

# An integrated genome research network for studying the genetics of alcohol addiction

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## ABSTRACT

Alcohol drinking is highly prevalent in many cultures and contributes to the global burden of disease. In fact, it was shown that alcohol constitutes 3.2% of all worldwide deaths in the year 2006 and is linked to more than 60 diseases, including cancers, cardiovascular diseases, liver cirrhosis, neuropsychiatric disorders, injuries and foetal alcohol syndrome. Alcoholism, which has been proven to have a high genetic load, is one potentially fatal consequence of chronic heavy alcohol consumption, and may be regarded as one of the most prevalent neuropsychiatric diseases afflicting our society today. The aim of the integrated genome research network '*Genetics of Alcohol Addiction*'—which is a German inter-/trans-disciplinary life science consortium consisting of molecular biologists, behavioural pharmacologists, system biologists with mathematicians, human geneticists and clinicians—is to better understand the genetics of alcohol addiction by identifying and validating candidate genes and molecular networks involved in the aetiology of this pathology. For comparison, addictive behaviour to other drugs of abuse (e.g. cocaine) is studied as well. Here, we present an overview of our research consortium, the current state of the art on genetic research in the alcohol field, and list finally several of our recently published research highlights. As a result of our scientific efforts, better insights into the molecular and physiological processes underlying addictive behaviour will be obtained, new targets and target networks in the addicted brain will be defined, and subsequently, novel and individualized treatment strategies for our patients will be delivered.

**Keywords** Alcoholism, drug addiction, genome-wide association study (GWAS), glutamate, imaging genetics, QTL analysis.

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## INFRASTRUCTURE AND AIMS OF THE GERMAN INTEGRATED GENOME RESEARCH NETWORK FOR STUDYING THE GENETICS OF ALCOHOL ADDICTION

Following 6 years of substantial funding within the framework of the *Nationales Genomforschungsnetz* (NGFN) for genetic studies in alcohol addiction, and having realized major achievements in this research area (see Research Highlights), we have now obtained further funding for an inter-/trans-disciplinary life science consortium consisting of molecular biologists, behavioural pharmacologists, system biologists with mathematicians, human geneticists and clinicians. In four project areas, 15 subprojects have been implemented in a highly integrative manner. Twenty-five principal investigators and almost 40 full-time researchers and PhD students now work in our German integrated genome research network *Genetics of Alcohol Addiction* (see <http://www.ngfn-alkohol.de>). The continuation of NGFN funding will ultimately help in consolidating excellent addiction research clusters in Germany (in Mannheim, Bonn, Berlin and Munich) (Mann 2010).

Our consortium takes advantage of the entire spectrum of state-of-the-art methodologies to study the genetics of alcoholism. Because of the fact that excellent animal models are available in this particular research field (Sanchis-Segura and Spanagel, 2006; Crabbe 2010) identification and functional characterization of genes using forward and reverse genetic approaches are applied. We are also using a variety of neuroimaging techniques to define endophenotypes in alcohol-dependent patients and laboratory animals, and will correlate them with genetic variations. We therefore employ in our consortium extensive functional characterization in alcohol-related endophenotypes and use appropriate animal models to test whether genes suggested through genome-wide association (GWA) studies influence excessive alcohol drinking behaviour and dependence. For functional validation of candidate genes, reverse genetic approaches are used. The most common reverse genetic approach is the generation of a conventional mouse knockout model and its subsequent behavioural analysis. However, the generation of a conventional knockout model is time consuming, cost-intensive, has no tissue specificity, and because the gene is ablated early in development, numerous compensatory mechanisms may ensue. Through more advanced techniques such as Cre/loxP and tetracycline-inducible systems, a gene of interest can be expressed or inactivated in a tissue-specific and time-controlled manner (Gavériaux-Ruff & Kieffer 2007). Although those conditional knockout models are of very high value for the neuroscience community, they still do

not provide a good rationale for large-scale functional validation of candidate genes because there is still an enormous effort to generate those models. As an alternative, the use of viral vectors for gene delivery offer many advantages for rapid functional validation studies. In particular, the advent of adeno-associated virus (AAV) vectors carrying cDNA for—or short hairpin RNA against—specific genes allows the rapid manipulation of gene function (Klugmann & Szumlanski 2008).

The overall theme of our research consortium is on the genetics of alcohol addiction, with the aim of identifying and validating candidate genes and molecular networks involved in the aetiology of this pathology. Importantly, this genetic and molecular information will guide us in the development of new medication strategies for alcohol-dependent patients.

Two research strategies are implemented: first, we are using transcriptomic, genetic and neuroimaging data sets derived from our previous NGFN and European Union-funded projects [i.e. TARGALC (Gebicke-Haerter & Sommer 2005), GENADDICT (<http://www.genaddict.co.uk>) and IMAGEN (Schumann *et al.* 2010)] and several GWA studies (GWAS) to extend our research projects into a systematic approach to identify more genes and molecular networks involved in excessive alcohol consumption and addiction. We are submitting all relevant information from our consortium, spanning from genomic data to endophenotyping and phenotyping data, to our computational unit on mathematical modelling to simulate disease progression. One goal of this systems genomic approach is to provide an understanding of the complex mapping relationship between the genome and disease by investigating intermediate endophenotypes. Hence, we will strictly follow a systems-oriented perspective in which the interactions and dynamics of genetic factors, transmitter release, neuronal network activity and pathological behaviour are centrally integrated (Gebicke-Haerter & Tretter 2008; Spanagel 2009; Kalant 2010; Spanagel 2010a). Beyond simulation of disease progression, we also believe that the datasets we generate can be integrated into *in silico* tools that allow prediction of therapeutic targets (*in silico* psychopharmacology).

In addition, we are taking a hypothesis-driven strategy, in which the involvement of the glutamatergic system in addictive behaviour is studied in great detail. In this context, genetically engineered mouse and rat models are used to study key elements of the glutamatergic system in alcohol drinking, seeking and relapse behaviour. Several mutant mouse and rat lines will be further tested for intravenous cocaine self-administration, food responding and reinstatement of drug- or food-seeking behaviour in order to establish a more comprehensive understanding of the functional involvement of a particular gene in addictive behaviour.

For measuring these behaviours, we are employing highly standardized and validated animal models (Sanchis-Segura and Spanagel, 2006; Crabbe 2010).

## CURRENT STATE OF THE ART ON GENETIC RESEARCH IN THE ALCOHOL FIELD

A genetic component of vulnerability to addiction has long been established. Twin, adoption and sibling studies have shown that genetic influences are directly responsible for some of the inter-individual differences observed in the predisposition to addictive disorders. A meta-analysis, which included 9987 monozygotic and dizygotic twin pairs, estimated a heritability of alcoholism to lie at around 50–60% (Goldman, Oroszi & Ducci 2005). Typically, alcoholism, as most psychiatric disorders, is a complex disorder that shows no obvious Mendelian transmission pattern and provides no evidence for common variants with main effects. Thus, the contribution of single genes to the clinical phenotype(s) of alcoholism is rather small.

### Imaging genetics in alcohol-dependent patients

Detailed endophenotype characterization of alcohol-dependent patients in particular, by means of various neuroimaging techniques, has become a very powerful approach to identify the neural circuitry of alcohol craving and relapse vulnerability (Heinz *et al.* 2009). Endophenotypes are defined as the intermediates between an observed pathological symptom and the underlying biological process. With vast developments in imaging techniques such as functional magnetic resonance imaging and magnetic resonance spectroscopy, it has become possible to define neurobiologically relevant endophenotypes and to correlate them with gene variations, so-called imaging genetics (Meyer-Lindenberg & Weinberger 2005). There is now increasing evidence that the contribution of single genes to an alcohol-related endophenotype is bigger than the clinical phenotype.

### Systematic GWAS in alcoholism

Systematic GWAS play a crucial role in candidate gene discovery. Until now, four GWAS on alcohol dependence have been conducted: (1) our recent study on alcohol dependence (Treutlein *et al.* 2009) is the first GWAS and follow-up study that identified a genome-wide significant association in alcohol dependence (see Research Highlights). (2) A further GWAS on a case-control sample drawn from the families in the Collaborative Study on the Genetics of Alcoholism (Edenberg *et al.* 2010) did not identify SNPs that met genome-wide criteria for significance; however several SNPs nominated as candidates in

our GWAS study (Treutlein *et al.* 2009) were replicated in this sample (e.g. GATA4). (3) An Australian/Dutch GWAS on alcohol dependence (Lind *et al.* 2010), where none of the SNPs achieved genome-wide significance, but a gene network diagram based on the top-results revealed overrepresentation of genes coding for ion-channels and cell adhesion molecules. (4) In a further American/German study, genetic influences on alcohol dependence were also explored (Bierut *et al.* 2010), but none of the findings survived in two independent replication series.

In conclusion, GWAS in the alcohol field provided first interesting results. However, given that much of the variance is driven by societal, lifestyle and behavioural influences—and in addition, there are also problems related to DSM-based diagnostic criteria (Miller 2010)—larger sample sizes, inclusion of endophenotypes and convergent genomic approaches (Niculescu and Le-Niculescu 2010) are warranted.

### The glutamate hypothesis of alcoholism

In recent years, the glutamate hypothesis of alcoholism and addictive behaviour has emerged as a major theory in the addiction research field (Tsai & Coyle 1998; Kalivas 2009; Spanagel 2009). In fact, alcohol affects the glutamatergic system on the molecular, synaptic and cellular level, and one hypothesis within the framework of the glutamate theory proposes that alcohol consumption may lead to an enhanced activity of the glutamatergic system in alcohol-dependent patients (Tsai & Coyle 1998; Spanagel 2009). This glutamate-induced hyperexcitability within the central nervous system (CNS) becomes uncovered during alcohol withdrawal. Furthermore, it has been suggested that augmented glutamatergic activity during protracted abstinence may contribute to craving and relapse behaviour, thus providing the rationale for using anti-glutamatergic compounds such as acamprosate for relapse prevention (Spanagel & Kiefer 2008). In our forward genetic approaches, we gathered additional support for the involvement of a variety of glutamate-related genes in addictive behaviour (e.g. Schumann *et al.* 2008). It is therefore of crucial importance to understand better the contribution of glutamatergic genes in the aetiology of alcohol addiction.

## RESEARCH HIGHLIGHTS OF THE GERMAN INTEGRATED GENOME RESEARCH NETWORK 'GENETICS OF ALCOHOL ADDICTION'

### Quantitative trait loci (QTL)-analysis of alcohol-related traits

Evidence for genetic linkage to alcohol-related phenotypes in the human and mouse genome have now been reported

**Table 1** Position, *P* values and LOD scores of all significant quantitative trait loci (QTL) revealed via QTL analysis. QTL analyses were performed either combined for both sexes or separately for female and male mice.

Trait	All ( <i>n</i> = 534)			
	Chr.	Location (cM)	P value (LOD score)	95% CI (cM)
Ethanol-induced hypothermia	1	85	< 0.001 (6.6)	81–87
	7	10	0.04 (3.6)	3.4–10.5
Ethanol tolerance	3	55	0.013 (4.1)	11.2–62.6
	6	24.7	0.014 (4.1)	18.7–30.7
	13	39	0.014 (4.1)	39–45
Ethanol-induced activity	1	65	< 0.001 (10.5)	63–71
Withdrawal-induced activity	1	65	< 0.001 (10.3)	59–71
	7	50	0.007 (4.4)	42.5–57.5
	11	43.1	0.02 (4.1)	33–47.1
Anxiety level during ethanol exposure	5	42	< 0.001 (13.2)	39–64
	12	18	< 0.001 (5.3)	13–22
Withdrawal-induced anxiety	1	79	< 0.001 (6.5)	39–83
	5	59	< 0.001 (15.0)	49–63
	12	21	0.04 (3.6)	3–32
Mean ethanol preference	16	31.4	0.009 (4.3)	17.4–35.4
Mean ethanol consumption	16	19.4	0.002 (5.1)	15.4–33.4
Ethanol preference before shock	16	33	0.02 (4.1)	13.4–36.5
Ethanol consumption before shock	16	29.4	0.002 (4.8)	7.4–35.4
Mean ethanol consumption	1	109	0.02 (3.9)	87–112
	2	102	0.01 (4.3)	80.9–108
	5	29	0.03 (3.9)	20–43
	10	2	0.003 (5.0)	2–21
	15	49	0.001 (5.2)	6.7–56.7

with some consistency across studies (Ehlers *et al.* 2010). We performed a large-scale analysis of quantitative traits related to alcohol-induced behaviours. A serial behavioural phenotyping of 534 animals from the second filial (F2) generation of a C57BL/6J and C3H/HeJ mice intercross was conducted. Traits assessed comprised of acute and chronic ethanol effects, ethanol consumption and preference, as well as ethanol withdrawal-related traits. Genotypes of all mice were assessed by microsatellite marker mapping. For this purpose, 264 markers with an average marker distance of 5.56 cM were genotyped, which represents a high-density whole genome coverage. QTL were subsequently identified using univariate analysis performed with the R/qtl tool, which is an extensible, interactive environment for mapping QTL in experimental crosses. QTL that have already been published were found, thus validating the serial phenotyping protocol, and several novel loci were identified as well. Our analysis further shows that the various responses to ethanol are regulated by independent groups of genes (Drews & Zimmer 2010; Drews *et al.* 2010; Table 1).

### The first GWAS on alcohol addiction

Our consortium published the first GWAS for alcohol dependence worldwide based on individual genotyping

(Treutlein *et al.* 2009). This GWAS was performed in 487 patients and 1358 controls. The best 139 SNPs were then analysed in a follow-up study of 1024 patients and 996 controls. The GWAS identified 121 SNPs with nominal  $P < 10E-4$ . These SNPs were then genotyped in the follow-up sample, together with 19 additional SNPs from homologues of rat genes showing differential expression (i.e. a convergent genomic approach; CGA). CGAs integrate genomic information (e.g. from microarray analysis) from animal models with a candidate gene or GWAS approach in humans. Especially, the explanatory power of genetic findings is enhanced by such a convergent approach (Bertsch *et al.* 2005) and as a result, a priority gene list for functional validation is obtained. Fifteen SNPs showed significant association with the same allele as in the GWAS. In the combined analysis, two closely linked SNPs in the 3' flanking region of the peroxisomal trans-2-enoyl-coA reductase (*PECR*) gene achieved genome-wide significance (rs7590720,  $P = 9.72 \times 10E-9$ ; rs1344694,  $P = 1.69 \times 10E-8$ ). These SNPs are located in chromosome region 2q35, which has been implicated in linkage studies for alcohol phenotypes. Candidate genes supported through our convergent rodent transcriptomic/human genetic approach included cadherin 13 (*CDH13*), alcohol dehydrogenase 1c (*ADH1C*) and GATA binding protein 4 (*GATA4*).

A total of four GWAS of alcohol dependence have now been published, and our group was involved in two: the first was conducted on our own data set (Treutlein *et al.* 2009), and the second was performed with one of our collaboration partners (Bierut *et al.* 2010). In the latter analysis, our data were used for replication purposes. The most recently published GWA study by Edenberg *et al.* (2010) replicated our *GATA4* finding.

#### Impact of newly identified risk variants on relapse behaviour and treatment response

Predicting the risk of relapse and the effect of treatment response (e.g. for naltrexone and acamprosate) in alcohol-dependent patients using genetic indicators (Oslin, Berrettini & O'Brien 2006; Ooteman *et al.* 2009) is a major challenge in the alcohol research field. We used a reverse genetic translational approach to test the impact of the most promising SNPs derived from our CGA (see Treutlein *et al.* 2009) on relapse and treatment response. In particular, we tested whether the top 15 SNPs were associated with relapse behaviour and pharmacological treatment response in 374 alcohol-dependent subjects who were included in a randomized, double-blind, placebo-controlled trial with acamprosate, naltrexone or placebo. The SNP rs13273672, which is located in *GATA4*, was associated with relapse within the 90-day medical treatment period ( $P < 0.01$ ). Subsequent pharmacogenetic analyses showed that this association was mainly attributable to patients treated with acamprosate ( $P < 0.01$ ) (Kiefer *et al.* 2010). In accordance with the observation that natriuretic peptide promoters are modulated by *GATA4*, we found a significant gene dose effect on the variance of atrial natriuretic peptide (ANP) plasma concentration in the different *GATA4* genotypes ( $P < 0.01$ ) (Kiefer *et al.* 2010). Hence, genetic variation in *GATA4* might influence relapse and response to treatment with acamprosate in alcohol-dependent patients via modulation of ANP plasma levels. These results will help to identify alcohol-dependent patients who are at an increased risk of relapse and who may respond better to treatment with acamprosate. Using a large and well-characterized clinical sample (PREDICT study; Mann *et al.* 2009) we will finally pin down the influence of specific genetic variations in respect to relapse risk and treatment response.

#### Gene–environment interactions in alcohol research

Alcohol use disorders are the result of cumulative responses to alcohol exposure, the genetic make-up of an individual and environmental perturbations over time (Spanagel 2009). Understanding how environmental influences moderate genetic risk (gene  $\times$  environment

interactions;  $G \times E$ ) is crucial for the elucidation of mechanisms underlying alcohol use disorders.

#### Serotonin transporter length polymorphism–stress interaction

Studies in non-human primate models of alcohol consumption have suggested that the serotonin transporter gene plays a role in the aetiology of excessive alcohol consumption (Heinz *et al.* 2003) and this genetic influence is dependent on the rearing conditions (Barr *et al.* 2004). To follow up these findings, we used a candidate gene approach with the Mannheim Study of Children at Risk (MARS), an epidemiological cohort study examining the outcome of early risk factors from infancy into adulthood (Laucht, Esser & Schmidt 1997). Adolescents took part in structured interviews to assess age at first drinking, current drinking and recent stressful life events. Life events during childhood and child psychopathology were measured using a standardized parent interview. We then analysed  $G \times E$  interactions in alcohol-related phenotypes. When exposed to high psychosocial adversity, male individuals in our study with the LL genotype of the serotonin transporter length polymorphism exhibited more hazardous drinking behaviour than those carrying the S allele or those without exposure to adversity. While the L allele relates more strongly to early-onset alcoholism, the S allele may be more closely linked to alcohol use that is associated with anxiety and depression (Laucht *et al.*, 2009). Other monoaminergic transporter systems may also contribute to excessive alcohol consumption; especially when taking the age at onset of alcohol use into consideration, genetic variations of the dopamine transporter gene may influence the extent of later excessive consumption (Schmid *et al.* 2009).

#### Corticotropin releasing hormone receptor 1 (*CRHR1*) and alcoholism

The hypothalamic pituitary adrenal axis and the extra-hypothalamic brain stress system mediate behavioural and neuroendocrine reactions to stress in animals and humans (de Kloet, Joels & Holsboer 2005; McEwen 2007). One important environmental risk factor for the development of alcohol use disorders is psychosocial stress (Uhart & Wand 2009) and the *CRHR1* seems to be the molecular mediator for this  $G \times E$  interaction (Heilig & Koob 2007; Clarke *et al.* 2008). In the course of our previous studies, we have discovered this crucial  $G \times E$  interaction by using *CRHR1* knockout mice (Sillaber *et al.* 2002). In these knockout mice, stress led to enhanced alcohol intake. This stress-induced alcohol drinking behaviour appeared with a delay and persisted throughout life. It was associated with an upregulation of the NMDA (N-methyl-D-aspartate) receptor subunit NR2B,

providing the first evidence of a link between these systems (Sillaber *et al.* 2002). With this study, we concluded that alterations in the *CRHR1* gene and adaptational changes in NR2B subunits may constitute a genetic risk factor for stress-induced alcohol drinking and alcohol addiction. These findings could then be translated to humans (Treutlein *et al.* 2006). An association of variations of the *CRHR1* gene with specific patterns of alcohol consumption could be demonstrated in two independent studies, especially when a high stress load was present (Blomeyer *et al.* 2008; Schmid *et al.* 2009). In yet another study, it was shown that individuals, who are sexually abused as children, are more likely to develop alcohol problems if they carry the risk variant of a *CRHR1* gene polymorphism (Nelson *et al.* 2010). In summary, *CRHR1* gene variation in conjunction with adverse life events and alcohol-related problems provide the most consistent gene–environment interaction in psychiatry genetics.

#### *Clock genes and alcoholism*

Another striking finding, namely the involvement of clock (*Period*; *Per*) genes in alcohol consumption and addictive-like behaviour arose from our excessive gene expression profiling experiments in the nucleus accumbens of alcohol-dependent animals. For the functional validation, we found that the clock gene *Per2* plays a crucial role in the regulation of excessive alcohol consumption. Interestingly, the *Per2* gene modulates, via the glutamate transporter GLAST, extracellular glutamate levels, thereby leading to hyperexcitability and enhanced consumption of alcohol (Spanagel *et al.* 2005). We now extended these findings by demonstrating a central role of the *Per2* gene in inhibiting alcohol sensitivity (Perreault *et al.* 2009). Most importantly, in the context of our working hypothesis that clock genes may play a major role in the aetiology of alcohol addiction, we have now developed a new treatment strategy by interfering with a drug targetable component of the clock machinery. By blocking a specific enzyme (which controls the phosphorylation status of several clock genes components) we can persistently suppress relapse behaviour.

In terms of clock G × E interactions, we found that the psychosocial stress moderates the effect of the circadian rhythm gene *Per1* on excessive alcohol drinking. We show that mouse mutants lacking a functional *Per1* gene respond to repeated exposure to adverse psychosocial stress with enhanced alcohol consumption, when compared with wild-type littermates. This finding translates into two independent human samples: in the MARS sample of 273 adolescents, we observed a gene–environment interaction where the association of a functional genetic variation in the promoter of *hPer1* with

frequency of heavy drinking is moderated by psychosocial stress. In an adult sample of 1006 patients and 1178 control individuals, this specific *hPer1* SNP is associated with alcohol dependence. In a functional *in vitro* characterization of this SNP in human, B-lymphocyte cell lines we found reduced cortisol-induced transcriptional activation of *hPer1* in cells with the risk genotype of this SNP. We further observed decreased binding affinity of the transcription factor snail1 to the risk allele of this specific *hPer1* SNP. Our findings indicate that the *Per1* gene does not influence alcohol consumption under baseline condition (Zghoul *et al.* 2007), but regulates alcohol drinking behaviour during stressful conditions and may thereby also contribute to the risk of developing alcohol addiction.

#### **Glutamate receptors and addictive behaviour**

Using a large systematic candidate gene approach on glutamate-transmission-related genes, we could demonstrate the importance of ionotropic and metabotropic receptors in the aetiology of alcohol addiction (Schumann *et al.* 2008). By means of conditional mouse mutant models, we were then studying the basic contribution of various glutamate receptors on drug-induced synaptic plasticity on the level of the ventral tegmental area (VTA) and the nucleus accumbens. By combining *ex-vivo* electrophysiology in acute brain slices with testing drug-induced sensitization, reinforcement and incubation of drug-seeking, we showed that the duration of the drug-evoked synaptic plasticity in the VTA is gated by metabotropic glutamate receptor 1 (mGluR1). Overriding mGluR1 *in vivo* made the potentiation in the VTA persistent. This led to synaptic plasticity in the nucleus accumbens, which contributed to incubation of drug-seeking behaviour after protracted abstinence. Impaired mGluR1 function in vulnerable individuals could therefore represent a first step in the recruitment of the neuronal network that underlies compulsion and addiction (Mameli *et al.* 2009).

The metabotropic glutamate receptors, mGluR5, also plays a central role in several forms of striatal synaptic plasticity (Spanagel 2010b). Using cell-specific RNA interference, we have studied a novel mouse line with a selective knockdown of mGluR5 in dopamine D1 receptor (D1R)-expressing neurons. Although mutant mice self-administer cocaine, we show that reinstatement of cocaine-seeking maintained by a cocaine-paired stimulus is impaired. By examining different aspects of associative learning, we identify deficits in specific incentive learning processes that enable a reward-paired stimulus to directly reinforce behaviour and to become attractive, thus eliciting approach towards it. Our findings indicate that targeting synaptic plasticity mediated by mGluR5 on striatal

DIR-expressing neurons may offer fruitful therapies for drug addiction through the disruption of specific incentive learning processes underlying relapse (Novak *et al.*, 2010).

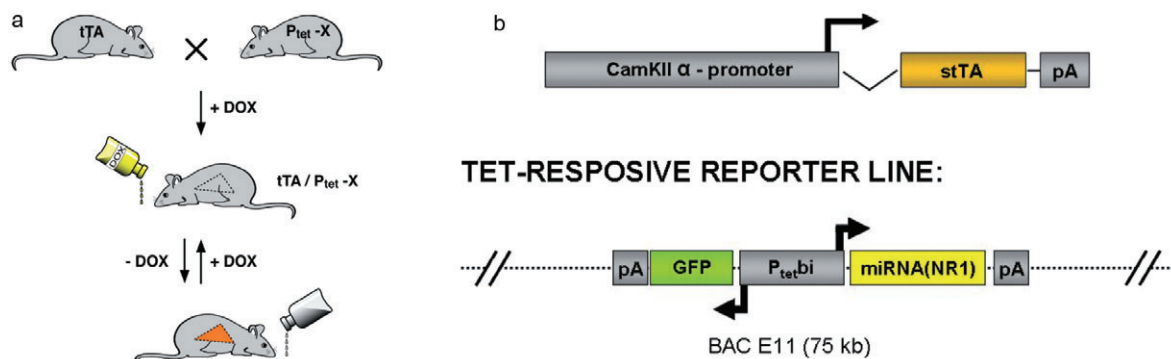
Drug-induced synaptic plasticity in dopaminergic cells is also mediated by GluR1 and NR1-containing receptors. These physiological processes underlie the persistence of drug-seeking behaviour (Engblom *et al.* 2008). Blockade of the NMDA receptor is, therefore, a promising avenue to intervene with relapse behaviour (Vengeliene *et al.* 2005). However, the application of NMDA receptor antagonists, especially at high doses, shows a strong unwanted pharmacological side-effect profile (Vengeliene *et al.* 2005). In order to interfere with NMDA receptor function, we have therefore chosen an alternative approach by targeting the modulatory glycine binding site at the NMDA receptor. We hypothesized that the blockade of glycine transporter 1 (GlyT1) might interfere through its effect on the glycine binding site at the NMDA receptor with compulsive alcohol consumption and relapse behaviour. We used an animal model of alcoholism—long-term compulsive alcohol consumption with repeated deprivation phases in rats (Rodd *et al.* 2009; Vengeliene *et al.* 2009)—to study the effects of a selective blocker of GlyT1 (Org25935). Subsequently, we examined alterations in dorsal striatal gene expression caused by chronic ethanol (EtOH) consumption, focusing on glycinergic and glutamatergic signaling-related genes. Gene expression profiles of Org25935 treated EtOH-drinking rats were compared with vehicle-treated EtOH-drinking versus age-matched EtOH-naïve rats. We found that repeated treatment with Org25935 reduced persistently compulsive relapse-like drinking without the development of tolerance. This persistent effect was paralleled by a reversal of altered expression levels of a set of glycinergic and glutamatergic signalling-related genes to

the levels found in EtOH-naïve control rats. Hence, the persistent anti-relapse effect might result from a restoration of normal glycinergic and glutamatergic signalling function (Vengeliene *et al.* 2010). Although no association of alcohol dependence with glycine transporter polymorphisms has been detected thus far (Koller *et al.* 2009), a prospective, double-blind, placebo-controlled trial investigating the efficacy and safety of Org25935 in relapse prevention in subjects with alcohol dependence (ClinicalTrials.gov Identifier: NCT00764660) seems to be justified on the basis of our preclinical data.

#### Generation of transgenic rats with conditional, tet-system based NMDA receptor knockdown

Rats are better suited to study complex behavioural patterns of addictive behaviour (Sanchis-Segura and Spanagel 2006). In order to study the contribution of NMDA receptors in compulsive drug use and relapse in more detail, we have generated genetically modified rats with conditional knockdown of the NR1 subunit of the NMDA receptors. First, we developed a concept of rationally designed, tetracycline-controlled miRNAs (Berger *et al.* 2010) and applied this strategy for designing miRNA targeting the NR1 subunit. The knockdown *in vitro* in cell culture system was above 90%. Next, we have generated transgenic rats with conditional, doxycycline-repressible knockdown of NMDA receptors (Fig. 1).

We could successfully establish doxycycline repressible, conditional transgenic expression of the NR1 miRNA *in vitro* and *in vivo*. First, we have generated transgenic rats with the tTA activator under the control of CaMKII $\alpha$  promoter. Expression of tTA activator was detected in the neurons of the forebrain in five founder lines, using tetO-lacZ reporters established in our laboratory. Next, we have generated a bicistronic expression vector based



**Figure 1** (a) Generation of transgenic rats with conditional, tet-system based NMDA receptor knockdown. The tet-system allows tissue-specific, conditional manipulation of gene expression in the rat brain by feeding animals with doxycycline. (b) Schematic drawing of the DNA constructs used for the generation of transgenic NR1 knockdown rat lines. The conditional knockdown of the NR1 mRNAs is restricted to the brain by tissue specific expression of stTA driven by CaMKII $\alpha$  promoter. The inducible bidirectional expression cassette controls expression of eGFP and the NR1 miRNA simultaneously

on the  $p_{tetbi}$  promoter expressing the NR1miRNA and eGFP mirroring each others expression. Transgenic rats were generated by microinjection of a DNA construct containing this unit embedded in a vector based on a BAC E11 (Schönig *et al.* 2010). Next, both the  $stTa$  and the  $p_{tetbi}$  rat lines were bred together and six double transgenic lines were analysed by immunohistochemistry. One of the lines showed expression of miRNA selectively targeted to medium spiny neurons of the striatum. Following further analysis, we conclude that we have obtained a functional knockdown of the NR1 subunit of the NMDA receptor in medium spiny neurons in transgenic rats, thus providing an ideal tool to study addictive behaviour.

#### *Discovery of Cystine Knot AMPA receptor Modifying Proteins (CKAMPs)*

In recent years, the discovery of AMPA and NMDA receptor channel-associated proteins was a major discovery indicating that receptor-associated proteins can play a dominant role in channel function and plasticity of neurotransmission. We made now use of a proteomic analysis of crude AMPA receptor purification to identify novel receptor-associated proteins. We were able to identify one novel postsynaptic membrane protein (CKAMP44) that modulates AMPA receptor function in *Xenopus oocytes* as well as in the mouse brain (von Engelhardt *et al.* 2010). Hippocampal CA1 pyramidal neurons express CKAMP44 at low levels, and over-expression of CKAMP44 leads in these cells to stronger and faster AMPA receptor desensitization, slower recovery from desensitization and a reduction in the paired-pulse ratio of AMPA currents. In contrast, dentate gyrus granule cells exhibit strong CKAMP44 expression, and here, CKAMP44 knockout increases the paired-pulse ratio of AMPA currents in lateral and medial perforant path-granule cell synapses. These results identify a novel molecular player that modulates short-term plasticity at specific excitatory synapses. A database analysis identified CKAMP44 as member of an entire new class of membrane proteins composed of CKAMP39, 44, 52 and 59 and characterized by a unique extracellular Cys-knot-containing domain and a C-terminal PSDII-type interaction motif. Their shared exon/intron structure points to the common evolutionary origin at a later time point in evolution, as CKAMPs can not be found in lower organisms. CKAMP39 was identified in mammals only indicating that the CKAMP's diversity increases with the complexity of the CNS. Both CKAMP44 and 39 can inhibit the AMPA receptor response in oocytes. Together with our studies on our AMPA and NMDA receptor knockout mice (Engblom *et al.* 2008), there is strong reason to believe that the CKAMP proteins are important modulators involved in mood disorders and drug abuse.

#### **Opioid peptides and alcoholism**

When comparing ethanol preference in opioid peptide knockouts, we found that ethanol consumption of  $\beta$ -endorphin knockout mice was significantly lower compared with wild-type and enkephalin mutant mice, while dynorphin knockouts showed an increased preference for ethanol. This genotype effect in  $\beta$ -endorphin knockouts was particularly pronounced in female animals. To evaluate the clinical relevance of these findings, we also performed an association analysis in a German and a Swedish case-control sample. We found a two-marker haplotype in the human  $\beta$ -endorphin gene (POMC) that was associated with dependence in females in both cohorts. Thus, our animal and human studies point to a gender-specific regulation of ethanol consumption by  $\beta$ -endorphin. We also observed that wild-type mice showed a higher increase in ethanol consumption after exposure to a foot shock stressor than any of the opioid knockout strains (Racz *et al.* 2008). This finding is consistent with our demonstration that endogenous opioids orchestrate stress responses. We have now begun to evaluate cFos induction in specific brain region after exposing mice to ethanol under high-stress and low-stress condition. Our findings strongly indicate that ethanol is highly effective in reducing the stress-related induction of cFos expression in wild-type mice in relevant brain regions such as the paraventricular nucleus. This effect is lacking in opioid receptor knockouts. Thus, the ethanol-induced release of opioid peptides, which has been demonstrated in many studies (Drews & Zimmer 2010), may contribute to modulation of stress responses by ethanol.

Another important opioid-like peptide that may contribute to enhanced vulnerability for alcohol addiction is nociceptin. Not only a variety of preclinical studies do in fact show this, but association studies of genetic variants of this peptide also provide a link with alcohol addiction (Huang *et al.* 2008; Xuei *et al.* 2008).

#### **Key transcription factors and addictive behaviour**

The persistent nature of addiction has been associated with activity-induced plasticity of neurons within the striatum and nucleus accumbens. In order to identify the molecular processes leading to these adaptations, we performed Cre/loxP-mediated genetic ablations of three key regulators of gene expression in response to activity: the  $Ca^{2+}$ /calmodulin-dependent protein kinase IV (CaMKIV) and its postulated main target, the cyclic adenosine monophosphate responsive element binding protein (CREB) and the serum response factor (SRF). We found that acute cocaine-induced gene expression in the striatum was largely unaffected by the loss of CaMKIV. On the behavioural level, mice lacking CaMKIV in dopaminocep-

tive neurons displayed increased sensitivity to cocaine, as evidenced by augmented expression of locomotor sensitization, and enhanced conditioned place preference and reinstatement after extinction. However, the loss of CREB in the forebrain had no effect on either of these behaviours, even though it robustly blunted acute cocaine-induced transcription. To test the relevance of these observations for addiction in humans, we performed an association study of CAMK4 and CREB promoter polymorphisms with cocaine addiction in a large sample of addicts. We found that a SNP in the CAMK4 promoter was significantly associated with cocaine addiction, whereas variations in the CREB promoter regions did not correlate with drug abuse. These findings reveal a critical role for CaMKIV in the development and persistence of cocaine-induced behaviours, through mechanisms dissociated from acute effects on gene expression and CREB-dependent transcription (Bilbao *et al.* 2008). Finally, SRF is essential for acute cocaine-induced stimulation of *Egr1*, 2 and 4, but does not affect cocaine reward (Parkitna *et al.* 2010).

### Acknowledgements

Our research consortium is funded within the framework of NGFNplus by the Bundesministerium für Bildung und Forschung (FKZ: 01GS08152).

### Authors Contribution

All authors are principal investigators of the consortium. RS has written in his function as a coordinator of this manuscript.

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