aspiration pneumonia, asphyxia
147	control	female	80	1	acute respiratory insufficiency, senile, hypertensive arteriosclerosis
151	control	male	47	2	pneumonia, earlier myocardial infarction, cirrhosis
152	suicide	male	32	6	hanging - asphyxia
154	control	female	72	4	acute myocardial infarction, earlier heart failure, arteriosclerosis
155	suicide	female	83	6	hanging - asphyxia
156	control	male	47	1	heart failure, coronary sclerosis
157	suicide	female	48	7	drug overdose
158	suicide	male	71	1	jumping from a height
159	suicide	male	48	6	hanging - asphyxia
160	control	male	50	2	myocardial infarction
162	control	male	83	5	Alzheimer-like dementia
163	control	female	81	5	global vascular dementia
164	control	male	85	3	heart failure
165	control	female	88	3	cardiac insufficiency, heart failure
167	suicide	female	65	5	hanging - asphyxia

Continued on next page

Table A.1 – continued from previous page

Brain ID	Classification	Sex	Age (years)	PMI[1] (hours)	Cause of death and other noteworthy medical conditions
168	control	female	94	6	dementia
169	control	female	81	6	dementia with Lewy bodies
170	control	male	37	8	electric shock
171	suicide	male	31	8	hanging - asphyxia
174	suicide	male	57	16	hanging - asphyxia
175	suicide	female	49	6	drug overdose
176	suicide	male	35	2	drug overdose
177	control	female	89	2	cerebral arteriosclerosis, dementia
178	suicide	male	43	4	hanging
180	control	male	73	6	acute cardiac failure
181	suicide	female	42	11	drug overdose
185	control	male	80	6	stroke
186	control	female	56	5	myocardial infarction
187	control	male	62	6	respiratory and cardiac insufficiency
188	control	female	86	5	Alzheimer's disease, cardiovascular-respiratory insufficiency
189	control	female	79	5	cardiac and respiratory insufficiency
190	control	female	71	7	stroke - cardiovascular-pulmonary insufficiency
191	control	female	93	7	Alzheimer's disease, cardiovascular-respiratory insufficiency
192	control	male	81	5	heart failure, cardiogenic shock
194	control	male	64	4	pneumonia
195	control	male	83	6	respiratory and cardiac insufficiency
197	control	male	64	6	myocardial infarction
198	control	female	68	3	pneumonia, respiratory insufficiency
199	control	female	76	6	heart failure
200	control	female	93	6	cardiorespiratory insufficiency, pulmonary embolism
209	control	male	52	5	myocardial infarction
211	control	female	56	6	cardiac and respiratory insufficiency
212	control	female	87	6	dementia, myocardial insufficiency
213	control	male	75	5	bipolar affective psychosis
214	control	female	86	4	dementia, myocardial insufficiency
215	control	female	44	5	myocardial infarction
216	control	male	53	5	pulmonary embolism
217	control	male	60	5	myocardial infarction

[1]) PMI = post-mortem interval, i.e. time between death and autopsy

Table A.2.: Annotation of brains from the **New South Wales Tissue Resource Centre** (all subjects were male)

Brain ID	**Classi-fication**	**Age** (years)	**PMI**[1] (hours)	**Clinical cause of death**	**Toxicological analysis**
182	Alcoholic	66	11.5	pneumonia	no blood alcohol
234	Alcoholic	39	24	aortic stenosis	not available
243	Alcoholic	70	33.5	respiratory failure	no blood alcohol
259	Alcoholic	56	15	ischaemic heart disease, emphysema	no blood alcohol, nordiazepam < 0.1 mg/l
287	Alcoholic	50	17	ischaemic heart disease	not available
417	Alcoholic	58	20	ischaemic heart disease, cirrhosis	no blood alcohol, guaifenesin 8.5 mg/l, ibuprofen 3.5 mg/l, paracetamol 16 mg/l,
430	Alcoholic	43	29	intra-abdominal haemorrhage, sepsis, hepatitis	not available
522	Alcoholic	58	21.5	acute and chronic pancreatitis	no blood alcohol, no drugs
547	Alcoholic	73	19	ischaemic bowel disease, cardiovascular disease	not available
591	Alcoholic	45	18.5	not available	not available
97	Control	38	13.5	cardiovascular disease	not available
190	Control	68	22	suicide by hanging, asphyxia	citalopram 0.4 mg/l, thioridazine 0.6 mg/l,
277	Control	50	19	ischaemic heart disease	no blood alcohol
295	Control	34	20.5	acute exacerbation of asthma	not available
301	Control	53	16	dilated cardiomyopathy	no blood alcohol, lidocaine 0.9 mg/l, sotalol 3.8 µmol/l
329	Control	48	24	ischaemic heart disease	not available
367	Control	57	18	ischaemic heart disease	HIV negative
510	Control	60	21.5	ischaemic heart disease	not performed
518	Control	66	23	ischaemic and hypertensive heart disease	irbesartan 0.6 mg/l, sulphapyridine detected
598	Control	56	19	arteriosclerotic coronary disease	no blood alcohol, no drugs

[1]) PMI = post-mortem interval, i.e. time between death and autopsy

Bibliography

H. Abdi. *Encyclopedia of Measurement and Statistics*. Thousand Oaks (CA, USA): Sage, 2007.

A. Acheson, J.L. Robinson, D.C. Glahn, W.R. Lovallo, and P.T. Fox. Differential activation of the anterior cingulate cortex and caudate nucleus in persons with a family history of alcoholism during a gambling simulation: Studies from the Oklahoma Family Health Patterns Project. *Drug Alcohol Depend*, 100(1-2):17–23, 2009.

A. Achiron and M. Gurevich. Peripheral blood gene expression signature mirrors central nervous system disease: the model of multiple sclerosis. *Autoimmun Rev*, 5(8):517–22, 2006.

G. Acosta, D.P. Freidman, K.A. Grant, and S.E. Hemby. Alternative splicing of AMPA subunits in prefrontal cortical fields of cynomolgus monkeys following chronic ethanol self-administration. *Front Psychiatry*, 2:72, 2011.

G.W. Ainslie. Impulse control in pigeons. *J Exp Anal Behav*, 21(3):485–9, 1974.

K. Alexander-Kaufman, G. James, D. Sheedy, C. Harper, and I. Matsumoto. Differential protein expression in the prefrontal white matter of human alcoholics: a proteomics study. *Mol Psychiatry*, 11(1):56–65, 2006.

K. Alexander-Kaufman, S. Cordwell, C. Harper, and I. Matsumoto. A proteome analysis of the dorsolateral prefrontal cortex in human alcoholic patients. *Proteomics Clin Appl*, 1(1):62–72, 2007.

R.P. Allen and A.M. Wagman. Do sleep patterns relate to the desire for alcohol? *Adv Exp Med Biol*, 59:495–508, 1975.

J.M. Allman, A. Hakeem, J.M. Erwin, E. Nimchinsky, and P. Hof. The anterior cingulate cortex. The evolution of an interface between emotion and cognition. *Ann N Y Acad Sci*, 935:107–17, 2001.

F.H. Allport and G.W. Allport. Personality traits: their classification and measurement. *J Abnorm Soc Psychol*, 16:6–40, 1921.

J. Alsiö, K. Nordenankar, E. Arvidsson, C. Birgner, S. Mahmoudi, B. Halbout, C. Smith, G.M. Fortin, L. Olson, L. Descarries, L.É. Trudeau, K. Kullander, D. Lévesque, and A. Wallén-Mackenzie. Enhanced sucrose and cocaine self-administration and cue-induced drug seeking after loss of VGLUT2 in midbrain dopamine neurons in mice. *J Neurosci*, 31(35):12593–603, 2011.

S. Amadio, C. Montilli, R. Magliozzi, G. Bernardi, R. Reynolds, and C. Volonté. P2Y12 receptor protein in cortical gray matter lesions in multiple sclerosis. *Cereb Cortex*, 20 (6):1263–73, 2010.

O. Ameisen. Complete and prolonged suppression of symptoms and consequences of alcohol-dependence using high-dose baclofen: a self-case report of a physician. *Alcohol Alcohol.*, 40(2):147–50, 2005.

American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR Fourth Edition*. American Psychiatric Pub, 2000.

O. Andersson, A. Stenqvist, A. Attersand, and G. von Euler. Nucleotide Sequence, Genomic Organization, and Chromosomal Localization of Genes Encoding the Human NMDA Receptor Subunits NR3A and NR3B. *Genomics*, 78(3):178–84, 2001.

R.F. Anton, G. Oroszi, S. O'Malley, D. Couper, R. Swift, H. Pettinati, and D. Goldman. An evaluation of mu-opioid receptor (OPRM1) as a predictor of naltrexone response in the treatment of alcohol dependence: results from the Combined Pharmacotherapies and Behavioral Interventions for Alcohol Dependence (COMBINE) study. *Arch Gen Psychiatry*, 65(2):135–44, 2008.

O. Arias-Carrión, M. Stamelou, E. Murillo-Rodríguez, M. Menéndez-González, and E. Pöppel. Dopaminergic reward system: a short integrative review. *Int Arch Med*, 3:24, 2010.

E. Arikawa, Y. Sun, J. Wang, Q. Zhou, B. Ning, S.L. Dial, L. Guo, and J. Yang. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC Genomics*, 9:328, 2008.

A. Arjona and D.K. Sarkar. Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells. *J Immunol*, 174(12):7618–24, 2005.

A. Arjona, N. Boyadjieva, and D.K. Sarkar. Circadian rhythms of granzyme B, perforin, IFN-gamma, and NK cell cytolytic activity in the spleen: effects of chronic ethanol. *J Immunol*, 172(5):2811–7, 2004.

R. Asheychik, T. Jackson, H. Baker, R. Ferraro, T. Ashton, and J. Kilgore. The efficacy of L-tryptophan in the reduction of sleep disturbance and depressive state in alcoholic patients. *J Stud Alcohol*, 50(6):525–32, 1989.

B. Ataman, J. Ashley, M. Gorczycy, P. Ramachandran, W. Fouquet, S.J. Sigrist, and V. Budnik. Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. *Neuron*, 57(5):705–18, 2008.

M.N. Baek, K.H. Jung, D. Halder, M.R. Choi, B.H. Lee, B.C. Lee, M.H. Jung, I.G. Choi, M.K. Chung, D.Y. Oh, and Y.G. Chai. Artificial microRNA-based neurokinin-1 receptor gene silencing reduces alcohol consumption in mice. *Neurosci Lett*, 475(3):124–8, 2010.

S.M. Bankston, D.D. Carroll, S.G. Cron, L.K. Granmayeh, M.T. Marcus, F.G. Moeller, J.M. Schmitz, P. Swank, and P.R. Liehr. Substance abuser impulsivity decreases with a nine-month stay in a therapeutic community. *Am J Drug Alcohol Abuse*, 35(6):417–20, 2009.

N.L. Barbosa-Morais, M.J. Dunning, S.A. Samarajiwa, J.F. Darot, M.E. Ritchie, A.G. Lynch, and S. Tavaré. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic acids research*, 38(3):e17, 2010.

A. Bari, J.W. Dalley, and T.W. Robbins. The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. *Nature Protocols*, 3(5):759–67, 2008.

F. Bedogni, R.D. Hodge, B.R. Nelson, E.A. Frederick, N. Shiba, R.A. Daza, and R.F. Hevner. Autism susceptibility candidate 2 (Auts2) encodes a nuclear protein expressed in developing brain regions implicated in autism neuropathology. *Gene Expr Patterns*, 10(1):9–15, 2010.

Y. Benjamini and Y. Hochberg. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Statist Soc Ser B*, 57(1):289–300, 1995.

K.C. Berridge. The depate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology (Berl.)*, 191(3):391–431, 2007.

S. Bezdjian, L.A. Baker, and C. Tuvblad. Genetic and environmental influences on impulsivity: a meta-analysis of twin, family and adoption studies. *Clin Psychol Rev*, 31(7): 1209–23, 2011.

I. Biaggioni. Circadian clocks, autonomic rhythms, and blood pressure dipping. *Hypertension*, 52(5):797–8, 2008.

C. Birgner, K. Nordenankar, M. Lundblad, J.A. Mendez, C. Smith, M. le Grevès, D. Galter, L. Olson, A. Fredriksson, L.E. Trudeau, K. Kullander, and A. Wallén-Mackenzie. VGLUT2 in dopamine neurons is required for psychostimulant-induced behavioral activation. *Proc Natl Acad Sci U S A*, 107(1):389–94, 2010.

K. Björk, A.C. Hansson, and W. H. Sommer. Genetic variation and brain gene expression in rodent models of alcoholism implications for medication development. *Int Rev Neurobiol*, 91:129–71, 2010.

B.M. Bolstad, R.A. Irizarry, M. Astrand, and T.P. Speed. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, 19:185–193, 2003.

M. Bots and J.P. Medema. Granzymes at a glance. *J Cell Sci*, 119(Pt 24):5011–4, 2006.

M.P. Broca. Remarques sur le siège de la faculté de langage articulé, suivies d'une observation d'aphémie (perte de parole). *Bulletins de la Société anatomique de Paris*, 6: 330–357, 1861.

K. Brodmann. *Vergleichende Lokalisationslehre der Großhirnrinde in ihren Prinzipien dargestellt auf Grund des Zellenbaues.* Verlag von Johann Ambrosius Barth, 1909.

K.H. Brower, M.S. Aldrich, E.A. Robinson, R.A. Zucker, and J.F. Greden. Insomnia, self-medication, and relapse to alcoholism. *Am J Psychiatry*, 158(3):399–404, 2001.

H. Bräuner-Osborne and P. Krogsgaard-Larsen. Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPRC5B, a family C receptor with a short amino-terminal domain. *Genomics*, 65(2):121–8, 2000.

R.M. Bujis, C.G. van Eden, V.D. Goncharuk, and A. Kalsbeek. The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. *J Endocrinol*, 177(1):17–26, 2003.

G. Bush, P. Luu, and M.I. Posner. Cognitive and emotional influences in anterior cingulate cortex. *Trends Cogn Sci*, 4(6):215–222, 2000.

L. Carim-Todd, M. Escarceller, X. Estivill, and L. Sumoy. Cloning of the novel gene TM6SF1 reveals conservation of clusters of paralogous genes between human chromosomes 15q24–>q26 and 19p13.3–>p12. *Cytogenet Cell Genet*, 90(3-4):255–60, 2000.

D.B. Carr and A.W. Lipkowski. Neuropeptides and pain. *Agressologie*, 31(4):173–7, 1990.

F.X. Castellanos and R. Tannock. Neuroscience of attention-deficit/hyperactivity disorder: the search for endophenotypes. *Nat Rev Neurosci*, 3(8):617–28, 2002.

C. Cheadle, M.P. Vawter, W.J. Freed, and K.G. Becker. Analysis of microarray data using Z score transformation. *J Mol Diagn*, 5(2):73–81, 2003.

C.P. Chen, P. Kuhn, J.P. Advis, and D.K. Sarkar. Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *J Neurochem*, 88(6):1547–54, 2004.

C.P. Chen, P. Kuhn, J.P. Advis, and D.K. Sarkar. Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *J Neurochem*, 97(4):1026–33, 2006.

P. Chomczynski and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162(1):156–159, 1987.

A. Christakou, T.W. Robbins, and B.J. Everitt. Prefrontal cortical-ventral striatal interactions involved in affective modulation of attentional performance: implications for corticostriatal circuit function. *J Neurosci*, 24(4):773–80, 2004.

Y. Chudasama, F. Passetti, S.E. Rhodes, D. Lopian, A. Desai, and T.W. Robbins. Dissociable aspects of performance on the 5-choice serial reaction time task following lesions of the dorsal anterior cingulate, infralimbic and orbitofrontal cortex in the rat: differential effects on selectivity, impulsivity and compulsivity. *Behav Brain Res*, 146(1-2):105–19, 2003.

J.D. Colbert, A. Plechanovová, and C. Watts. Glycosylation directs targeting and activation of cystatin f from intracellular and extracellular sources. *Traffic*, 10(4):425–37, 2009.

R.T. Cook. Alcohol abuse, alcoholism, and damage to the immune system–a review. *Alcohol Clin Exp Res*, 22(9):1927–42, 1998.

T.G. Costa Dias, V.B. Wilson, D.R. Bathula, S.P. Iyer, K.L. Mills, B.L. Thurlow, C.A. Stevens, E.D. Musser, S.D. Carpenter, D.S. Grayson, S.H. Mitchell, J.T. Nigg, and D.A. Fair. Reward circuit connectivity relates to delay discounting in children with attention-deficit/hyperactivity disorder. *Eur Neuropsychopharmacol*, S0924-977X(12): 3006–9, 2012.

J.C. Crabbe, R.L. Bell, and C.L. Ehlers. Human and laboratory rodent low response to alcohol: is better consilience possible? *Addict Biol*, 15(2):125–44, 2010.

D.K. Crawford, J.R. Trudell, E.J. Bertaccini, K. Li, D.L. Davies, and R.L. Alkana. Evidence that ethanol acts on a target in Loop 2 of the extracellular domain of alpha1 glycine receptors. *J Neurochem*, 102(6):2097–109, 2007.

C.P. Cross, L.T. Copping, and A. Campbell. Sex differences in impulsivity: a meta-analysis. *Psychol Bull.*, 137(1):97–130, 2011.

C.A. Czeisler, J.F. Duffy, T.L. Shanahan, E.N. Brown, J.F. Mitchell, D.W. Rimmer, J.M. Ronda, E.J. Silva, J.S. Allan, J.S. Emens, D.J. Dijk, and R.E. Kronauer. Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science*, 284 (5423):2177–81, 1999.

M. Dai, P. Wang, A. D. Boyd, G. Kostov, B. Athey, E. G. Jones, W. E. Bunney, R. M. Myers, T. P. Speed, H. Akil, S. J. Watson, and F. Meng. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res*, 33(20):e175, 2005.

J.W. Dalley, B.J. Everitt, and T.W. Robbins. Impulsivity, compulsivity, and top-down cognitive control. *Neuron*, 69(4):680–94, 2011.

M.H. Dao-Castellana, Y. Samson, F. Legault, J.L. Martinot, H.J. Aubin, C. Crouzel, L. Feldman, D. Barrucand, G. Rancurel, A. Féline, and A. Syrota. Frontal dysfunction in neurologically normal chronic alcoholic subjects: metabolic and neuropsychological findings. *Psychol Med.*, 28(5):1039–48, 1998.

M. Daoust, E. Legrand, M. Gewiss, C. Heidbreder, P. DeWitte, G. Tran, and P. Durbin. Acamprosate modulates synaptosomal GABA transmission in chronically alcoholised rats. *Pharmacology, biochemistry, and behavior*, 41(4):669–74, 1992.

M.I. Davis. Ethanol-BDNF interactions: Still More Questions than Answers. *Pharmacol Ther*, 118(1):36–57, 2008.

I. Dedova, A. Harding, D. Sheedy, T. Garrick, N. Sundqvist, C. Hunt, J. Gillies, and C.G. Harper. The importance of brain banks for molecular neuropathological research: The New South wales tissue resource centre experience. *Int J Mol Sci*, 10(1):366–84, 2009.

M. Devaney, D. Graham, and J. Greeley. Circadian variation of the acute and delayed response to alcohol: investigation of core body temperature variations in humans. *Pharmacol Biochem Behav*, 75(4):881–7, 2003.

S.J. Dickman and D.E. Meyer. Impulsivity and speed-accuracy tradeoffs in information processing. *J Pers Soc Psychol*, 54(2):274–90, 1988.

Die Drogenbeauftragte der Bundesregierung. *Drogen- und Suchtbericht Mai 2012*. Druckerei Conrad Gmbh, Berlin, 2012.

H. Dong, P. Zhang, I. Song, R.S. Petralia, D. Liao, and R.L. Huganir. Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. *J Neurosci*, 19(16): 6930–41, 1999.

G. Donmez and T.F. Outeiro. SIRT1 and SIRT2: emerging targets in neurodegeneration. *EMBO Mol Med*, 5(3):344–52, 2013.

N. Doran, B. Spring, D. McChargue, M. Pergadia, and M. Richmond. Impulsivity and smoking relapse. *Nicotine Tob Res*, 6(4):641–7, 2004.

F. Dosseville, S. Laborde, and R. Lericollais. Validation of a Chronotype Questionnaire Including an Amplitude Dimension. *Chronobiol Int*, 30(5):639–48, 2013.

G.E. Duffield, J.D. Best, B.H. Meurers, A. Bittner, J.J. Loros, and J.C. Dunlap. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. *Curr Biol*, 12(7):551–7, 2002.

J. Durana and P. Barnes. *The Impulsive Client: Theory, Research, and Treatment*, chapter A neurodevelopmental view of impulsivity and its relationship to the superfactors of personality. American Psychological Association (APA), 1996.

D.M. Eagle, A. Bari, and T.W. Robbins. The neuropsychopharmacology of action inhibition: cross-species translation of the stop-signal and go/no-go tasks. *Psychopharmacology (Berl.)*, 199(3):439–56, 2008.

A.M. Egbert. The older alcoholic: recognizing the subtle clinical clues. *Geriatrics*, 48(7): 63–6, 1993.

L.A. Ehrman, M.T. Williams, T.L. Schaefer, G.A. Gudelsky, T.M. Reed, A.A. Fienberg, P. Greengard, and C.V. Vorhees. Phosphodiesterase 1B differentially modulates the effects of methamphetamine on locomotor activity and spatial learning through DARPP32-dependent pathways: evidence from PDE1B-DARPP32 double-knockout mice. *Genes Brain Behav*, 5(7):540–51, 2006.

M.A. Enoch, Z. Zhou, M. Kimura, D.C. Mash, Q. Yuan, and D. Goldman. GABAergic gene expression in postmortem hippocampus from alcoholics and cocaine addicts; corresponding findings in alcohol-naïve P and NP rats. *PLoS One*, 7(1):e29369, 2012.

K.D. Ersche, A.J. Turton, S. Pradhan, E.T. Bullmore, and T.W. Robbins. Drug Addiction Endophenotypes: Impulsive Versus Sensation-Seeking Personality Traits. *Biol Psychiatry*, 68(8):770–773, 2010.

J.L. Evenden. Varieties of impulsivity. *Psychopharmacology (Berl.)*, 46(4):348–61, 1999.

J.L. Evenden and C.N. Ryan. The pharmacology of impulsive behaviour in rats: the effects of drugs on response choice with varying delays of reinforcement. *Psychopharmacology (Berl.)*, 128(2):161–70, 1996.

B.J. Everitt, J.A. Parkinson, M.C. Olmstead, M. Arroyo, P. Robledo, and T.W. Robbins. Associative processes in addiction and reward. The role of amygdala-ventral striatal subsystems. *Ann N Y Acad Sci*, 877:412–38, 1999.

Y.Z. Farnell, G.C. Allen, S.S. Nahm, N. Neuendorff, J.R. West, W.J. Chen, and D.J. Earnest. Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. *Alcohol Clin Exp Res*, 32(3):544–52, 2008.

S. Fasano and R. Brambilla. Ras-ERK Signaling in Behavior: Old Questions and New Perspectives. *Front Behav Neurosci*, 5:79, 2011.

S. Fasano, A. D'Antoni, P.C. Orban, E. Valjent, E. Putignano, H. Vara, T. Pizzorusso, M. Giustetto, B. Yoon, P. Soloway, R. Maldonado, J. Caboche, and R. Brambilla. Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) controls activation of extracellular signal-regulated kinase (ERK) signaling in the striatum and long-term behavioral responses to cocaine. *Biol Psychiatry*, 66(8):758–68, 2009.

D.T. Fei, H. Paxton, and A.B. Chen. Difficulties in precise quantitation of CD4+ T lymphocytes for clinical trials: a review. *Biologicals*, 21(3):221–31, 1993.

P.B. Fitzgerald, T.J. Oxley, A.R. Laird, J. Kulkarni, G.F. Egan, and Z.J. Daskalakis. An analysis of functional neuroimaging studies of dorsolateral prefrontal cortical activity in depression. *Psychiatry Res*, 148(1):33–45, 2006.

G. Freund and K.J. Anderson. Glutamate receptors in the frontal cortex of alcoholics. *Alcohol Clin Exp Res*, 20(7):1165–72, 1996.

E. Fries, L. Dettenborn, and C. Kirschbaum. The cortisol awakening response (CAR): facts and future directions. *Int J Psychophysiol*, 72(1):67–73, 2009.

C. Fukuhara and G. Tosini. Analysis of daily and circadian gene expression in the rat pineal gland. *Neurosci Res*, 60(2):192–198, 2008.

T. Furmark, L. Appel, A. Michelgård, K. Wahlstedt, F. Ahs, S. Zancan, E. Jacobsson, K. Flyckt, M. Groph, M. Bergström, E.M. Pich, L.G. Nilsson, M. Bani, B. Långström, and M. Fredrikson. Cerebral blood flow changes after treatment of social phobia with the neurokinin-1 antagonist GR205171, citalopram, or placebo. *Biol Psychiatry*, 58(2): 132–42, 2005.

M.E. Futschik and H. Herzel. Are we overestimating the number of cell-cycling genes? The impact of background models on time-series analysis. *Bioinformatics*, 24(8):1063–9, 2008.

L. Gautier, L. Cope, B.M. Bolstad, and R.A. Irizarry. affy—analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*, 20(3):307–315, 2004.

D.T. George, J. Gilman, J. Hersh, A. Thorsell, D. Herion, C. Geyer, X. Peng, W. Kielbasa, R. Rawlings, J.E. Brandt, D.R. Gehlert, J.T. Tauscher, S.P. Hunt, D. Hommer, and M. Heilig. Neurokinin 1 receptor antagonism as a possible therapy for alcoholism. *Science*, 319(5869):1536–9, 2008.

M.S. George, R.F. Anton, C. Bloomer, C. Teneback, D.J. Drobes, J.P. Lorberbaum, Z. Nahas, and D.J. Vincent. Activation of prefrontal cortex and anterior thalamus in alcoholic subjects on exposure to alcohol-specific cues. *Arch Gen Psychiatry*, 58(4):345–52, 2001.

J. R. Gerstner and J. C. Yin. Circadian rhythms and memory formation. *Nat. Rev. Neurosci.*, 11(8):577–588, 2010.

J.E. Gibbs, J. Blaikley, S. Beesley, L. Matthews, K.D. Simpson, S.H. Boyce, S.N. Farrow, K.J. Else, D. Singh, D.W. Ray, and A.S. Loudon. The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *Proc Natl Acad Sci U S A*, 109(2):582–7, 2012.

J.M. Gilman, A.R. Smith, V.A. Ramchandani, R. Momenan, and D.W. Hommer. The effect of intravenous alcohol on the neural correlates of risky decision making in healthy social drinkers. *Addict Biol*, 17(2):465–78, 2012.

R.Z. Goldstein and N.D. Volkow. Drug Addiction and Its Underlying Neurobiological Basis: Neuroimaging Evidence for the Involvement of the Frontal Cortex. *Am J Psychiatry*, 159:1642–1652, 2002.

H. Gray. *Anatomy of the human body*. Lea & Febiger, 20th edition, 1918.

A.M. Graybiel. The basal ganglia: learning new tricks and loving it. *Curr Opin Neurobiol*, 15(6):638–44, 2005.

A.C. Hall, F.R. Lucas, and P.C. Salinas. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell*, 100(5):525–35, 2000.

G. Hamilton, J.D. Colbert, A.W. Schuettelkopf, and C. Watts. Cystatin F is a cathepsin C-directed protease inhibitor regulated by proteolysis. *EMBO J*, 27(3):499–508, 2008.

N.K. Hansell, A. Agrawal, J.B. Whitfield, K.I. Morley, S.D. Gordon, P.A. Lind, M.L. Pergadia, G.W. Montgomery, P.A. Madden, R.D. Todd, A.C. Heath, and N.G. Martin. Can we identify genes for alcohol consumption in samples ascertained for heterogeneous purposes? *Alcohol Clin Exp Res*, 33(4):729–39, 2009.

E.S. Hansen, S. Hasselbalch, I. Law, and T.G. Bolwig. The caudate nucleus in obsessive-compulsive disorder. Reduced metabolism following treatment with paroxetine: a PET study. *Int J Neuropsychopharmacol*, 5(1):1–10, 2002.

Y. Harada, C. Yokota, R. Habas, D.C. Slusarski, and X. He. Retinoic Acid-Inducible G Protein-Coupled Receptors Bind to Frizzled Receptors and May Activate Non-canonical Wnt Signaling. *Biochem Biophy Res Commun*, 358(4):968–75, 2007.

B.P. Hasler, L.J. Smith, J.C. Cousins, and R.R. Bootzin. Circadian rhythms, sleep, and substance abuse. *Sleep Med Rev*, 16(1):67–81, 2012.

M.H. Hastings. Central clocking. *Trends Neurosci*, 20(10):459–64, 1997.

M.L. Hastings, H.A. Ingle, M.A. Lazar, and S.H. Munroe. Post-transcriptional regulation of thyroid hormone receptor expression by cis-acting sequences and a naturally occurring antisense RNA. *J Biol Chem*, 275(15):11507–13, 2000.

S.E. Haynes, G. Hollopeter, G. Yang, D. Kurpius, M.E. Dailey, W.B. Gan, and D. Julius. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*, 9(12):1512–9, 2006.

L. Heimer and R.D. Wilson. The subcortical projections of the allocortex: similarities in the neural associations of the hippocampus, the piriform cortex, and the neocortex. In M. Santini, editor, *Golgi Centennial Symposium*, pages 177–193. Raven Press, New York, 1975.

F. Heinrich, A.and Nees, A. Lourdusamy, J. Tzschoppe, S. Meier, S. Vollstädt-Klein, M. Fauth-Bühler, S. Steiner, C. Bach, L. Poustka, T. Banaschewski, G.J. Barker, C. Büchel, P.J. Conrod, H. Garavan, J. Gallinat, A. Heinz, B. Ittermann, E. Loth, K. Mann, E. Artiges, T. Paus, C. Lawrence, Z. Pausova, M.N. Smolka, A. Ströhle, M. Struve, S.H. Witt, G. Schumann, H. Flor, M. Rietschel, and The IMAGEN consortium. From gene to brain to behavior: schizophrenia-associated variation in AMBRA1 alters impulsivity-related traits. *Eur J Neurosci*, [Epub ahead of print], 2013.

P. Heiser, M. Heinzel-Gutenbrunner, J. Frey, J. Smidt, J. Grabarkiewicz, S. Friedel, W. Kühnau, J. Schmidtke, H. Remschmidt, and J. Hebebrand. Twin study on heritability of activity, attention, and impulsivity as assessed by objective measures. *J Atten Disord*, 9(4):575–81, 2006.

A. Herz. Endogenous opioid systems and alcohol addiction. *Psychopharmacology (Berl.)*, 129(2):99–111, 1997.

S.P. Hinshaw. Impulsivity, emotion regulation, and developmental psychopathology: specificity versus generality of linkages. *Ann N Y Acad Sci*, 1008:149–59, 2003.

B. Hoang, M.Jr. Moos, S. Vukicevic, and F.P. Luyten. Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis. *J Biol Chem*, 271(42):26131–7, 1996.

A. Holmes, M. Heilig, N.M. Rupniak, T. Steckler, and G. Griebel. Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. *Trends Pharmacol Sci*, 24(11):580–8, 2003.

M.C. Huang, C.W. Ho, C.H. Chen, S.C. Liu, C.C. Chen, and S.J. Leu. Reduced expression of circadian clock genes in male alcoholic patients. *Alcohol Clin Exp Res*, 34(11):1899–904, 2010.

W. Huang da, B.T. Sherman, and R.A. Lempicki. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*, 4(1):44–57, 2009a.

W. Huang da, B.T. Sherman, and R.A. Lempicki. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*, 37 (1):1–13, 2009b.

B.L. Hungund and B.S. Basavarajappa. Role of endocannabinoids and cannabinoid CB1 receptors in alcohol-related behaviors. *Ann N Y Acad Sci*, 1025:515–27, 2004.

M. Iijima, T. Nikaido, M. Akiyama, T. Moriya, and S. Shibata. Methamphetamine-induced, suprachiasmatic nucleus-independent circadian rhythms of activity and mPer gene expression in the striatum of the mouse. *Eur J Neurosci*, 16(5):921–9, 2002.

International Molecular Genetic Study of Autism Consortium (IMGSAC). A genomewide screen for autism: strong evidence for linkage to chromosomes 2q, 7q, and 16p. *Am J Hum Genet*, 69(3):570–81, 2001.

R.A. Irizarry, B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, and T.P. Speed. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, 4(2):249–64, 2003.

S. Itzkovitz and A. van Oudenaarden. Validating transcripts with probes and imaging technology. *Nat Methods*, 8(4 Suppl):S12–9, 2011.

M. Jacobsen, D. Repsilber, A. Gutschmidt, A. Neher, K. Feldmann, H.J. Mollenkopf, A. Ziegler, and S.H. Kaufmann. Ras-associated small GTPase 33A, a novel T cell factor, is down-regulated in patients with tuberculosis. *J Infect Dis*, 192(7):1211–8, 2005.

B.J. Janssen, C.M. Tyssen, H. Duindam, and W.J. Rietveld. Suprachiasmatic lesions eliminate 24-h blood pressure variability in rats. *Physiol Behav*, 55(2):307–11, 1994.

R.D. Jindal. Insomnia in patients with depression: some pathophysiological and treatment considerations. *CNS Drugs*, 23(4):309–29, 2009.

K.H. Joe, Y.K. Kim, T.S. Kim, S.W. Roh, S.W. Choi, Y.B. Kim, H.J. Lee, and D.J. Kim. Decreased plasma brain-derived neurotrophic factor levels in patients with alcohol dependence. *Alcohol Clin Exp Res*, 31(11):1833–8, 2007.

B.A. Johnson. Update on neuropharmacological treatments for alcoholism: scientific basis and clinical findings. *Biochem Pharmacol*, 75(1):34–56, 2008.

B.A. Johnson, N. Rosenthal, J.A. Capece, F. Wiegand, L. Mao, K. Beyers, A. McKay, N. Ait-Daoud, R.F. Anton, D.A. Ciraulo, H.R. Kranzler, K. Mann, S.S. O'Malley, R.M. Swift, Topiramate for Alcoholism Advisory Board, and Topiramate for Alcoholism Study Group. Topiramate for treating alcohol dependence: a randomized controlled trial. *JAMA*, 298(14):1641–51, 2007.

E.M. Jones, D. Knutson, and D. Haines. Common problems in patients recovering from chemical dependency. *Am Fam Physician*, 68(10):1971–8, 2003.

P.W. Kalivas. The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci*, 10 (8):561–72, 2009.

P.W. Kalivas and N.D. Volkow. The Neural Basis of Addiction: A Pathology of Motivation and Choice. *Am J Psychiatry*, 162:1403–1413, 2005.

L.S. Kaplow. Cytochemical heterogeneity of human circulating monocytes. *Acta Cytol*, 19 (4):358–65, 1975.

F. Kasanetz, V. Deroche-Gamonet, N. Berson, E. Balado, M. Lafourcade, O. Manzoni, and P.V. Piazza. Transition to addiction is associated with a persistent impairment in synaptic plasticity. *Science*, 328(5986):1709–12, 2010.

Y. Kawano, C.S. Pontes, H. Abe, S. Takishita, and T. Omae. Effects of alcohol consumption and restriction on home blood pressure in hypertensive patients: serial changes in the morning and evening records. *Clin Exp Hypertens*, 24(1-2):33–9, 2002.

A. Kawasaki, Y. Shinkai, H. Yagita, and K. Okumura. Expression of perforin in murine natural killer cells and cytotoxic T lymphocytes in vivo. *Eur J Immunol*, 22(5):1215–9, 1992.

F. Kiefer, H. Jahn, T. Tarnaske, H. Helwig, P. Briken, R. Holzbach, P. Kämpf, R. Stracke, M. Baehr, D. Naber, and K. Wiedemann. Comparing and combining naltrexone and acamprosate in relapse prevention of alcoholism: a double-blind, placebo-controlled study. *Arch Gen Psychiatry*, 60(1):92–9, 2003.

H.W. Kim, S.I. Rapoport, and J.S. Rao. Altered expression of apoptotic factors and synaptic markers in postmortem brain from bipolar disorder patients. *Neurobiol Dis*, 37 (3):596–603, 2010.

T. Kishi, T. Kitajima, K. Kawashima, T. Okochi, Y. Yamanouchi, Y. Kinoshita, H. Ujike, T. Inada, M. Yamada, N. Uchimura, I. Sora, M. Iyo, N. Ozaki, and N. Iwata. Association Analysis of Nuclear Receptor Rev-erb Alpha Gene (NR1D1) and Japanese Methamphetamine Dependence. *Curr Neuropharmacol*, 9(1):129–32, 2011.

E.B. Klermann, D.W. Rimmer, D.J. Dijk, R.E. Kronauer, J.F. Rizzo, and C.A. Czeisler. Nonphotic entrainment of the human circadian pacemaker. *Am J Physiol*, 274(4 Pt 2): R991–6, 1998.

C.H. Ko and J.S. Takahashi. Molecular components of the mammalian circadian clock. *Hum Mol Genet*, 15(2):R271–7, 2006.

G.F. Koob. Theoretical frameworks and mechanistic aspects of alcohol addiction: alcohol addiction as a reward deficit disorder. *Curr Top Behav Neurosci*, 13:3–30, 2013.

G.F. Koob and N.D. Volkow. Neurocircuitry of Addiction. *Neuropsychopharmacology*, 35: 217–38, 2010.

E. Kowalska and S.A. Brown. Peripheral clocks: keeping up with the master clock. *Cold Spring Harb Symp Quant Biol*, 72:301–5, 2007.

T.P. Laine, A. Ahonen, P. Räsänen, and J. Tiihonen. Dopamine transporter availability and depressive symptoms during alcohol withdrawal. *Psychiatry Res*, 90(3):153–7, 1999.

H.P. Landolt and J.C. Gillin. Sleep abnormalities during abstinence in alcohol-dependent patients. Aetiology and management. *CNS Drugs*, 15(5):413–25, 2001.

R. Lape and J.A. Dani. Complex response to afferent excitatory bursts by nucleus accumbens medium spiny projection neurons. *J Neurophysio*, 92(3):1276–84, 2004.

B. Lemmer, K. Witte, H. Enzminger, S. Schiffer, and S. Hauptfleisch. Transgenic TGR(mREN2)27 rats as a model for disturbed circadian organization at the level of the brain, the heart, and the kidneys. *Chronobiol Int*, 20(4):711–38, 2003.

L. Leyns, T. Bouwmeester, S.H. Kim, S. Piccolo, and E.M. De Robertis. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell*, 88(6): 747–56, 1997.

J.Z. Li, B.G. Bunney, F. Meng, M.H. Hagenauer, D.M. Walsh, M.P. Vawter, S.J. Evans, P.V. Choudary, P. Cartagena, J.D. Barchas, A.F. Schatzberg, E.G. Jones, R.M. Myers, S.J.Jr. Watson, H. Akil, and W.E. Bunney. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc Natl Acad Sci U S A*, 110(24):9950–5, 2013.

S.X. Li, L.J. Liu, W.G. Jiang, and L. Lu. Morphine withdrawal produces circadian rhythm alterations of clock genes in mesolimbic brain areas and peripheral blood mononuclear cells in rats. *J Neurochem*, 109(6):1668–79, 2009.

S.X. Li, L.J. Liu, W.G. Jiang, L.L. Sun, S.J. Zhou, B. Le Foll, X.Y. Zhang, T.R. Kosten, and L. Lu. Circadian alteration in neurobiology during protracted opiate withdrawal in rats. *J Neurochem*, 115(2):353–62, 2010.

K. Lin, S. Wang, M.A. Julius, J. Kitajewski, M. Jr. Moos, and F.P. Luyten. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. *Proc Natl Acad Sci U S A*, 94(21):11196–200, 1997.

G.A. Lincoln, F.J. Ebling, and O.F. Almeida. Generation of melatonin rhythms. *Ciba Found Symp*, 117:129–48, 1985.

J. Littleton. Acamprosate in alcohol dependence: how does it work? *Addiction*, 90(9): 1179–88, 1995.

J. Liu, E. Walter, D. Stenger, and D. Thach. Effects of Globin mRNA Reduction Methods on Gene Expression Profiles from Whole Blood. *The Journal of Molecular Diagnostics*, 8(5):551–558, 2006.

M.K. Lobo and E.J. Nestler. The striatal balancing act in drug addiction: distinct roles of direct and indirect pathway medium spiny neurons. *Front Neuroanat*, 5(41):1–11, 2011.

M. Loos, S. van der Sluis, Z. Bochdanovits, I.J. van Zutphen, T. Pattij, O. Stiedl, Neuro-BSIK Mouse Phenomics consortium, A.B. Smit, and S. Spijker. Activity and impulsive action are controlled by different genetic and environmental factors. *Genes Brain Behav*, 8(8):817–28, 2009.

P.L. Lowrey and J.S. Takahashi. Genetics of circadian rhythms in Mammalian model organisms. *Adv Genet*, 74:175–230, 2011.

A. Manasco, S. Chang, J. Larriviere, L.L. Hamm, and M. Glass. Alcohol withdrawal. *South Med J*, 105(11):607–12, 2012.

H. Manev and T. Uz. Clock genes: influencing and being influenced by psychoactive drugs. *Trends Pharmacol Sci*, 27(4):186–9, 2006.

K. Mann, F. Kiefer, R. Spanagel, and J. Littleton. Acamprosate: recent findings and future research directions. *Alcohol Clin Exp Res*, 32(7):1105–10, 2008.

K. Mann, A. Bladström, L. Torup, A. Gual, and W. van den Brink. Extending the Treatment Options in Alcohol Dependence: A Randomized Controlled Study of As-Needed Nalmefene. *Biol Psychiatry*, S0006-3223(12):00942–0, 2012.

T.A. Manolio. Genomewide association studies and assessment of the risk of disease. *N Engl J Med*, 363(2):166–76, 2010.

C.G. Marfella, Y. Ohkawa, A.H. Coles, D.S. Garlick, S.N. Jones, and A.N. Imbalzano. Mutation of the SNF2 family member Chd2 affects mouse development and survival. *J Cell Physiol*, 209(1):162–71, 2006.

B.J. Mason, A.M. Goodman, S. Chabac, and P. Lehert. Effect of oral acamprosate on abstinence in patients with alcohol dependence in a double-blind, placebo-controlled trial: the role of patient motivation. *J Psychiatr Res*, 40(5):383–93, 2006.

S. Masubuchi, S. Honma, H. Abe, K. Ishizaki, M. Namihira, M. Ikeda, and K. Honma. Clock genes outside the suprachiasmatic nucleus involved in manifestation of locomotor activity rhythm in rats. *Eur J Neurosci*, 12(12):4206–14, 2000.

F. Matthäus, V.A. Smith, A. Fogtman, W.H. Sommer, F. Leonardi-Essmann, A. Lourdusamy, M.A. Reimers, R. Spanagel, and P.J. Gebicke-Haerter. Interactive molecular networks obtained by computer-aided conversion of microarray data from brains of alcohol-drinking rats. *Pharmacopsychiatry*, 42 Suppl1:S118–28, 2009.

M.M. Maxwell, E.M. Tomkinson, J. Nobles, J.W. Wizeman, A.M. Amore, L. Quinti, V. Chopra, S.M. Hersch, and A.G. Kazantsev. The Sirtuin 2 microtubule deacetylase is an abundant neuronal protein that accumulates in the aging CNS. *Hum Mol Genet*, 20 (20):3986–96, 2011.

R.R. McCrae and P.T. Costa. Validation of the five-factor model of personality across instruments and observers. *J Pers Soc Psychol*, 52(1):81–90, 1987.

R.E. McCullumsmith and J.H. Meador-Woodruff. Novel approaches to the study of postmortem brain in psychiatric illness: old limitations and new challenges. *Biol Psychiatry*, 69(2):127–33, 2011.

J.E. McCutcheon and M Marinelli. Age matters. *Eur J Neurosci*, 29(5):997–1014, 2009.

P.O. McGowan and M. Szyf. The epigenetics of social adversity in early life: implications for mental health outcomes. *Neurobiol Dis*, 39(1):66–72, 2010.

M.W. Meinhardt, A.C. Hansson, S. Perreau-Lenz, C. Bauder-Wenz, O. Stählin, M. Heilig, C. Harper, K.U. Drescher, R. Spanagel, and W. H. Sommer. Rescue of infralimbic mGluR2 deficit restores control over drug-seeking behavior in alcohol dependence. *J Neurosci*, 33(7):2794–806, 2013.

R.I. Melendez, J.F. McGinty, P.W. Kalivas, and H.C. Becker. Brain region-specific gene expression changes after chronic intermittent ethanol exposure and early withdrawal in C57BL/6J mice. *Addict Biol*, 17(2):351–64, 2012.

W.R. Miller, S.T. Walters, and M.E. Bennett. How effective is alcoholism treatment in the United States? *J Stud Alcohol*, 62(2):211–20, 2001.

F.G. Moeller, E.S. Barratt, D.M. Dougherty, J.M. Schmitz, and A.C. Swann. Psychiatric aspects of impulsivity. *Am J Psychiatry*, 158(11):1783–93, 2001.

J.A. Mohawk, C.B. Green, and J.S. Takahashi. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci*, 35:445–62, 2012.

M.P. Moisan, B. Llamas, M.N. Cook, and P. Mormède. Further dissection of a genomic locus associated with behavioral activity in the Wistar-Kyoto hyperactive rat, an animal model of hyperkinesis. *Mol Psychiatry*, 8(3):348–52, 2003.

A. Molander, H.H. Lidö, E. Löf, M. Ericson, and B. Söderpalm. The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male wistar rats. *Alcohol Alcohol*, 42(1):11–8, 2007.

P.E. Molina, K.I. Happel, P. Zhang, J.K. Kolls, and S. Nelson. Focus on: alcohol and the immune system. *Alcohol Res Health*, 33(1):97–108, 2010.

H.T. Mueller and J.H. Meador-Woodruff. NR3A NMDA receptor subunit mRNA expression in schizophrenia, depression and bipolar disorder. *Schizophr Res*, 71(2-3):361–70, 2004.

H. Muhle, S. von Spiczak, V. Gaus, S. Kara, I. Helbig, J. Hampe, A. Franke, Y. Weber, H. Lerche, A.A. Kleefuss-Lie, C.E. Elger, S. Schreiber, U. Stephani, and T. Sander. Role of GRM4 in idiopathic generalized epilepsies analysed by genetic association and sequence analysis. *Epilepsy Res*, 89(2-3):319–26, 2010.

M.K. Mulligan, I. Ponomarev, R.J. Hitzemann, J.K. Belknap, B. Tabakoff, R.A. Harris, J.C. Crabbe, Y.A. Blednov, N.J. Grahame, T.J. Phillips, D.A. Finn, P.L. Hoffman, V.R. Iyer, G.F. Koob, and S.E. Bergeson. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proc. Natl. Acad. Sci. U.S.A.*, 130(16): 6368–73, 2006.

E.R. Murphy, J.W. Dalley, and T.W. Robbins. Local glutamate receptor antagonism in the rat prefrontal cortex disrupts response inhibition in a visuospatial attentional task. *Psychopharmacology (Berl.)*, 179(1):99–107, 2005.

T. Nagatsu. Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology. *Essays Biochem*, 30:15–35, 1995.

P. Najt, J. Perez, M. Sanches, M.A. Peluso, D. Glahn, and J.C. Soares. Impulsivity and bipolar disorder. *Eur Neuropsychopharmacol*, 17(5):313–20, 2007.

K. Nakamura and O. Hikosaka. Role of Dopamine in the Primate Caudate Nucleus in Reward Modulation of Saccades. *J Neurosci*, 26(20):5360–9, 2006.

N. Nakaya, H.S. Lee, Y. Takada, I. Tzchori, and S.I. Tomarev. Zebrafish olfactomedin 1 regulates retinal axon elongation in vivo and is a modulator of Wnt signaling pathway. *J Neurosci*, 28(31):7900–10, 2008.

R.A. Nicholas. Identification of the P2Y12 Receptor: A Novel Member of the P2Y Family of Receptors Activated by Extracellular Nucleotides. *Mol Pharmacol*, 60(3):416–20, 2001.

S.L. Nichols and D.A. Waschbusch. A review of the validity of laboratory cognitive tasks used to assess symptoms of ADHD. *Child Psychiatry Hum Dev*, 34(4):2997–315, 2004.

F.A. Nielsen, D. Balslev, and L.K. Hansen. Mining the posterior cingulate: segregation between memory and pain components. *Neuroimage*, 27(3):520–32, 2005.

R.D. Oades and G.M. Halliday. Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. *Brain Res.*, 434(2):117–65, 1987.

K. Oishi, K. Sakamoto, T. Okada, T. Nagase, and N. Ishida. Antiphase circadian expression between BMAL1 and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem Biophy Res Commun*, 253(2):199–203, 1998.

M.F. Olive, H.N. Koenig, M.A. Nannini, and C.W. Hodge. Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine. *J Neurosci*, 21(23):RC184, 2001.

J.S. O'Neill and A.B. Reddy. Circadian clocks in human red blood cells. *Nature*, 469(7331): 498–503, 2011.

M. Otsuka and M. Yanagisawa. Pain and neurotransmitters. *Cell Mol Neurobiol*, 10(3): 293–302, 1990.

S. Pachernegg, N. Strutz-Seebohm, and M. Hollmann. GluN3 subunit-containing NMDA receptors: not just one-trick ponies. *Trends Neurosci*, 35(4):240–9, 2012.

M.G. Packard and B.J. Knowlton. Learning and memory functions of the basal ganglia. *Annu Rev Neurosci*, 25:563–93, 2002.

T.Y. Pang, T. Renoir, X. Du, A.J. Lawrence, and A.J. Hannan. Depression-related behaviours displayed by female C57BL/6J mice during abstinence from chronic ethanol consumption are rescued by wheel-running. *Eur J Neurosci*, 37(11):1803–10, 2013.

M.A. Patestas and L.P. Gartner. *A Textbook of Neuroanatomy*. Blackwell Publishers, 2006.

J.H. Patton, M.S. Stanford, and E.S. Barratt. Factor structure of the Barratt impulsiveness scale. *J Clin Psychol*, 51(6):768–74, 1995.

G. Paxinos and C. Watson. *The Rat Brain in Stereotaxic Coordinates*. Elsevier, 4 edition, 1998.

B. Peng, R.K. Yu, K.L. Dehoff, and C.I. Amos. Normalizing a large number of quantitative traits using empirical normal quantile transformation. *BMC Proc*, 1 Suppl 1:S156, 2007.

J.L. Perry and M.E. Carroll. The role of impulsive behavior in drug abuse. *Psychopharmacology (Berl.)*, 200(1):1–26, 2008.

C.Y. Pietersen, M.P. Lim, L. Macey, T.U. Woo, and K.C. Sonntag. Neuronal type-specific gene expression profiling and laser-capture microdissection. *Methods Mol Biol*, 755:327–43, 2011.

H.H. Pothuizen, A.L. Jongen-Rêlo, J. Feldon, and B.K. Yee. Double dissociation of the effects of selective nucleus accumbens core and shell lesions on impulsive-choice behaviour and salience learning in rats. *Eur J Neurosci*, 22(10):2605–16, 2005.

M. Qiang, A.D. Denny, and M.K. Ticku. Chronic intermittent ethanol treatment selectively alters N-methyl-D-aspertate receptor subunit surface expression in cultured cortical neurons. *Mol Pharmacol*, 72(1):95–102, 2007.

L. Quartara and C.A. Maggi. The tachykinin NK1 receptor. Part I: Ligands and mechanisms of cellular activation. *Neuropeptides*, 31(6):537–563, 1997.

J. Radua and D. Mataix-Cols. Voxel-wise meta-analysis of grey matter changes in obsessive-compulsive disorder. *Br J Psychiatry*, 195(5):393–402, 2009.

M. Randić, H. Hećimović, and P.D. Ryu. Substance P modulates glutamate-induced currents in acutely isolated rat spinal dorsal horn neurons. *Neurosci Lett*, 117(1-2):74–80, 1990.

R.C. Raymond, M. Warren, R.W. Morris, and J.B. Leikin. Periodicity of presentations of drugs of abuse and overdose in an emergency department. *J Toxicol Clin Toxicol*, 30(3): 467–78, 1992.

W. Renthal, A. Kumar, G. Xiao, M. Wilkinson, H.E.3rd. Covington, I. Maze, D. Sikder, A.J. Robison, Q. LaPlant, D.M. Dietz, S.J. Russo, V. Vialou, S. Chakravarty, T.J. Kodadek, M. Stack, A. Kabbaj, and E.J. Nestler. Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. *Neuron*, 62(3):335–48, 2009.

A.F. Ribeiro, D. Correia, A.A. Torres, G.R. Boas, A.V. Rueda, R. Camarini, S. Chiavegatto, R. Boerngen-Lacerda, and A.L. Brunialti-Godard. A transcriptional study in mice with different ethanol-drinking profiles: possible involvement of the GABA(B) receptor. *Pharmacol Biochem Behav*, 102(2):224–32, 2012.

M.J. Robbins, K.J. Charles, D.C. Harrison, and M.N. Pangalos. Localisation of the GPRC5B receptor in the rat brain and spinal cord. *Mol Brain Res*, 106(1-2):136–144, 2002.

T.W. Robbins. The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology (Berl.)*, 163(3-4):362–380, 2002.

Z.A. Rodd, R.L. Bell, H.J. Sable, J.M. Murphy, and W.J. McBride. Recent advances in animal models of alcohol craving and relapse. *Pharmacol Biochem Behav*, 79(3):439–50, 2004.

R.S. Ronimus and H.W. Morgan. Cloning and biochemical characterization of a novel mouse ADP-dependent glucokinase. *Biochem Biophy Res Commun*, 315(3):652–8, 2004.

A.M. Rosenwasser and M.C. Fixaris. Chronobiology of alcohol: Studies in C57BL/6J and DBA/2J inbred mice. *Physiol Behav*, 110-111:140–7, 2013.

A.M. Rosenwasser, M.E. Fecteau, and R.W. Logan. Effects of ethanol intake and ethanol withdrawal on free-running circadian activity rhythms in rats. *Physiol Behav*, 84(4): 537–42, 2005.

G.J. Royce and E.J. Laine. Efferent connections of the caudate nucleus, including cortical projections of the striatum and other basal ganglia: An autoradiographic and horseradish peroxidase investigation in the cat. *J Comp Neurol*, 226(1):28–49, 2004.

K. Rubia, T. Russell, S. Overmeyer, M.J. Brammer, E.T. Bullmore, T. Sharma, A. Simmons, S.C. Williams, V. Giampietro, C.M. Andrew, and E. Taylor. Mapping motor inhibition: conjunctive brain activations across different versions of go/no-go and stop tasks. *Neuroimage*, 13(2):250–61, 2001.

S. Rösner, A. Hackl-Herrwerth, S. Leucht, P. Lehert, S. Vecchi, and M. Soyka. Acamprosate for alcohol dependence. *Cochrane Database Syst Rev*, 8(9):CD004332, 2010.

L. Sachs and J. Hedderich. *Angewandte Statistik*. Springer, 12 edition, 2006.

A. Sahu. Intracellular leptin-signaling pathways in hypothalamic neurons: the emerging role of phosphatidylinositol-3 kinase-phosphodiesterase-3B-cAMP pathway. *Neuroendocrinology*, 93(4):201–10, 2011.

F. Saint-Preux, L.R. Bores, I. Tulloch, B. Ladenheim, R. Kim, P.K. Thanos, N.D. Volkow, and J.L. Cadet. Chronic co-administration of nicotine and methamphetamine causes differential expression of immediate early genes in the dorsal striatum and nucleus accumbens of Rats. *Neuroscience*, 243:89–96, 2013.

R. Sandberg and O. Larsson. Improved precision and accuracy for microarrays using updated probe set definitions. *BMC bioinformatics*, 8:48, 2007.

M. Sazdanović, P. Sazdanović, I. Zivanović-Macuzić, V. Jakovljević, D. Jeremić, A. Peljto, and J. Tosevski. Neurons of the human nucleus accumbens. *Vojnosanit Pregl*, 68(8): 655–60, 2011.

F.A. Scheer, C. Pirovano, E.J. Van Someren, and R.M. Buijs. Environmental light and suprachiasmatic nucleus interact in the regulation of body temperature. *Neuroscience*, 132(2):465–77, 2005.

C. Scheiermann, Y. Kunisaki, and P.S. Frenette. Circadian control of the immune system. *Nat Rev Immunol*, 13(3):190–8, 2013.

M. Schmitz, R. Frey, P. Pichler, H. Röpke, P. Anderer, B. Saletu, and S. Rudas. Sleep quality during alcohol withdrawal with bright light therapy. *Prog Neuropsychopharmacol Biol Psychiatry*, 21(6):965–77, 1997.

G. Schumann, L. J. Coin, A. Lourdusamy, P. Charoen, K. H. Berger, D. Stacey, S. Desrivieres, F. A. Aliev, A. A. Khan, N. Amin, Y. S. Aulchenko, G. Bakalkin, S. J. Bakker, B. Balkau, J. W. Beulens, A. Bilbao, R. A. de Boer, D. Beury, M. L. Bots, E. J. Breetvelt,

S. Cauchi, C. Cavalcanti-Proenca, J. C. Chambers, T. K. Clarke, N. Dahmen, E. J. de Geus, D. Dick, F. Ducci, A. Easton, H. J. Edenberg, T. Esko, T. Esk, A. Fernandez-Medarde, T. Foroud, N. B. Freimer, J. A. Girault, D. E. Grobbee, S. Guarrera, D. F. Gudbjartsson, A. L. Hartikainen, A. C. Heath, V. Hesselbrock, A. Hofman, J. J. Hottenga, M. K. Isohanni, J. Kaprio, K. T. Khaw, B. Kuehnel, J. Laitinen, S. Lobbens, J. Luan, M. Mangino, M. Maroteaux, G. Matullo, M. I. McCarthy, C. Mueller, G. Navis, M. E. Numans, A. Nunez, D. R. Nyholt, C. N. Onland-Moret, B. A. Oostra, P. F. O'Reilly, M. Palkovits, B. W. Penninx, S. Polidoro, A. Pouta, I. Prokopenko, F. Ricceri, E. Santos, J. H. Smit, N. Soranzo, K. Song, U. Sovio, M. Stumvoll, I. Surakk, T. E. Thorgeirsson, U. Thorsteinsdottir, C. Troakes, T. Tyrfingsson, A. Tonjes, C. S. Uiterwaal, A. G. Uitterlinden, P. van der Harst, Y. T. van der Schouw, O. Staehlin, N. Vogelzangs, P. Vollenweider, G. Waeber, N. J. Wareham, D. M. Waterworth, J. B. Whitfield, E. H. Wichmann, G. Willemsen, J. C. Witteman, X. Yuan, G. Zhai, J. H. Zhao, W. Zhang, N. G. Martin, A. Metspalu, A. Doering, J. Scott, T. D. Spector, R. J. Loos, D. I. Boomsma, V. Mooser, L. Peltonen, K. Stefansson, C. M. van Duijn, P. Vineis, W. H. Sommer, J. S. Kooner, R. Spanagel, U. A. Heberlein, M. R. Jarvelin, and P. Elliott. Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. *Proc. Natl. Acad. Sci. U.S.A.*, 108(17):7119–7124, 2011.

W.J. Schwartz, A. Jr. Carpino, H.O. de la Iglesia, R. Baler, D.C. Klein, Y. Nakabeppu, and N. Aronin. Differential regulation of fos family genes in the ventrolateral and dorsomedial subdivisions of the rat suprachiasmatic nucleus. *Neuroscience*, 98(3):535–47, 2000.

C. Seneviratne, N. Ait-Daoud, J.Z. Ma, G. Chen, B.A. Johnson, and M.D. Li. Susceptibility locus in neurokinin-1 receptor gene associated with alcohol dependence. *Neuropsychopharmacology*, 34(11):2442–9, 2009.

P. Sengupta. A Scientific Review of Age Determination for a Laboratory Rat: How Old is it in Comparison with Human Age? *Biomedicine International*, 2:81–89, 2011.

D. Sheedy, T. Garrick, I. Dedova, C. Hunt, R. Miller, N. Sundqvist, and C. Harper. An Australian Brain Bank: a critical investment with a high return! *Cell Tissue Bank*, 9 (3):205–16, 2008.

H. Shibata, A. Tani, T. Chikuhara, R. Kikuta, M. Sakai, H. Ninomiya, N. Tashiro, N. Iwata, N. Ozaki, and Y. Fukumaki. Association study of polymorphisms in the group III metabotropic glutamate receptor genes, GRM4 and GRM7, with schizophrenia. *Psychiatry Res*, 167(1-2):88–96, 2009.

Y. Shigeri, R.P. Seal, and K. Shimamoto. Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain Res Brain Res Rev*, 45(3):250–65, 2004.

J.D. Sinclair. Evidence about the use of naltrexone and for different ways of using it in the treatment of alcoholism. *Alcohol Alcohol.*, 36(1):2–10, 2001.

J.H. Sitz, K. Baumgärtel, B. Hämmerle, C. Papadopoulos, P. Hekerman, F.J. Tejedor, W. Becker, and B. Lutz. The Down syndrome candidate dual-specificity tyrosine phosphorylation-regulated kinase 1A phosphorylates the neurodegeneration-related septin 4. *Neuroscience*, 157(3):596–605, 2008.

J.A. Siuciak, S.A. McCarthy, D.S. Chapin, T.M. Reed, C.V. Vorhees, and D.R. Repaske. Behavioral and neurochemical characterization of mice deficient in the phosphodiesterase-1B (PDE1B) enzyme. *Neuropharmacology*, 53(1):113–24, 2007.

Y. Smith and A. Parent. Differential connections of caudate nucleus and putamen in the squirrel monkey (Saimiri sciureus). *Neuroscience*, 18(2):347–71, 1986.

R. Spanagel. Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol Rev*, 89(2):649–705, 2009.

R. Spanagel and M. Heilig. Addiction and its brain science. *Addiction*, 100(12):1813–22, 2005.

R. Spanagel and S. M. Hölter. Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism? *Alcohol Alcohol.*, 34(2):231–243, 1999.

R. Spanagel and F. Kiefer. Drugs for relapse prevention of alcoholism: ten years of progress. *Trends Pharmacol Sci*, 29(3):109–15, 2008.

R. Spanagel and F. Weiss. The dopamine hypothesis of reward: past and current status. *Trends Neurosci*, 22(11):521–7, 1999.

R. Spanagel, G. Pendyala, C. Abarca, T. Zghoul, C. Sanchis-Segura, M.C. Magnone, J. Lascorz, M. Depner, D. Holzberg, M. Soyka, S. Schreiber, F. Matsuda, M. Lathrop, G. Schumann, and U. Albrecht. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med*, 11(1):35–42, 2005.

S.L. Spurgeon, R.C. Jones, and R. Ramakrishnan. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. *PLoS One*, 3(2):e1662, 2008.

D. Stacey, A. Bilbao, M. Maroteaux, T. Jia, A. C. Easton, S. Longueville, C. Nymberg, T. Banaschewski, G. J. Barker, C. Buchel, F. Carvalho, P. J. Conrod, S. Desrivieres, M. Fauth-Buhler, A. Fernandez-Medarde, H. Flor, J. Gallinat, H. Garavan, A. L. Bokde, A. Heinz, B. Ittermann, M. Lathrop, C. Lawrence, E. Loth, A. Lourdusamy, K. F. Mann, J. L. Martinot, F. Nees, M. Palkovits, T. Paus, Z. Pausova, M. Rietschel, B. Ruggeri, E. Santos, M. N. Smolka, O. Staehlin, M. R. Jarvelin, P. Elliott, W. H. Sommer, M. Mameli, C. P. Muller, R. Spanagel, J. A. Girault, G. Schumann, and the IMAGEN Consortium. RASGRF2 regulates alcohol-induced reinforcement by influencing mesolimbic dopamine neuron activity and dopamine release. *Proc. Natl. Acad. Sci. U.S.A.*, 18 (51):21128–21133, 2012.

A.D. Stan, S. Ghose, X.M. Gao, R.C. Roberts, K. Lewis-Amezcua, K.J. Hatanpaa, and Tamminga C.A. Human Postmortem Tissue: What Quality Markers Matter? *Brain Res.*, 1123(1):1–11, 2006.

P.F. Sullivan, C. Fan, and C.M. Perou. Evaluating the comparability of gene expression in blood and brain. *Am J Med Genet B Neuropsychiatr Genet*, 141B(3):261–8, 2006.

R. Sultana, C.E. Yu, J. Yu, J. Munson, D. Chen, W. Hua, A. Estes, F. Cortes, F. de la Barra, D. Yu, S.T. Haider, B.J. Trask, E.D. Green, W.H. Raskind, C.M. Disteche, E. Wijsman, G. Dawson, D.R. Storm, G.D. Schellenberg, and E.C. Villacres. Identification of a novel gene on chromosome 7q11.2 interrupted by a translocation breakpoint in a pair of autistic twins. *Genomics*, 80(2):129–34, 2002.

R.M. Swift. Naltrexone and nalmefene: any meaningful difference? *Biol Psychiatry*, 73 (8):700–1, 2013.

C. Taccioli, J. Tegnér, V. Maselli, D. Gomez-Cabrero, G. Altobelli, W. Emmett, F. Lescai, S. Gustincich, and E. Stupka. ParkDB: a Parkinson's disease gene expression database. *Database (Oxford)*, 2011:bar007, 2011.

Y. Takeda, K.B. Chou, J. Takeda, B.S. Sachais, and J.E. Krause. Molecular cloning, structural characterization and functional expression of the human substance P receptor. *Biochem Biophy Res Commun*, 179(3):1232–40, 1991.

R. Taylor. Interpretation of the Correlation Coefficient: A Basic Review. *JDMS*, 1:35–39, 1990.

M. Teboul, M.A. Barrat-Petit, X.M. Li, B. Claustrat, J.L. Formento, F. Delaunay, F. Lévi, and G. Milano. Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. *J Mol Med (Berl)*, 83(9):693–9, 2005.

SW Thompson. "Daimon Drink": Ancient Greek and Roman Explanations for Drunkenness. *Christian Spirituality and Science*, 8(1):1–19, 2010.

H. Tomita, M.P. Vawter, D.M. Walsh, S.J. Evans, P.V. Choudary, J. Li, K.M. Overman, M.E. Atz, R.M. Myers, E.G. Jones, S.J. Watson, H. Akil, and W.E.Jr. Bunney. Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. *Biol Psychiatry*, 55(4):346–52, 2004.

J.A. Trapani. Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B. *Aust N Z J Med*, 25(6):793–9, 1995.

M. Trepel. *Neuroanatomie: Struktur und Funktion.* Urban & Fischer Verlag/Elsevier GmbH, 4th edition, 2008.

P. Trifilieff and D. Martinez. Imaging addiction: D2 receptors and dopamine signaling in the striatum as biomarkers for impulsivity. *Neuropharmacology*, [Epub ahead of print]pii: S0028-3908(13):00300–6, 2013.

I. Ubeda-Bañon, A. Novejarque, A. Mohedano-Moriano, P. Pro-Sistiaga, C. de la Rosa-Prieto, R. Insausti, F. Martinez-Garcia, E. Lanuza, and A. Martinez-Marcos. Projections from the posterolateral olfactory amygdala to the ventral striatum: neural basis for reinforcing properties of chemical stimuli. *BMC Neurosci*, 8:103, 2007.

N.A. Uranova, V.M. Vostrikov, D.D. Orlovskaya, and V.I. Rachmanova. Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. *Schizophr Res*, 67(2-3):269–75, 2004.

T. Uz, M. Akhisaroglu, R. Ahmed, and H. Manev. The pineal gland is critical for circadian Period1 expression in the striatum and for circadian cocaine sensitization in mice. *Neuropsychopharmacology*, 28(12):2117–23, 2003.

H.H. van Rossum, F.P. Romijn, K.J. Sellar, N.P. Smit, P.J. van der Boog, J.W. de Fijter, and J. van Pelt. Variation in leukocyte subset concentrations affects calcineurin activity measurement: implications for pharmacodynamic monitoring strategies. *Clin Chem*, 54 (3):517–24, 2008.

J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, 3(7):RESEARCH0034, 2002.

G. Varga, A. Szekely, P. Antal, P. Sarkozy, Z. Nemoda, Z. Demetrovics, and M. Sasvari-Szekely. Additive effects of serotonergic and dopaminergic polymorphisms on trait impulsivity. *Am J Med Genet B Neuropsychiatr Genet*, 159B(3):281–8, 2012.

L.F. Vendruscolo, E. Terenina-Rigaldie, F. Raba, A. Ramos, R.N. Takahashi, and P. Mormède. A QTL on rat chromosome 7 modulates prepulse inhibition, a neuro-behavioral trait of ADHD, in a Lewis x SHR intercross. *Behav Brain Funct*, 12:2–21, 2006.

V. Vengeliene, F. Leonardi-Essmann, W.H. Sommer, H.M. Marston, and R. Spanagel. Glycine transporter-1 blockade leads to persistently reduced relapse-like alcohol drinking in rats. *Biol Psychiatry*, 68(8):704–11, 2010.

B.A. Vogt and D.N. Pandya. Cingulate cortex of the rhesus monkey: II. Cortical afferents. *J Comp Neurol*, 262(2):271–89, 1987.

B.A. Vogt and G. Paxinos. Cytoarchitecture of mouse and rat cingulate cortex with human homologies. *Brain Struct Funct*, [Epub ahead of print]:1–8, 2012.

B.A. Vogt, D.N. Pandya, and D.L. Rosene. Cingulate cortex of the rhesus monkey: I. Cytoarchitecture and thalamic afferents. *J Comp Neurol*, 262(2):256–70, 1987.

B.A. Vogt, E.A. Nimchinsky, L.J. Vogt, and P.R. Hof. Human cingulate cortex: Surface features, flat maps, and cytoarchitecture. *J Comp Neurol*, 359(3):490–506, 2004.

U.S. von Euler and J.H. Gaddum. An unidentified depressor substance in certain tissue extracts. *J Physiol*, 72:74–87, 1931.

M. Wan, K. Hejjas, Z. Ronai, Z. Elek, M. Sasvari-Szekely, F.A. Champagne, A. Miklósi, and E. Kubinyi. DRD4 and TH gene polymorphisms are associated with activity, impulsivity and inattention in Siberian Husky dogs. *Anim Genet*, [Epub ahead of print], 2013.

J.A. Wasielewski and F.A. Holloway. Alcohol's interactions with circadian rhythms. A focus on body temperature. *Alcohol Res Health*, 25(2):94–100, 2001.

M. Watanabe, A. Hida, S. Kitamura, M. Enomoto, Y. Ohsawa, Y. Katayose, K. Nozaki, Y. Moriguchi, S. Aritake, S. Higuchi, M. Tamura, M. Kato, and K. Mishima. Rhythmic expression of circadian clock genes in human leukocytes and beard hair follicle cells. *Biochem Biophys Res Commun*, 425(4):902–7, 2012.

D.R. Weaver. The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms*, 13 (2):100–12, 1998.

H. Weiner, R.D. Myers, C.W. Simpson, and M.S. Thurman. The Effect of Alcohol on Dopamine Metabolism in the Caudate Nucleus of an Unanesthetized Monkey. *Alcohol Clin Exp Res*, 4(4):427–30, 1980.

H. Wijnen. Circadian rhythms. A circadian loop asSIRTs itself. *Science*, 324(5927):598–9, 2009.

C.A. Winstanley, D.M. Eagle, and T.W. Robbins. Behavioral models of impulsivity in relation to ADHD: translation between clinical and preclinical studies. *Clin Psychol Rev*, 26(4):379–95, 2006.

R.A. Wise and M.A. Bozarth. Brain reward circuitry: four circuit elements "wired" in apparent series. *Brain Res Bull*, 12(2):203–8, 1984.

J.T. Wolstenholme, J.A. Warner, M.I. Capparuccini, K.J. Archer, K.L. Shelton, and M.F. Miles. Genomic analysis of individual differences in ethanol drinking: evidence for non-genetic factors in C57BL/6 mice. *PLoS One*, 6(6):e21100, 2011.

A.H. Wong, B.K. Lipska, O. Likhodi, E. Boffa, D.R. Weinberger, J.L. Kennedy, and H.H. Van Tol. Cortical gene expression in the neonatal ventral-hippocampal lesion rat model. *Schizophr Res*, 77(2-3):261–70, 2005.

H. Yan, L. Tian, W. Yan, J. Sun, Q. Liu, Y.B. Zhang, X.M. Li, Y.F. Zhang, and D. Zhang. Functional and anatomical connectivity abnormalities in cognitive division of anterior cingulate cortex in schizophrenia. *PLoS One*, 7(9):e45659, 2012.

Q.S. Yan, M.J. Feng, and S.E. Yan. Different expression of brain-derived neurotrophic factor in the nucleus accumbens of alcohol-preferring (P) and -nonpreferring (NP) rats. *Brain Res*, 1035(2):215–8, 2005.

J. Zalewska-Kaszubska, B. Bajer, D. Gorska, D. Andrzejczak, W. Dyr, and P. Bieńkowski. Effect of repeated treatment with topiramate on voluntary alcohol intake and beta-endorphin plasma level in Warsaw alcohol high-preferring rats. *Psychopharmacology (Berl.)*, 225(2):275–81, 2013.

A. Zavada, M.C. Gordijn, D.G. Beersma, S. Daan, and T. Roenneberg. Comparison of the Munich Chronotype Questionnaire with the Horne-Ostberg's Morningness-Eveningness Score. *Chronobiol Int*, 22(2):267–78, 2005.

M.L. Zeise, S.G. Madamba, G.R. Siggins, J. Putzke, and W. Ziegelgansberger. The anti-craving substance Acamprosate reduces glutamatergic synaptic transmission and a high threshold calcium current in neocortical and hippocampal pyramidal neurons. *Alcohol Clin Exp Res*, 18(2):36A, 1994.

H. Zhang, H.R. Kranzler, B.Z. Yang, X. Luo, and J. Gelernter. The OPRD1 and OPRK1 loci in alcohol or drug dependence: OPRD1 variation modulates substance dependence risk. *Mol Psychiatry*, 13(5):531–43, 2008.

J. Zhang, X. Dong, Y. Fujimoto, and H. Okamura. Molecular signals of Mammalian circadian clock. *Kobe J Med Sci*, 50(3-4):101–9, 2004.

J. Zhang, J. Baran, A. Cros, J.M. Guberman, S. Haider, J. Hsu, Y. Liang, E. Rivkin, J. Wang, B. Whitty, M. Wong-Erasmus, L. Yao, and A. Kasprzyk. International Cancer Genome Consortium Data Portal–a one-stop shop for cancer genomics data. *Database (Oxford)*, 2011:bar026, 2011.

B. Zheng, D.W. Larkin, U. Albrecht, Z.S. Sun, M. Sage, G. Eichele, C.C. Lee, and A. Bradley. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature*, 400(6740):169–73, 1999.

Publications with content from this thesis

Pitzoi S, Petretto E, Mar AC, Theobald DE, Yolanda Pena O, Jordan ER, Atanur S, Srivastava P, Saar K, Huebner N, Sommer WH, Staehlin O, Spanagel R, Robinson ES, Schuhmann G, Everitt BJ, Robbins T, Schumann G, Aitman TJ, Dalley JW (-) Integrated transcriptional profiling and linkage analysis of high impulsive behaviour in rats. Status: in preparation.

Stacey D, Bilbao A, Maroteaux M, Jia T, Easton AC, Longueville S, Nymberg C, Banaschewski T, Barker GJ, Büchel C, Carvalho F, Conrod P, Desrivières S, Fauth-Buehler M, Fernandez-Medarde A, Flor H, Gallinat J, Garavan H, Bokde A, Heinz A, Ittermann B, Lathrop M, Lawrence C, Loth E, Lourdusamy A, Mann K, Martinot J-L, Nees F, Palkovits M, Paus T, Pausova Z, Rietschel M, Ruggeri B, Santos E, Smolka MN, Staehlin O, Jarvelin MJ, Elliott P, Sommer WH, Mameli M, Müller CP, Spanagel R, Girault JA, Schumann G, and the IMAGEN consortium (`www.imagen-europe.com`)(2012) RASGRF2 regulates alcohol-induced reinforcement by influencing mesolimbic dopamine neuron activity and dopamine release. *Proc Natl Acad Sci U.S.A.* 109(51):21128-33.
doi: 10.1073/pnas.1211844110

G. Schumann, L. J. Coin, A. Lourdusamy, P. Charoen, K. H. Berger, D. Stacey, S. Desriv- ieres, F. A. Aliev, A. A. Khan, N. Amin, Y. S. Aulchenko, G. Bakalkin, S. J. Bakker, B. Balkau, J. W. Beulens, A. Bilbao, R. A. de Boer, D. Beury, M. L. Bots, E. J. Breetvelt, S. Cauchi, C. Cavalcanti-Proenca, J. C. Chambers, T. K. Clarke, N. Dahmen, E. J. de Geus, D. Dick, F. Ducci, A. Easton, H. J. Edenberg, T. Esko, T. Esk, A. Fernandez-Medarde, T. Foroud, N. B. Freimer, J. A. Girault, D. E. Grobbee, S. Guarrera, D. F. Gudbjartsson, A. L. Hartikainen, A. C. Heath, V. Hesselbrock, A. Hofman, J. J. Hot- tenga, M. K. Isohanni, J. Kaprio, K. T. Khaw, B. Kuehnel, J. Laitinen, S. Lobbens, J. Luan, M. Mangino, M. Maroteaux, G. Matullo, M. I. McCarthy, C. Mueller, G. Navis, M. E. Numans, A. Nunez, D. R. Nyholt, C. N. Onland-Moret, B. A. Oostra, P. F. O'Reilly, M. Palkovits, B. W. Penninx, S. Polidoro, A. Pouta, I. Prokopenko, F. Ric- ceri, E. Santos, J. H. Smit, N. Soranzo, K. Song, U. Sovio, M. Stumvoll, I. Surakk, T. E. Thorgeirsson, U. Thorsteinsdottir, C. Troakes, T. Tyrfingsson, A. Tonjes, C. S. Uiterwaal, A. G. Uitterlinden, P. van der Harst, Y. T. van der Schouw, O. Staehlin, N. Vogelzangs, P. Vollenweider, G. Waeber, N. J. Wareham, D. M. Waterworth, J. B. Whitfield, E. H. Wichmann, G. Willemsen, J. C. Witteman, X. Yuan, G. Zhai, J. H. Zhao, W. Zhang, N. G. Martin, A. Metspalu, A. Doering, J. Scott, T. D. Spector, R. J. Loos, D. I. Boomsma, V. Mooser, L. Peltonen, K. Stefansson, C. M. van Duijn, P. Vineis, W. H. Sommer, J. S. Kooner, R. Spanagel, U. A. Heberlein, M. R. Jarvelin, and P. Elliott (2011) Genome-wide association

and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. *Proc Natl Acad Sci U.S.A.* 108(17):7119-24. doi: 10.1073/pnas.1017288108

Acknowledgments

My sincere gratitude goes to Professor Spanagel for accepting me at the Institute of Psychopharmacology and for giving me the opportunity to pursue a PhD in the field of addiction biology. I wish to thank Dr. Wolfgang Sommer for the overall guidance throughout my PhD studies and the abundance of scientific input he has given me. I'm also very thankful to Prof. Gebicke-Härter for sharing his ideas with me and letting me participate in the study on circadian rhythms in human blood.

I'm particularly grateful to Elisabeth, our technical assistant, for all her support and advise. I owe a debt of gratitude to Stéphanie Perreau-Lenz who introduced me so enthusiastically to circadian rhythm research.

My deepest thanks go to all the people who shared the office with me: Marcus Meinhardt, Laura Broccoli, Natalie Hirth, Stefanie Uhrig and all the others students and guests who shared their ideas so wonderfully and helped me stay upbeat. I'm also indebted to Fernando Leonardi-Essmann who introduced me to the statistical software R and who has been a great partner in many fruitful discussions about my projects and addiction research in general.

I also wish to thank all the other people of my research group. Valentina Vengeliene, Anita Hansson, Rick Bernardi, Ainhoa Bilbao Leis, Miriam Schneider, Chris Friemel, Peggy Schneider, Hamid Noori and Briac Halbout helped me in so many ways and I'm deeply thankful for that.

Special thanks go to Josef Frank, whose readiness to share his statistical and R programming skills was of inestimable value to me. My sincere gratitude goes also to Franziska Matthäus and Lily Villarín Pildaín from the Interdisciplinary Center for Scientific Computing in Heidelberg who provided vital input to the circadian rhythm studies, shared their technical expertise and taught me how to set up a proper LaTeX document. The numerous consultations with Dr. Jennen-Steinmetz from the department of Biostatistics at the Central Institute of Mental Health have always been very helpful. I'm very thankful for this excellent support.

I also wish to express my thanks to the staff of the microarray core facility of the DKFZ and the respective unit of the university medical centre in Mannheim. Sabine Henze, Oliver Heil, Melanie Bewerunge-Hudler and Maria Sailer were all very helpful in overcoming technical hurdles so that we could obtain the different gene expression data sets.

There surely would be many more names which I should mention here. I hope their missing will not be regarded as a sign of ingratitude and that you will indulgently overlook the shortcomings of my memory.

Last but not least I wish to thank my family and friends for their ongoing support.